Aus der Medizinischen Klinik und Poliklinik III-Großhadern-der
Ludwig-Maximilians-Universität München,
Direktor: Prof. Dr. med. Wolfgang Hiddemann

Proteomic analysis of acute promyelocytic leukemia: PML-RARα promotes mitotic exit by increased expression and decreased phosphorylation of OP18 at serine 63

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Vorgelegt von
Mulu Geletu Heye
Aus
Addis Ababa, Ethiopia
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From the Department of Internal Medicine III
Ludwig-Maximilians-University, Munich
Direktor: Prof. Dr. med. Wolfgang Hiddemann

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Mulu Geletu Heye
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Mit Genehmigung der Medizinischen Fakultät
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1. Berichterstatter:	Prof. Dr. med. W. Hiddemann
Mitberichterstatter:	Prof. Dr. B.Emmerich
	Prof. Dr. J. Johnson
Mitbetreuung durch den promovierten Mitarbeiter:	PD. Dr. Gerhard Behre
Dekan:	Prof. Dr. med. D. Reinhardt
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1. Supervisor/Examiner:	Prof. Dr. med. W. Hiddemann
Co-Examiners:	Prof. Dr. B.Emmerich
	Prof. Dr. J. Johnson
Co-Supervisor:	PD. Dr. Gerhard Behre
Dean:	Prof. Dr. med. D. Reinhardt
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Dedicated To My Father Late Geletu Heye
&
Father in Law Late Nigussie Demeke

Table of Contents

Abbreviations

ALL	Acute Lymphoid Leukemia
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
ATRA	All-Trans-Retinoic Acid
BSA	Bovine Serum Albumin
CDK	Cyclin-Dependent Kinases
C/EBPα	CCAAT/Enhancer Binding Protein α
CHAPS	3[(3-Cholamidopropyl) dimethylammonio] propanesulfonic acid
CHCA	α-Cyano-4-Hydroxycinnamic Acid
CK	Complex Kryotype
CLL	Chronic Lymphocytic Leukemia
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	Hemopoietic Stem Cells
HDAC	Histon Deacetylase
IB	Immunoblot
IPG	Immobilized pH Gradient
IRF-1	Interferon Regulatory Factor-1
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
MTs	Microtubules

MAPs	Microtubule-Associated Protein
MAPK	Mitogen-Activated Protein Kinase
MI	Mitotic Index
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
OA	Okadaic Acid
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
PI	Propidium Iodide
pH	Potential Hydrogen
PODs	PML Oncogenic Domains

PTM	Posttranslational Modification
PMF	Peptide Mass Fingerprinting
RA	Retinoic Acid
RARα	Retinoic Acid Receptor α
RARE	Retinoic Acid Response Element
Rb	Retinoblastoma
RIPA	Radioimmunoprecipitation Assay
SDS	Sodium Dodecyl Sulphate
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 Hematopoiesis and acute myeloid leukemia (AML)

Hematopoiesis is a regulated developmental cascade for progressive restriction of cell fate potentials from hematopoietic stem cells (HSCs) to lineage-restricted progenitors that produce all lineages of mature blood cells (Miyamoto et al., 2004; Shizuru et al., 2005). The HSCs, characterized by pluripotency and self-renewal capacity are the basis of the hematopoietic hierarchy that gives rise to long-term (LT) and short-term (ST) HSCs, which then develop into myeloid and lymphoid progenitor cells. Subsequently, these progenitor cells give rise to distinct mature blood cells as depicted in Figure 1.

Acute myeloid leukemia (AML) is a malignancy of the hematopoietic cells characterized by an abnormal proliferation of myeloid precursor cells, an inefficient activation of the apoptotic program and an arrest in cellular differentiation (Bonnet and Dick, 1997; Jansen et al., 1999; Lowenberg et al., 1999; Tenen et al., 1997). A variety of leukemia also arise from mutations that accumulate in HSCs to cause their malignant transformation at the stage of stem cells or their progeny (Ferreira et al., 2005; O'Neill and Schaffer, 2004; Reya et al., 2001; Weissman and Baltimore, 2001). Increasing evidences suggest that the leukemias are sustained by leukemic stem cells (Rosenbauer et al., 2005). The leukemic-stem cell (LSC) hypothesis represents a modern-day interpretation of the proposal made by Rudolph Virchow and Julius Cohnheim 150 years ago that the cancer results from the activation of dormant

embryonic-tissue remnants (Huntly and Gilliland, 2005). The LSCs share important stem-cell function with normal HSCs such as self-renewal, initial differentiation, and survival (Figure 2).

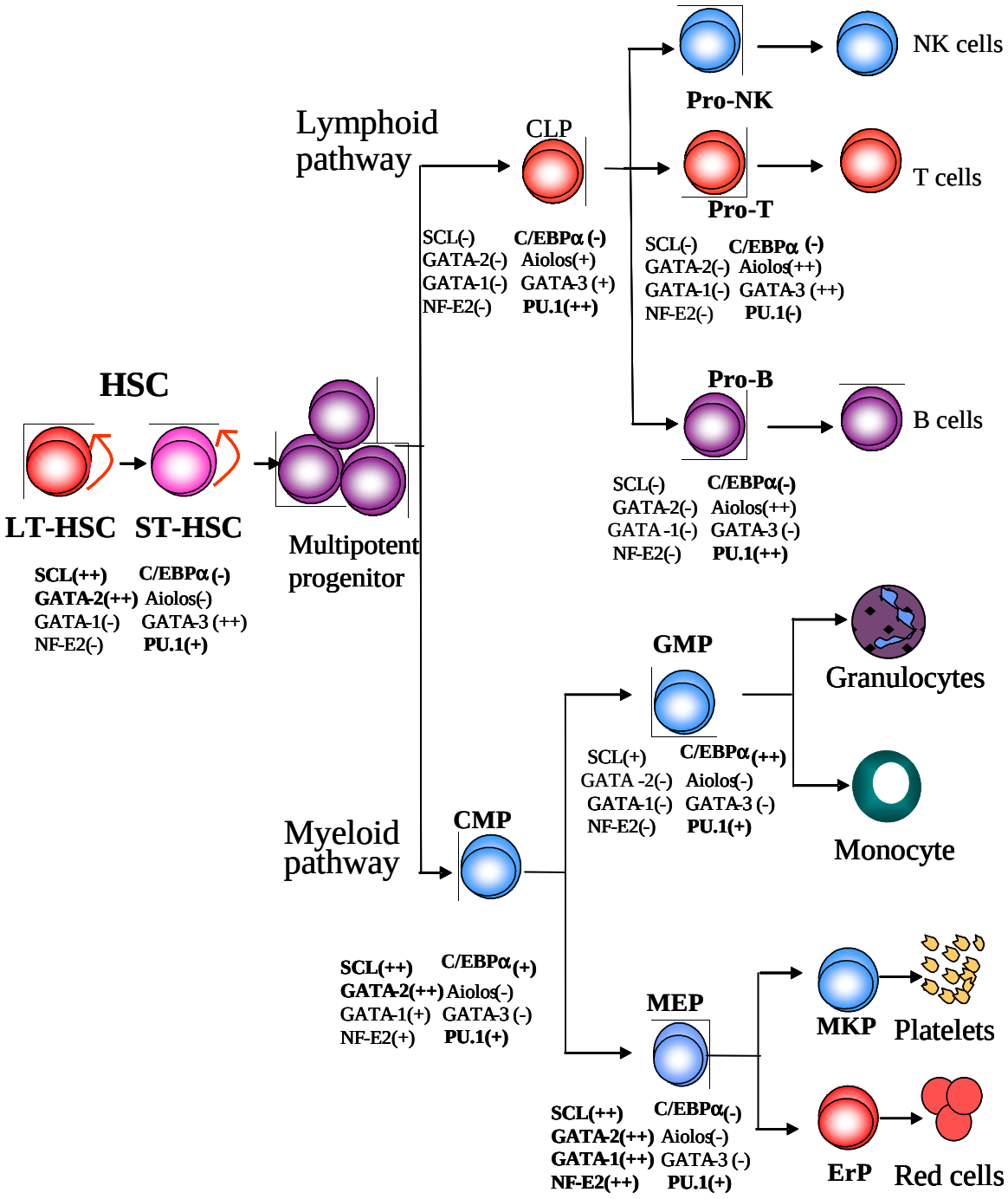


Figure 1: Schematic representation of the main lineage commitment steps in hematopoiesis and transcription factors which are involved in hematopoietic development. The hematopoietic stem cell (HSC) is the basis of the hematopoietic hierarchy and gives rise to LT-HSC and ST- HSC and which gives multilineage progenitors

(MLP), which can differentiate into all the hematopoietic lineages. MLPs become lineage restricted to the lymphoid and myeloid lineages in the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP), respectively. CLPs can give rise to B and T cells, while CMPs can give rise to megakaryocyte-erythrocyte progenitors (MEP) and granulocyte-monocyte progenitors (GMP). (Reya et al., 2001; Weissman and Baltimore, 2001).

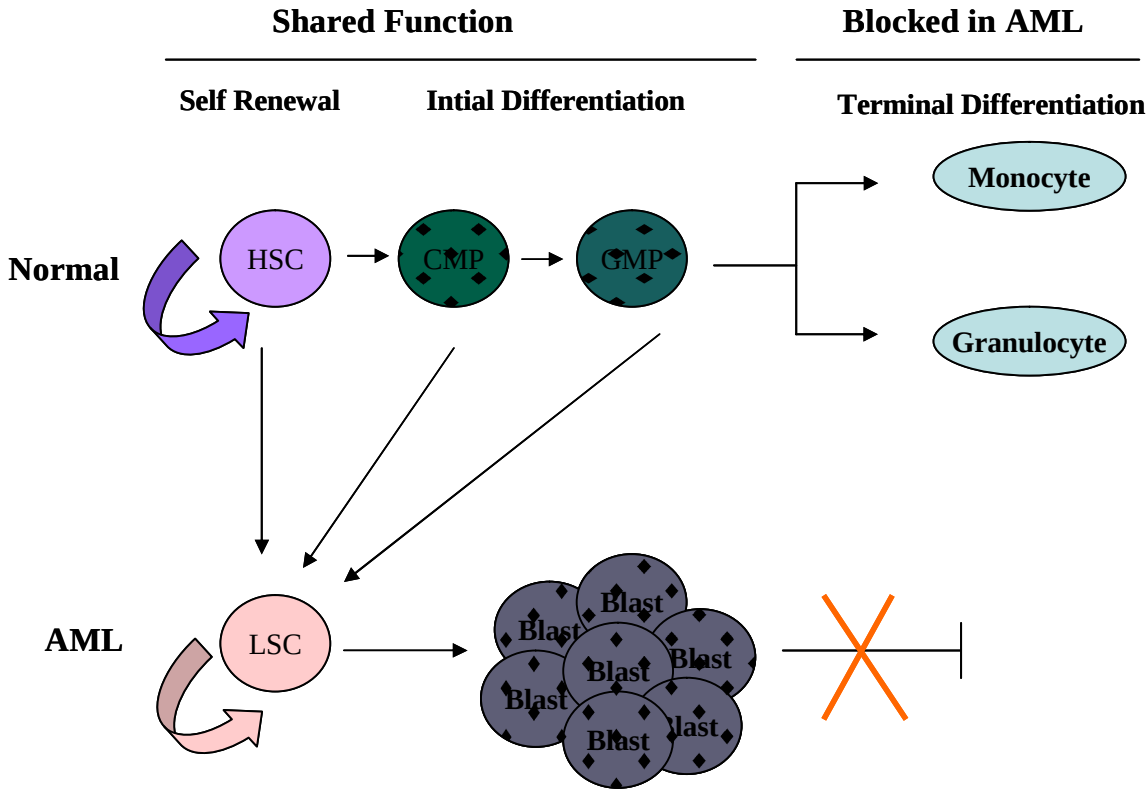


Figure 2: Leukemic stem-cell hypothesis. Both normal HSCs and neoplastic LSCs have the ability to self-renew and initially differentiate into less pluripotent daughter cells. However, HSCs produce short-lived progenitors such as CMPs and GMPs, which terminaly differentiate into mature monocyte and granulocytes, In contrast, LSCs give rise to leukemic blasts, which harbour a block in their terminal differentiation (Rosenbauer et al., 2005)

AML is not a single disease but a group of neoplasms with diverse genetic abnormalities and variable responses to treatment. Based on cytogenetic and molecular analyses three major groups of AML subtypes can be discriminated. One comprises AML with balanced chromosomal

aberrations, such as t(8;21), inv(16) and t(15;17). The second group includes cases with unbalanced, mostly numeric chromosomal aberrations frequently involving chromosomes 5, 7 and 8. The third group consists of patients with no detectable abnormalities (Hiddemann W; 2003). Balanced chromosomal translocations are the most specific genetic lesions in AML and may represent critical, early events in the genesis of the leukemic clone. The most common translocations are listed in the Table 1. In addition to the chromosomal abnormalities, aberrant expression of the lineage specific transcription factors plays an important role in the pathogenesis of AML through activation or repression of genes regulating proliferation and differentiation (Mills et al., 2000).

Table 1. Common translocations in AML (Lowenberg et al., 1999)

Translocation	**Genes Involved**	**Morphology**
t(8;21)(q22;q22)	AML1/ETO	M2 (90%), Mild narrow eosinophilia
t(16;21)(q24;q22) t(3;21)(q26;q22)	AML1/MTG16 AML1/EV11	Variable Variable
Inv(16)(p13;q22)	CFBβ/MYH11	M4Eo (almost exclusively), rarely M4, M5, M2 also with abnormal marrow eosinophilia
t(16;16)(p13;q22) del(16)(q22)	CFBβ/MYH11 CFBβ?	As for inv (16) M4, M2 (probably no) M4Eo with out CFBβ/MYH1
t(15;17)(q22;q12)	PML/RARα	M3 (exclusively)
t(11;17)(q23;q12)	PLZF/RARα	M3 (exclusively)
t(5;17)(q35;q12) t(11;17)(q13;q12) t(17;17)(q11;q12) t(4;11)(q21;q23)	NPM/ RARα NuMA/ RARα STAT5b/RARα MLL/AF4	M3 (exclusively) M3 (exclusively) M3 (exclusively) Most commonly associated with infant ALL
t(6;11)(q27;q23)	MLL/AF6	M4 or M5 (& T-ALL)
t(9;11)(q22;q23)	MLL/AF9	M4 or M5

t(11;19)(q23;p13;3)	MLL/ENL	Biphenotypic; Pre-B ALL; M4 or M5
t(11;19)(q23;p13.1) t(11;19)(q23;p13.3) t(11;16)(q23;p13)	MLL/ELL MLL/EEN MLL/CBP	M4 or M5 M4 or M5 M4 or M5 ,occasional dyserythropoiesis
t(11;22)(q23;p13)	MLL /p300	
t(7;11)(p15;p15)	NUP98/HOXA9	M2 or M4
t(2;11)(q31;p15)	NUP98/HOXD13	Variable
t(1;11)(q24;p15)	NUP98/PMX1	M2
inv(11)(p15;q22) t(6,9)(p23;q34)	NUP98/DDX10 DEK/CAN (NUP214)	M2 or M4, bone marrow basophilia, myelodysplasia with ringed sideroblasts
t(18;16)(p11;p13)	MOZ/CBP	FAB M4 or M5, bone marrow erythropagocytosis
t(9;22)(q34;q11) t(3;3)(q21;q26)	BCR/ABL EVI-1 (overexpression)	M1 or M2 Megakaryocytic dysplasia and often trilineage dysplasia
inv(3)(q21;q26)	EVI-1 (overexpression)	Megakaryocytic dysplasia and often trilineage dysplasia
t(16;21)(p11;q22)	TLS/ERG	Variable FAB; extensive bone marrow hemophagocytosis
del(17p)	p53 mutations	Characteristic dysgranulopoiesis

1.2 Acute promyelocytic leukemia (APL) and role of PML-RARα in leukemogenesis

Acute promyelocytic leukemia (APL), which represents 10% of acute myeloid leukemia, is characterized by an accumulation of malignant promyelocytes that fail to differentiate into normal granulocytes. (Douer, 2002; Warrell et al., 1994). APL is specifically associated with the chromosomal translocation t(15;17)(q22;q21), which generates a PML-RARα fusion between the genes of a nuclear protein, promyelocytic leukemia (PML), and that of a transcription factor, retinoic acid receptor α (RARα) (Lallemand-Breitenbach et al., 1999; Warrell, 1993; Warrell et al., 1993). PML-RARα which is the molecular signature of APL, plays a crucial role in leukemogenesis by acting

as a dominant-negative factor thereby disrupting the wild-type functions of both the PML and the RARα pathways (Kakizuka et al., 1991; Melnick and Licht, 1999). Both the *in-vivo* analysis in transgenic animals and the *in-vitro* analysis in hematopoietic cells have demonstrated that the PML-RARα fusion protein contributes to the leukemic phenotype by inhibiting differentiation and promoting survival of hematopoietic precursor cells (Wang et al., 1998b; Zhong et al., 2000).

Different mechanisms are proposed to explain how PML-RARα blocks differentiation. The fusion protein is able to silence natural RAR targets which are involved in the hematopoietic differentiation. The capacity to silence those genes depends on its capacity to bind DNA, recruit chromatin modifiers (histone deacetylase (HDACs) and DNA/histone methyltransferases) and induce changes in chromatin structure that are not permissive for transcription (Grignani et al., 1998; Lin et al., 1998; Minucci et al., 2001). Recently, it has been shown that the PML-RARα inhibits p53 by a mechanism independent from its transcriptional properties. The PML-RARα associates with p53, deacetylates later by recruiting HDACs and subsequently degrades p53 via the proteasome/MDM2 pathway in hematopoietic precursor cells (Insinga et al., 2005). Furthermore, PML-RARα physically interacts with PML, disrupts the PML nuclear bodies (NB) in a dominant negative manner. This induces the delocalisation of PML as well as the other NB component into an aberrant nuclear subdomains, thus potentially impairing PML function (Koken et al.,

1994; Zhong et al., 2000). PML-RARα does not rely only on its ability to affect myeloid differentiation. Recent studies shows that the transgenic animals expressing the PML-RARα fusion protein have a myeloproliferative syndrome with elevated numbers of myeloid cells in the marrow and the spleen (Kelly et al., 2002). This suggests that the PML-RARα fusion protein leads to an increased proliferative rate of the leukemic cells. Additionally, It has also been reported from PML-RARα transgenic mice that the leukemic cells actively proliferate and can form an increased number of colonies in *in-vitro* bone marrow cultures (He et al., 1998). These data would suggest an indirect role of PML-RARα in cell cycle. However this has not been investigated so far. In fact, a dominant negative action of PML-RARα on PML could result in shortening of the transition through the cell cycle (Pandolfi PP, 2001). *Pml-/-* cells have an increased proliferative potential in view of faster transition through the G1 phase of the cell cycle (Wang et al., 1998a).

A limited number of APL cases have been described with alternative chromosomal translocations, like the t(11;17) which generates a distinct fusion gene PLZF-RARα, the t(11;17) which gives rise to NuMA-RARα fusion gene and the t(5;17) that gives rise to the NPM-RARα fusion gene as depicted in Figure 3 (Corey et al., 1994; Licht et al., 1995; Look, 1997; Redner et al., 1996).

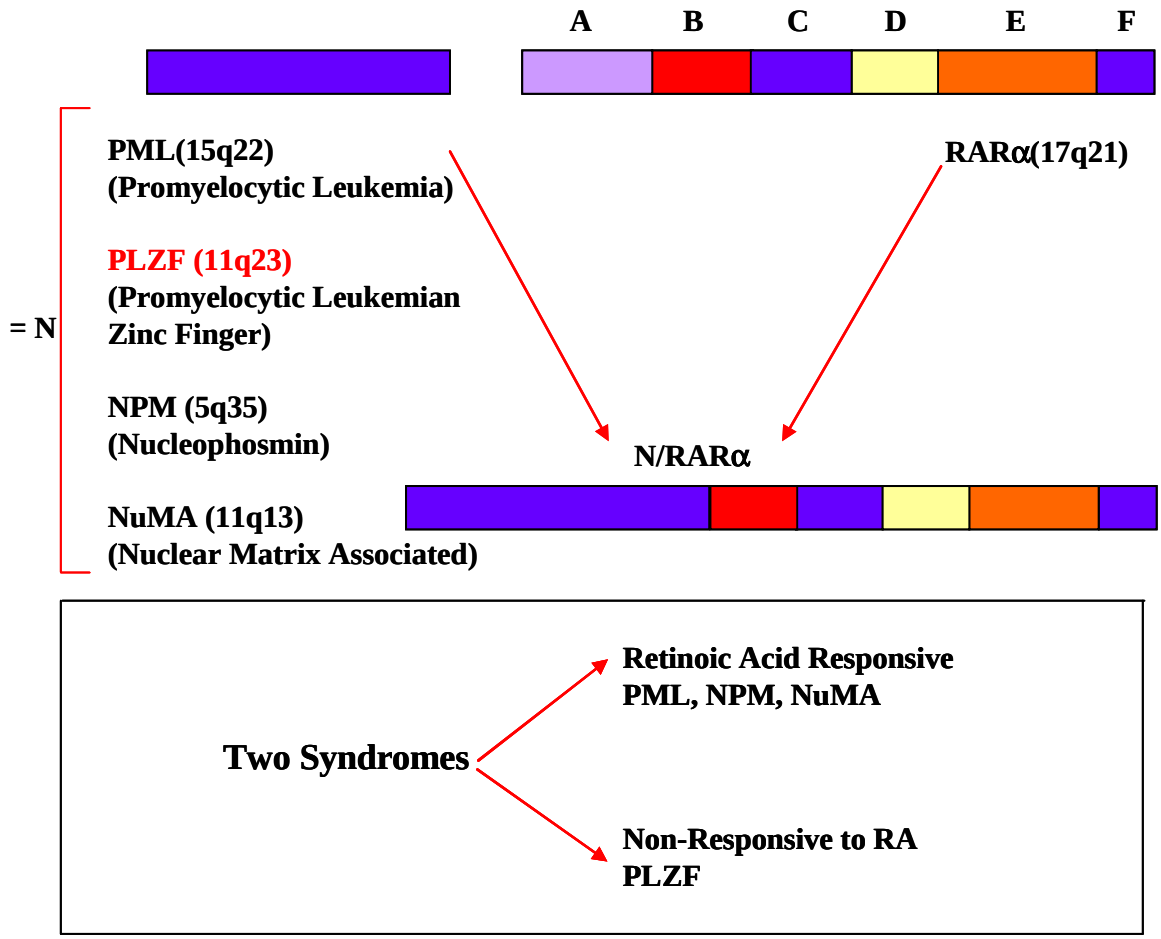


Figure 3: Graphical representation of N (PML, PLZF, NPM, and NuMA), RARα and N/RARα (PML-RARα) fusion protein. The four chromosomal translocations associated with APL results in fusion proteins in which the B through F domains of RARα, including the DNA binding and ligand binding domains of proteins, are linked C-terminal to four different nuclear proteins containing self-association domain. The t(11;7) APL syndrome linking PLZF and RARα is unique among these forms of APL in its resistance to differentiation therapy with ATRA or conventional chemotherapy(A = Transcriptional Activation AF-1, B = Transcriptional Activation AF-1, C = DNA Binding (RARE) Zn Fingers, D = Hinge Region, E = Ligand Binding Hetrodimerization Transcriptional Activation AF-2, N-Cor/SMRT Binding, TIF-1/TIF-2/CBP Associate and F = Function Unknown) (Melnick and Licht, 1999).

1.3 The effect of ATRA in APL and its role in the degradation of PML-RARα

APL is the first human malignancy that can be efficiently treated with a cell differentiation inducer All-Trans-Retinoic Acid (ATRA) and chemotherapy in

both remission induction and maintenance therapy (Douer, 2002). Treatment of APL with ATRA leads to complete remission (CR) in about 90% of patients, which is attributable to proteolysis of PML-RARα, re-formation of PML-NB, and terminal differentiation of APL cells into normal granulocytes (Breitman et al., 1981; Chambon, 1996). Restored PML protein and RARα overcomes the differentiation block and causes commitment of APL cells into granulocyte differentiation (Grignani et al., 1993a; Grignani et al., 1993b; Yoshida et al., 1996). In addition to the release of transcriptional repression with pharmacological ATRA level (10^{-6}-10^{-7}M), other possible mechanisms involved in ATRAs effectiveness in myeloid cell differentiation include expression of different gene classes, including induction of p21, cyclin-dependent kinase inhibitor, C/EBP-α, β, and ε, interferon regulatory factor 1 (IRF-1); and the regulation of localization of PML oncogenic domains (PODs) (Weis et al., 1994).

The success of retinoic acid (RA) as a standard therapy for the induction of remissions in patients with APL led to the notion that a defect in the RA signaling pathway could be responsible for the differentiation block in APL (Zhong et al., 2000). A report by Kastner *et al.* (Wang et al., 1998b) indicated that disruption in the RA signaling pathway is not sufficient to provide an explanation for the differentiation block since granulocytic lineage commitment is not impaired in RARα and RARγ-deficient mice. The global analysis of multiple gene expression patterns using cDNA microarray

technologies has provided additional information at the transcriptional level in response to ATRA- induced cell differentiation (Lee et al., 2002; Liu et al., 2000; Yang et al., 2003). These studies have established the gene networks in the NB4 cells that define phenotypic changes during decrease in cellular proliferation and promotion of granulocytic maturation by up-regulation of p19, GADD153, BTG1 and src-like adaptor (Liu et al., 2000).

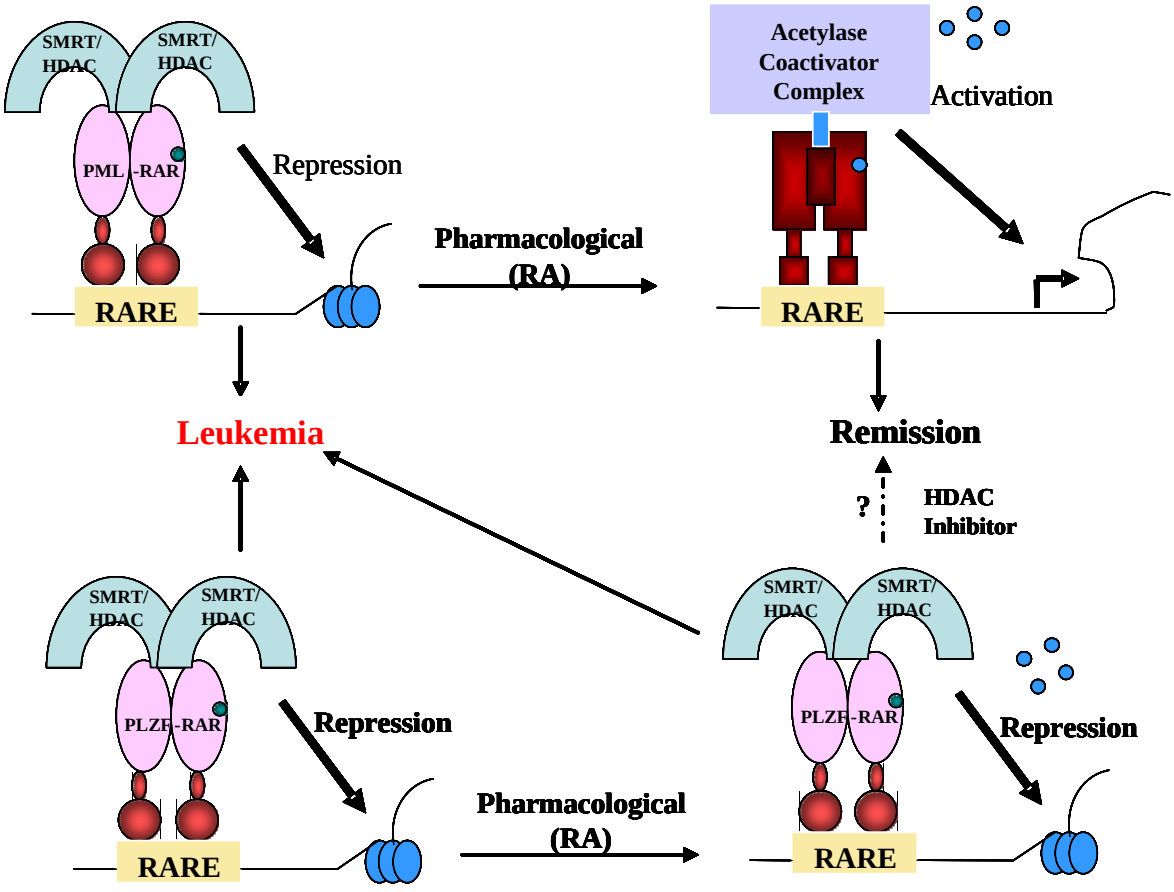


Figure 4: Model of transcription repression in the pathogenesis and hormonal response of APL. Under physiological concentrations of RA, the expression of RA target genes is repressed by X-RARα proteins through the action of the corepressor-HDAC complex. This results in the block of myeloid differentiation. Under pharmacological doses of RA, PML-RARα but not PLZF-RARα, dissociates the corepressor-HDAC complex and activates differentiation target genes and induces clinical remission in APL patients. The

combination of RA and HDAC inhibitors may be able to overcome RA resistance and trigger remission in PLZF-RARα-positive APL as indicated by a dashed line (Lin et al., 2001).

1.4 Proteomics and its role in understanding the biology and the mechanism of a diseased state

The array of proteins found within the cell, their interactions and modifications hold the key to understand the biological systems. This is encapsulated in the term “Proteome”. It can be defined as the total sum of protein of a cell, characterized in terms of localization, posttranslational modifications (PTM), interaction, and turnover, at any given time.

Before the advent of advanced mass spectrometry and proteomic technology, DNA microarray analysis was applied to identify molecular markers of human haematological malignancies. This technology readily allows measurement of the expression levels of thousands of genes simultaneously (Duggan et al., 1999). Expression profiling with microarrays has thus, made it possible, for example, to distinguish acute myeloid leukemia (AML) from acute lymphoid leukemia (ALL) (Golub et al., 1999), to define novel subgroups of leukemias and lymphomas (Alizadeh and Staudt, 2000; Armstrong et al., 2002), and to identify candidate genes leading to leukemogenesis (Makishima et al., 2002; Ohmine et al., 2001). Despite its potential for identifying genes important in leukemogenesis, microarry analysis is not able to provide direct information of proteins. Furthermore the transcriptional activity of a given gene is not always a major determinant of

the expression level of the translated protein. The characterization of leukemogenesis thus, requires a direct determination of the accompanying changes not only in the amount of cellular mRNAs but also in the protein abundance. The activities of many proteins are influenced by posttranslational modifications, such as phosphorylation, glycosylation, ubiqitination and sumoylation, which can only be assessed by MS based proteomics. Since the introduction of the term “proteomics” in 1995 (Wasinger et al., 1995), the proteomic analysis has gained considerable interest and is presently being used as a major tool for profiling proteins and biomarker for drug-discovery (Larsen et al., 2001).

The acute promyelocytic leukemia (APL) model system has turned out to be a useful tool to identify molecular pathways that could be manipulated for cancer therapy. Post-genomic strategies are being applied to identify genes and their encoded proteins that function in disease progression. A large amount of gene expression data and retinoic acid-responsive genes are available for APL (Walter et al., 2004). However, the need for protein expression data becomes inevitable. Therefore, global high-throughout functional proteomics could lead to new insight into the network of protein-protein interactions and target proteins involved in leukemia as well as provide new targets for pathogenesis based therapies of leukemia.

1.5 Cell cycle and its role in leukemogenesis

All cells reproduce by performing an orderly sequence of events in the cell cycle phase and duplicate its contents and then divide into two. Upon activation of mitogenic signalling cascades, cells commit to entry into a series of four sequential steps allowing traverse of the cell cycle: G1 (first gap), S (synthetic phase), G2 (second gap), and M (mitosis). Synthesis of DNA (genome duplication), also known as S phase, is followed by separation into two daughter cells (chromatid separation or M phase). During the G2 phase (the time between the S and M phases) cells can repair errors that occur during DNA duplication, preventing the propagation of these errors to daughter cells. In contrast, the G1 phase the time between the M and S phase represents the period of commitment to cell cycle progression. In order for the cell to continue cycling to the next phase, the prior phase has to be properly completed; otherwise, “fail safe” mechanisms, also known as “cell cycle checkpoints”, are elicited (Paulovich et al., 1997). The cell cycle machinery is controlled by the sequential activation of genetic programs including the synthesis of cyclins, the activation of the cyclin-dependent kinases (CDK) and cofactors, including cyclins and cdk inhibitors (CDKIs). The main function of the CDKs is the phosphorylation of substrates required for the cell cycle progression (Morgan, 1997; Muchardt and Yaniv, 2001).

Cell cycle regulation plays an important role for cell growth and differentiation. During hematopoiesis, cell cycle control genes are

distinctively regulated in a lineage dependent manner, reflecting the cell cycle characteristics of each lineage (Dao and Nolta, 1999; Furukawa, 1998; Steinman, 2002). Alterations in cell cycle machinery, especially in the G1/S phase regulation are known to be linked to the development of solid tumours as well as hematological malignant diseases (Hangaishi et al., 1996; Ogawa et al., 1994). For example, overexpression and prognostic significance for cyclin E has been reported in chronic lymphocytic leukemias and lymphomas (Erlanson et al., 1998; Wolowiec et al., 1995), overexpression of cyclin E has been observed in AML (Iida et al., 1997; Radosevic et al., 2001) and expression of p27 protein is significantly correlated with the response to chemotherapy (Radosevic et al., 2001). The role of cyclin A in development of AML has been demonstrated in transgenic mice, in which a targeted overexpression of the cyclin A1 in early myeloid cells initiated AML (Liao et al., 2001).

1.6 Microtubule dynamics during the cell cycle

When a cell divides into two daughter cells, the chromosomes must segregate prior to cell division. This process is mediated by a complex structure, the mitotic spindle, which is composed of microtubule polymers consisting of α/β tubulin heterodimers (Desai and Mitchison, 1997). The movement of chromosomes on the mitotic spindle is dependent on the dynamic instability of microtubules, a characteristic property of microtubles that allows them to switch abruptly between the state of elongation and rapid shortening (Desai

and Mitchison, 1997). The transition frequencies from a state of growth to a state of shortening is called ‘catastrophe’ and from a state of shortening to growth is called ‘rescue’ (Desai and Mitchison, 1997). The dynamics of microtubule polymerization and depolymerization during the cell cycle are regulated by a balance between the activities of two major classes of proteins, the microtubule-stabilizing microtubule-associated proteins (MAPs), which stabilize the assembled microtubules by suppressing catastrophe (Cassimeris, 1999), and destabilizing the protein OP18 (Belmont and Mitchison, 1996), the KinI family of kinesin-related protein (XKCM1 and XKIF2) (Desai and Hyman, 1999) , and the microtubule severing proteins (Katanin, p56 and EF1a) (McNally and Vale, 1993; Shiina et al., 1994). OP18 has been shown to stimulate transition from elongation to shortening of MTs (Walczak, 2000).

1.7 OP18

OP18 is a highly conserved 19kDa cytopalsmic and soluble phospho-protein, also designated as Stathmin, p19, prosolin, p18 or OP18, oncoprotein18 and pp20-pp21-pp23 (Cooper et al., 1991; Luo et a*l.*, 1991; Marklund et al., 1993; Peyron et al., 1989; Schubart et al., 1992). The OP18 family contains phosphorylation-controlled MT destabilizing proteins that are important for proper cell cycle progression in many types of proliferating eucaryotic cells (Andersen, 2000; Cassimeris, 2002; Gavet et al., 1998; Lawler, 1998; Sobel, 1991), crucial for the development of the nervous system in drosophila (Ozon et al., 2002) and are implicated in a wide variety of cancers (Bieche et al.,

1998; Melhem et al., 1997). The monomeric OP18 protein consists of an N-terminal capping domain (Steinmetz et al., 2000) and a C-terminal helical interaction domain (Belmont et al., 1996). The N-terminal region of OP18 is required for catastrophe-promotion while the C-terminal region is required to inhibit the MT-polymerization rate *in-vitro* (Holmfeldt et al., 2001).

Originally, OP18 was described as a protein that binds to tubulin dimers and increases the catastrophe frequency of MT *in-vitro*. Subsequent studies identified two putative mechanisms for OP18: (i) tubulin dimer sequestration that slows the MT growth rate (Curmi et al., 1997; Jourdain et al., 1997) and (ii) direct stimulation of MT plus-end catastrophes as depicted in Figure 5 (Arnal et al., 2000; Holmfeldt et al., 2001; Howell et al., 1999; Larsson et al., 1999; Segerman et al., 2003; Tournebize et al., 1997). Besides microtubule depolymerization activity, OP18 is regulated by changes in its level of phosphorylation that occur during cell cycle progression as depicted in Figure 6 (Mistry and Atweh, 2002). OP18 is phosphorylated in multiple and complex ways in response to a variety of extra cellular stimuli on four Serines (S16, S25, S38, and S63) (Amat et al., 1991; Curmi et al., 1999; Doye et al., 1990; Sobel et al., 1989). Phosphorylation of OP18 is regulated by cyclin dependent kinases (CDKs).

1.8 Role of OP18 in the regulation of cell cycle, microtubule dynamics and mitotic phase of the cell cycle

The first hint that OP18 may play a role in the regulation of cell cycle progression came from the observation that the level of phosphorylation of OP18 increases when erythroleukemic K562 cells enter the mitotic phase of the cell cycle (Luo et al., 1994). The level of OP18 phosphorylation is significantly lower in cells blocked in the G1/S phases of the cell cycle compared to proliferating cells and peaks in mitosis in both jurkat T cell and HeLa cells (Brattsand et al., 1994). The fact that both overexpression (Marklund et al., 1994a) and inhibition of OP18 expression (Luo et al., 1994) result in mitotic arrest generated a paradox that was resolved by the independent identification of OP18 as a cellular factor involved in the regulation of microtubule dynamics (Belmont and Mitchison, 1996). Belmont and Mitchison proposed the first model to explain the mechanism by which OP18 exerts its mitotic effect through an increase in the rate of catastrophe. Subsequently, a different model was proposed by Jourdain et al (Jourdain et al., 1997) in which the microtubule-depolymerizing activity of OP18 is mediated through direct sequestration of tubulin. This model is based on the observation that OP18 binds two unpolymerized tubulin heterodimers and forms a ternary OP18-tubulin complex (T2S). In conclusion, these studies demonstrate that OP18 is capable of binding both polymerized and unpolymerized tubulin and can prevent polymerization of α/β heterodimers

under some conditions and/or promote depolymerization of microtubules under others.

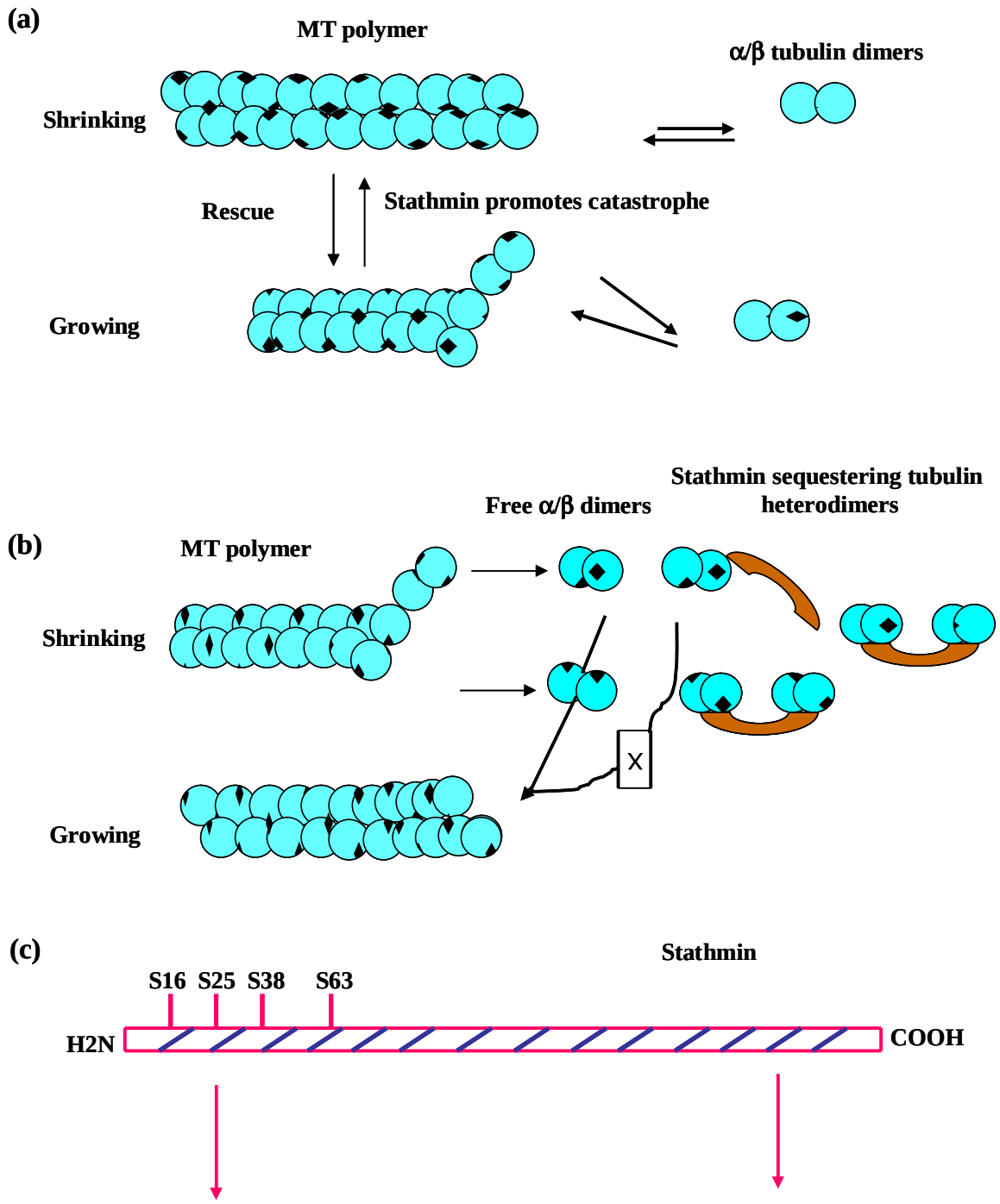


Figure 5: Models for OP18 induced destabilization of microtubules. (a) The dynamic instability of microtubules. **(b)** Tubulin sequestering activity of OP18. **(c)** The dual activity of OP18 (Mistry and Atweh, 2002).

Although the exact molecular mechanisms by which OP18 exerts its mitotic function are still unknown, there is wide consensus that its microtubule-depolymerizing activity is essential for orderly progression through the cell cycle. As cells proceed through anaphase and telophase, fluctuations between microtubule polymerization and depolymerization lead to the structural reorganization and eventually the disassembly of the spindle, which is followed by the exit from mitosis and cytokinesis. Microtubules then reorganize into a new interphase cytoskeleton upon entry into a new cell cycle (Rubin and Atweh, 2004). Fig 6 depicts a schematic representation of role of OP18 during cell cycle progression.

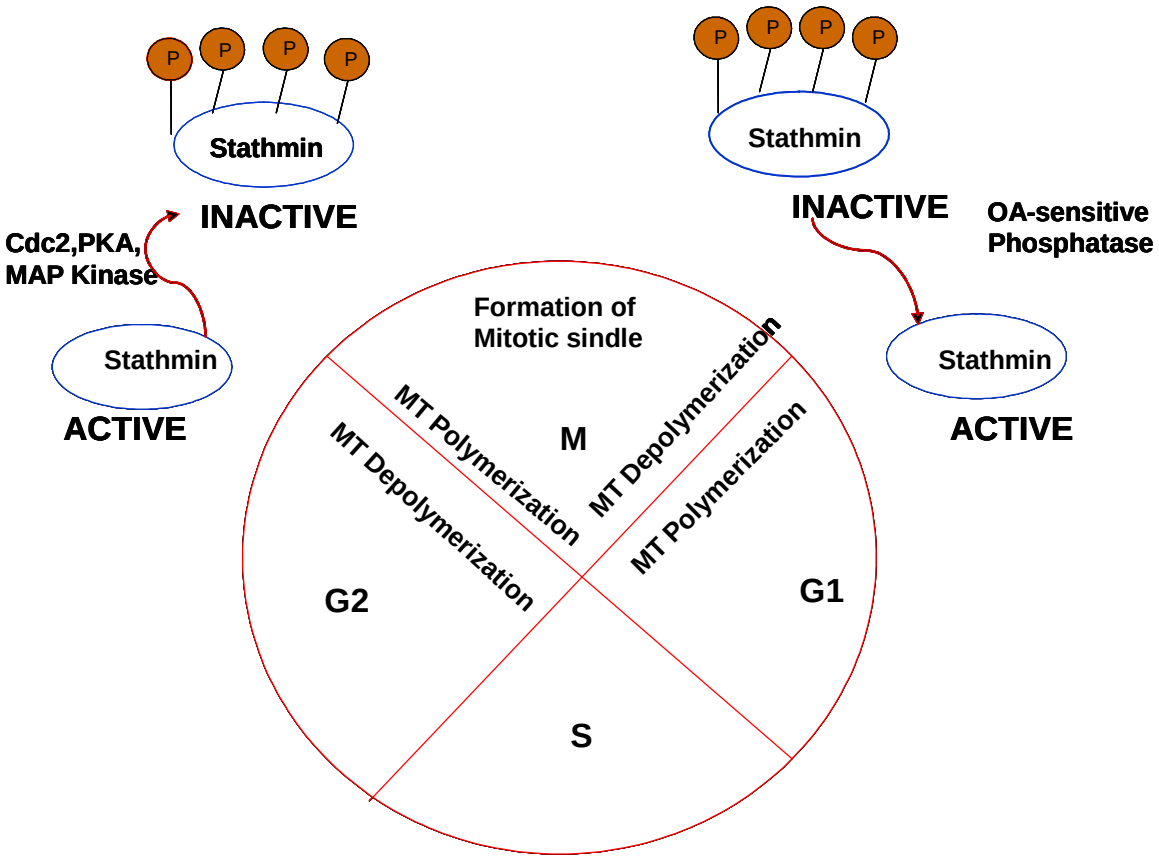


Figure 6: Regulation of OP18 activity during cell cycle progression. The unphosphorylated form of OP18 in late interphase promotes depolymerization of microtubules, and phosphorylation of stathmin early in mitosis turns off its activity, allowing the mitotic spindle to form. Dephosphorylation of OP18 during late mitosis is necessary for spindle disassembly and exit from mitosis (Mistry and Atweh, 2002).

1.9 Role of OP18 in cell proliferation

A growing set of arguments indicate that OP18 may participate in the control of cell proliferation. Data from development, tissue regeneration, cell biology or cancer studies indicate that there are differences in expression patterns of OP18 (Doye et al., 1992; Koppel et al., 1990; Koppel et al., 1993). For example, during development OP18 expression is high in undifferentiated multipotent cells of the early embryo (Doye et al., 1990) and in most tissues at the neonatal period (Doye et al., 1989; Schubart et al., 1992). In addition it is strongly decreased in the adult, remaining abundant mostly in the nervous system and in the testis (Koppel et al., 1990; Peschanski et al., 1993). Moreover, studies in several leukemic cell lines and in primary acute leukemia cells showed that OP18 expression is drastically decreased when the cells decrease to proliferate upon exposure to a variety of differentiation agents (Chen et al., 2003; Feuerstein and Cooper, 1983; Hanash et al., 1988; Luo et al., 1991). Furthermore, OP18 expression was markedly increased when normal lymphocytes are induced to proliferate by exposure to mitogenic stimuli (Hanash et al., 1988). Interestingly, the level of OP18 expression in leukocytes from patients with chronic myelogenous leukemia was also shown

to increase significantly when the disease progresses in to the more proliferative stage known as “blast crisis” (Rubin and Atweh, 2004). A similar observation was made in undifferentiated multipotential embryonal carcinoma cell lines where OP18 expression was decreased upon induction of differentiation. Besides, in other solid tumours like breast and ovarian cancer, it was shown that poorly differentiated tumours with a high proliferative potential expressed higher levels of OP18 than more differentiated and less proliferative tumours (Brattsand, 2000). All these observation suggested a strong correlation between OP18 expression and cellular proliferation in both normal and malignant cell.

1.10 Aim of this study

The PML-RARα fusion protein has the potential to inhibit terminal differentiation of hematopoietic precursor cells thereby giving leukemic blast a proliferative and survival advantage. However, the precise mechanism by which the PML-RARα fusion protein causes an APL phenotype remains largely unknown. Recent developments in the proteomics technology and mass spectrometry enabled us to address this question and analyze the changes in global protein expression induced by the fusion protein PML-RARα.

The objective of the present study is to identify differentially regulated proteins of PML-RARα, the posttranslational modification of the proteins and

the biological characterization of identified proteins by applying proteomic technology (2D gel electrophoresis and mass spectrometry).

2. Materials and Methods

2.1 Materials

2.1.1 Mammalian cell line

NB4 a maturation inducible cell line with t(15;17) marker (from DSMZ Acc 207)

U937 (Human myeloid cell line, monoblastic)

P/R-U937 cells (PML-RARα stably transfected U937 zinc inducible cells), kindly provided by Dr. Pellici

Cells from APL patients (bone marrow), kindly provided by Leukemia Diagnostic Laboratory, Medicine III Klinikum Großhadern, Munich.

2.1.2 Antibody

Anti- β-tubulin (Boehringer Mannheim, Germany)

Anti-Cdk2 (Santa Cruz Biotechnology, CA, USA, sc-163)

Anti- Cdk4 (Santa Cruz Biotechnology, CA, USA, sc-260)

Anti-GRP75 (Santa Cruz Biotechnology, CA, USA, sc-13967)

Anti- HSP70 (Santa Cruz Biotechnology, CA, USA, sc-7298)

Anti-MPM2 (Upstate Biotechnology, CA, USA, 05-368)

Anti- OP18 (Santa Cruz Biotechnology, CA, USA, sc-20796)

Anti- Pin1 (Santa Cruz Biotechnology, CA, USA, sc-15340)

Anti- p21 (Santa Cruz Biotechnology, CA, USA, sc-817)

Anti- PP2A (Santa Cruz Biotechnology, CA, USA, sc-14020)

Anti- PP2BA (Santa Cruz Biotechnology, CA, USA, sc-9070)

Anti-pOP18 (Santa Cruz Biotechnology, CA, USA, sc-12949-R)

Anti-RARα (Santa Cruz Biotechnology, CA, USA, sc-551)

2.1.3 Mutagenesis

Effectene transfection reagent (Qiagen, Germany)

GFP (Invitrogen, Germany)

Nucleofector kit (AMAXA, Cologne, Germany)

PCEP4-Op18 plasmid (from Dr.Samir Hanash)

Site-directed PCR Mutagenesis kit (Stratagene)

Primers (forward/reverse) for the generation of OP18 (NM_203401)

Ser63/ala (phosphorylation deficient mutant) and Ser63/asp (constitutive active mutant) (Metabion)

2.1.4 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 Immunoblot analysis

Total cellular protein was extracted from PR9 cells and U937 cells before and after 100µM Zinc sulphate treatment using RIPA laysis buffer (50mM Tris pH 8.0, 150mM NaCl, 1% Nonidet p-40 (Np40), 0.5% sodium Deoxycholate, 0.1% SDS, 5mM EDTA, Phosphatase and proteinase inhibitors cocktail). Protein concentration was measured using BioRad protein assay reagent. Equal amount of protein were mixed with equal amount of 2X loading dye (0.5M tris pH 6.8, 10% SDS, 100% glycerol, 2-mercapto ethanol, pinch of bromophenol blue and dH_2O) and boiled to 95^oC for 5 minutes. The sample was cooled on ice, centrifuged briefly and subjected to electrophoresis on 10% SDS-PAGE gels and transferred into Immobilin-P transfer membrane

(Millipore, Massachusetts, and USA). The western blotting procedure was performed and the blots detected with the ECL system as described previously (Behre et al., 1999b). Immunoblot analysis was performed for RARα, OP18, HSP70, GRP75, Pin1, CDk2, CDk4, PP2A and PP2BA. Anti-β-tubulin and annti-γ-tubulin antibody (Boehringer Mannheim, Germany) was used as internal loading control on the same blot after stripping.

2.2.2 Quantitative two-dimensional gel electrophoresis

PR9 cells were induced for PML-RARα with 100 μM Zinc sulphate for 6h. The whole cell lysates were then prepared using urea laysis buffer (9.8M urea, 1% DTE, 4% CHAPS, 2.5mM EDTA and 2.5mM EGTA) and the total protein concentration determined by BioRad-assay as described previously (Zada et al., 2003). The isoelectric focusing was performed with 1 mg (for coomassie stained gel) or 300μg (for silver stained gel) of protein (total volume was 360μl, mixed with 2.25μl of resolyte and 2μl of 0.5% bromophenol-blue) samples by using immobilized pH gradient (IPG) strips pH 3-10 and pH 4-7 (Amersham). The IPG strips were incubated for 10 min with DTE and carbamylation buffer respectively, washed two times with ddH_2O and subjected to second dimension. The proteins were then separated in the second dimension by 12% SDS-PAGE and protein spots visualized by either colloidal coomassie blue staining or silver staining. 2D-DIGE was also performed with 50μg of protein using fluorescent dyes.

2.2.3 Data analysis by ProteomeWeaver

The gels were scanned as 16 bit images (UMAX , PowerLook 2100XL scanner) and analyzed with the ProteomWeaver software version 2.1 (Definiens, Munich, Germany). The protein pattern of three independent gels (both uninduced and induced conditions) was used to generate an average gel and the spots detected in comparison with one another in terms of their intensity on the gels. The pair match-based normalization function of the program enabled us to remove nonexpression related variations in the spot intensity. With the support of statistical analysis, we could detect and analyze differentially regulated proteins in induced condition as compared to uninduced condition.

2.2.4 Mass spectrometry

The spots were carefully excised from the gels with a pipette tip, 3 times washed with dH_2O for 15 minute each. The spot were incubated with 50% Acetonitrile and 50mM Ammonium bicarbonate (NH_4HCO_3) and repeated two times. Trypsin enzyme (5ng/µl) reconstituted with ammonium bicarbonate (NH_4HCO_3) was used for overnight digestion at 37°C. The tryptic peptides were extracted in 70% acetronitrile. The supernatant were dried using speedVac (SPD111V, Savant) and resuspended in 20% acetronitrile, 0.1% TFA and sonicated for 3 minutes before processing for mass spectrometry. The extracted peptides (0.6 µL) were loaded onto the anchor chip target plate by mixing the peptides with the matrix in a ratio of 1:1. We used DHB matrix,

which is prepared by mixing 20 mg/ml 2.5-dihydroxy-benzoic acid with 20 mg/ml 2-hydroxy-5-methoxy-benzoic acid (9:1 v/v respectively) and used for our analysis. Measurements were performed using a ReflexIII MALDI-TOF instrument (Bruker Daltonic, Germany), operated in reflector mode, with an accelerating voltage of 20 kV. The identification of the proteins proposed by PMF from MALDI-TOF analysis and differentially regulated by PML-RARα was also confirmed by using MS/MS with AB4700 GPS explorer Software (Applied Biosystems). We used CHCA matrix For MS MS analysis, which is prepared by mixing 8mg of CHCA (Sigma) in 200µl solvent solution (50%Acetonitrile and 0.05% TFA). 2/3 of solvent solution mixed in to 1/3 of CHCA solution and 0.5µl matrix and 0.5µl of sample were loaded on the plate and dried. After drying the analysis was performed. Peptide masses were searched against Mascot database (Matrix Science).

2.2.5 Gene expression profiles of clinical samples

Microarray analyses were performed with bone marrow sample of acute myeloid leukemia patients utilizing the U133A microarrays GeneChip System® (Affymetrix U95 Av2 and U133A Santa Clara, CA, USA). Target cRNA preparation from total RNA, hybridization to the microarray, washing and staining with the antibody amplification procedure, and scanning were all carried out according to the manufacturer's instruction on analysis technical manual as described previously (Schoch, Kohlmann et al., 2002). To compare different experiments, the global microarray intensities were scaled to

common target intensity. Each new human GenChip expression array features, 100 human maintenance gene that serve as a tool to normalize and scale the data before performing data comparisons. These 100 probe sets were used for normalization, as recommended by the manufacturer (U133A mask file. http://www.affymetrix.com\suport\technical\mask_filesaffx.). Scaling was performed to a target intensity of 5000. All probe sets representing genes of interest were functionally annotated by NetAffx database (affymetrix), and HGNC (HUGO Gene Nomenclature Committee) approved gene symbols were proven.

2.2.6 Transient transfections and reporter assays

Transfections in 293T cells were carried out with Lipofectamine (Invitrogen). The luciferase reporter construct for OP18 used was a kind gift from Dr. Herrera: pOP18-luc. Other expression plasmids used are: pSG5-PML-RARα, pSG5-RARαand pCDNA3-PML. Transient transfections were carried out with promoter/luciferase construct and cotransfected with expression plasmids for human PML-RARα, RARαPML and Renilla Luciferase-null. Promoter activities were determined by measuring the luciferase activity with the Dual Luciferase Assay System (Promega). Firefly Luciferase activities were normalized to the Renilla Luciferase values of pRL-0.

2.2.7 Immunofluorescence

PR9 cells ($3x10^5$) under uninduced or induced for PML-RARα conditions were cytocentrifuged on glass slides with coverslips, fixed using 1:1

methanol/acetone and permeabilized using 0.3% TritonX. After blocking in PBG (0.5% BSA, 0.045% Fish-gelatin in PBS) containing 5% FBS, the fixed cells were incubated with anti-OP18 (anti-rabbit sc-20796, Santa Cruz) and anti-α–tubulin (anti-mouse) antibodies followed by incubation with Alexa Fluor 488 chicken anti-rabbit and Alexa Fluor 594 chicken anti-mouse IgG secondary antibodies (Molecular Probes) respectively and 4´, 6´-diamidino-2-phenylindole dihydrochloride (DAPI, 1μg/ml) for 15 min. The cells were mounted in aqueous mounting medium and the images were acquired and analysed using a fluorescence microscope.

2.2.8 Cell cycle and mitotic index evaluation

We used propidium iodide (PI) staining to analyze the distribution of cells in the different phases of the cell cycle as described previously (Zada et al., 2003). Briefly, PR9 cells and U937 cells ($5x10^5$) under uninduced or induced for PML-RARα conditions were washed once in PBS 1X 1000RPM for 10 minutes at 4^0C. The cells were resuspended with 200μl PI lysis buffer (0.1% Sodium citrate, 0.1% Triton X -100, and 20μg/ml Propidium Iodide). The lysate were incubated on ice for 5 minutes and FACS analysis was done using the ModFit LT™ 3.0 software (Becton Dickinson). Standard statistical methods were used to analyse the cell cycle data including mean, standard deviation and standard error of the mean. P value was calculated from the student`s t-test by comparing the means of two different conditions in each experiments. For mitotic index, the cells under different conditions were

cytocentrifuged onto glass slides, fixed in methanol and then stained with either DAPI or Wright-Giemsa for 10 min. The mitotic cells were counted in different fields and represented as a percentage of total cells (mitotic index MI). 150-200 cells were counted for quantification.

2.2.9 *In- vitro* kinase assay

PR9 cells were washed with cold PBS and RIPA lysates prepared at different time points. Lysates were collected by centrifugation for 30 min and protein concentrations were quantified by the Bradford assay (Bio-Rad Laboratories, Germany). 200 µg of protein was incubated with 2 µg of anti-CDK2 or anti-CDK4 antibody at 4° C for 2 h with rotation. Protein A agarose beads (20 µl) was then added and the incubation continued for another 2 h. Immunocomplex beads were washed twice with PBS buffer and three times with kinase buffer (150 mM NaCl; 1 mM EDTA; 50 mM Tris-HCl, pH 7.5; 10 mM MgCl2; and 10 mM DTT). Kinase activity was assayed by incubating the beads at 37° C for 30 min with 25 µl kinase buffer, 3 µg histone H1 (Upstate, Germany; CDK2) or Rb-fusion protein (Santa Cruz; CDK4), 10 µM ATP, and 4 µCi [γ-32P] ATP (3000 Ci/mmol). Samples were then boiled for 5 min in 2 times sample buffer electrophoreses through a 12% SDS-polyacrylamide gel, dried, and phosphorylated histone H1 and Rb proteins were visualized by auto radiography. Also shown is an immunoblot IP control for CDK2 and CDK4.

2.2.10 RNA interference using siRNA

For knocking-down OP18 expression in PR9 cells, we used siRNA technology. The targeted sequence of OP18 was empirically determined in the OP18 coding sequence and was verified by BLAST searches. To ensure gene (or protein) specific effects, we designed 2 oligos corresponding to DNA target sequences CTG CCT GTC GCT TGT CTT CTA and TTG AGT TAG GTT AAT AAA TCA of the OP18 sequence (gene accession number NM_203401): oligo1, sense r (GCC UGU CGC UUG UCU UCU A) dTdT and antisense r (UAG AAG ACA AGC GAC AGG C) dAdA; oligo2, sense r (GAG UUA GGU UAA UAA AUC A) dTdT and antisense r (UGA UUU AUU AAC CUA ACU C) dAdA. The oligos were transfected into PR9 and U937 cells using RNAiFect essentially using manufacturer's instructions with minor modifications (Qiagen). A random DNA target sequence AAT TCT CCG AAC GTG TCA CGT with the siRNA duplex sequences, sense r (UUC UCC GAA CGU GUC ACG U) dTdT and antisense r (ACG UGA CAC GUU CGG AGA A) dAdA was used as a non-silencing siRNA control and siRNA conjugated to rhodamine was also used to visualize the transfection efficiency of the transfected siRNA sequences into the cells. For the transfection of siRNA into cells, RNAiFect (Qiagen) was used. 5-10 μg of each siRNA was used in each condition; the cells were analyzed for toxicity 18-24h post-transfection and then stimulated further with RA or Zn when needed. The effect of siRNA on protein expression of OP18 was analyzed by immunoblot.

2.2.11 Construction of OP18 mutants and transfections

The pCEP4-OP18 plasmid was a kind gift from Dr. Samir Hanash. PCR mutagenesis kit (Stratagene) was used for the generation of OP18 mutants from this plasmid. The primers used for the generation of OP18 Ser63/ala mutant were, forward 5`-GAA AGA CGC AAG GCC CAT GAA GCT GAG-3` and reverse 5`-CTC AGC TTC ATG GGC CTT GCG TCT TTC-3`and for OP18 Ser63/asp mutant were, forward 5`-GAA AGA CGC AAG GAC CAT GAA GCT GAG-3` and reverse 5`-CTC AGC TTC ATG GTC CTT GCG TCT TTC-3`. All the plasmid constructs were verified by sequencing. It is important to mention here that Serine/threonine-to alanine substitution has been shown to block phosphorylation in a number of proteins. On the other hand, conversion of the Serine/threonine to aspartic acid mimics phosphorylation of these residues since, aspartic acid has a negative charge and is enough to maintain the phosphorylation status of the protein (Hall et al., 1996; Huang and Erikson, 1994; Warnock et al., 2005). For the transfection of PR9, U937 and NB4 cells, effectene transfection reagent were used (Qiagen). For the transfection of APL patient samples, cell nucleofector kit (AMAXA) was used essentially as described by manufacturer. 5μg of plasmid DNA constructs were used for each transfection and the analysis ($3x10^5$) was performed 14h later. For analyzing transfection efficiency, a plasmid with GFP marker (2μg) was used.

3. Results

3.1 The PML-RARα fusion protein is induced after the addition of zinc in PR9 cells

In the present study, we used an *in-vitro* cell line model system, the PR9 cells, kindly provided by Dr. Pellici, which has PML-RARα cDNA under the control of the Zinc inducible metallothionein promoter. PR9 and U937 cells were induced with 100µM zinc sulphate at different time point. Expression of the PML-RARα fusion protein in PR9 cells (Fig. 7a) could be detected after zinc induction, which persisted till 12 h and then gradually decreased thereafter. However, expression of the fusion protein was maintained by continual addition of zinc to the medium after every 12 h time period of induction (data not shown). U937 cells under similar condition were used as a zinc control (Fig. 7b). The expression level of PML-RARα (Fig. 7a) has previously been shown to be comparable to those of APL blasts (Casini and Pelicci, 1999).

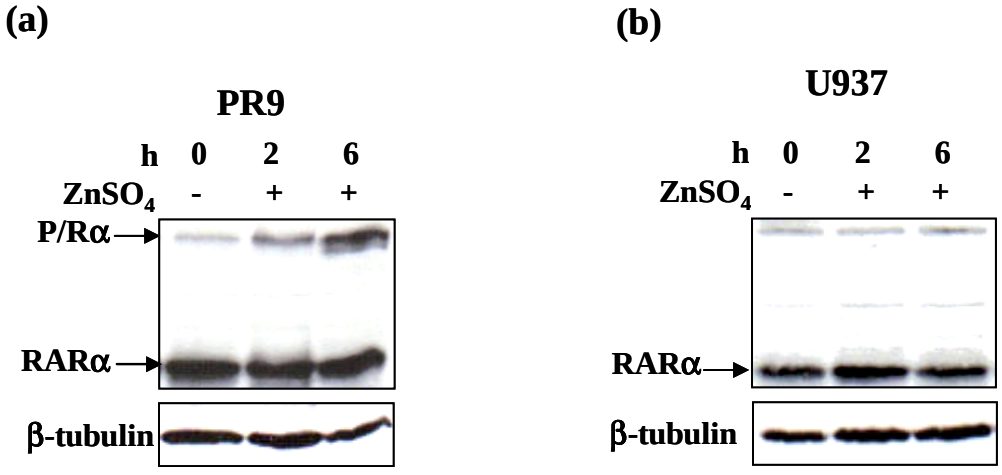


Figure 7: PML-RARα is induced after zinc induction in PR9 cells: (a) Immunoblot analysis showing the expression of PML-RARα in PR9 cells upon Zinc induction (100µM

$ZnSO_4$) using anti-RARα antibody (C-20, sc-551 Santa Cruz). **(b)** U937 cell were used as zinc control.

3.2 PML-RARα induction in PR9 cells is associated with changes in protein expression: two-dimensional gel analysis approach

No systematic identification and characterization of the global proteome and the corresponding cellular function of the PML-RARα fusion protein has been reported till date. To this end, we used a global proteomic approach to identify target proteins of the fusion protein in an *in vitro* cell line model system. Proteins from PR9 cells induced for PML-RARα (with treatment with $ZnSO_4$ for 6h) were separated using a pH 3-10 and a pH 4-7 immobilized pH gradient gel in the first dimension followed by SDS-PAGE. The 2D gels were stained with either colloidal coomassie blue (Fig. 8a) or silver (Fig. 8b) to obtain a spot pattern under each condition (without and with PML-RARα). The spot patterns were quantitatively analyzed by the ProteomWeaver software, which generated nonwarped and warped images corresponding to each gel, under uninduced (without PML-RARα) and induced (with PML-RARα) condition (Fig.8d). This allowed us to individually look for the differences in the protein expression corresponding to each spot in a single gel. The spot patterns were observed to be reproducible and the degree of reproducibility shown as a scatter plot between a reference gel and the two other gels (Fig. 8f) with correlation coefficients of 0.58 and 0.66, statistically calculated by the software.

Of the total of 224 proteins identified, PML-RARα in PR9 cells showed significant changes in 47 proteins that exhibited a 1.5-fold change or greater, of which the expression of 25 proteins was found to be increased and the expression of 22 proteins decreased upon PML-RARα induction (Table 2) compared to the uninduced control. As an illustration of changes occurring in the protein expression as a consequence of PML-RARα induction, we have compared changes without and with PML-RARα (Fig. 8c). To rule out the changes in protein expression as a result of zinc induction, we used the parental U937 cells as control (Fig. 8a bottom panel). In addition to coomassie and silver staining procedures, we also performed a more reliable quantitative fluorescent 2D DIGE staining methods using Cy3 and Cy5 labelling dyes (Amersham, Germany) in PR9 cells under uninduced and induced conditions (data not shown).

(a)

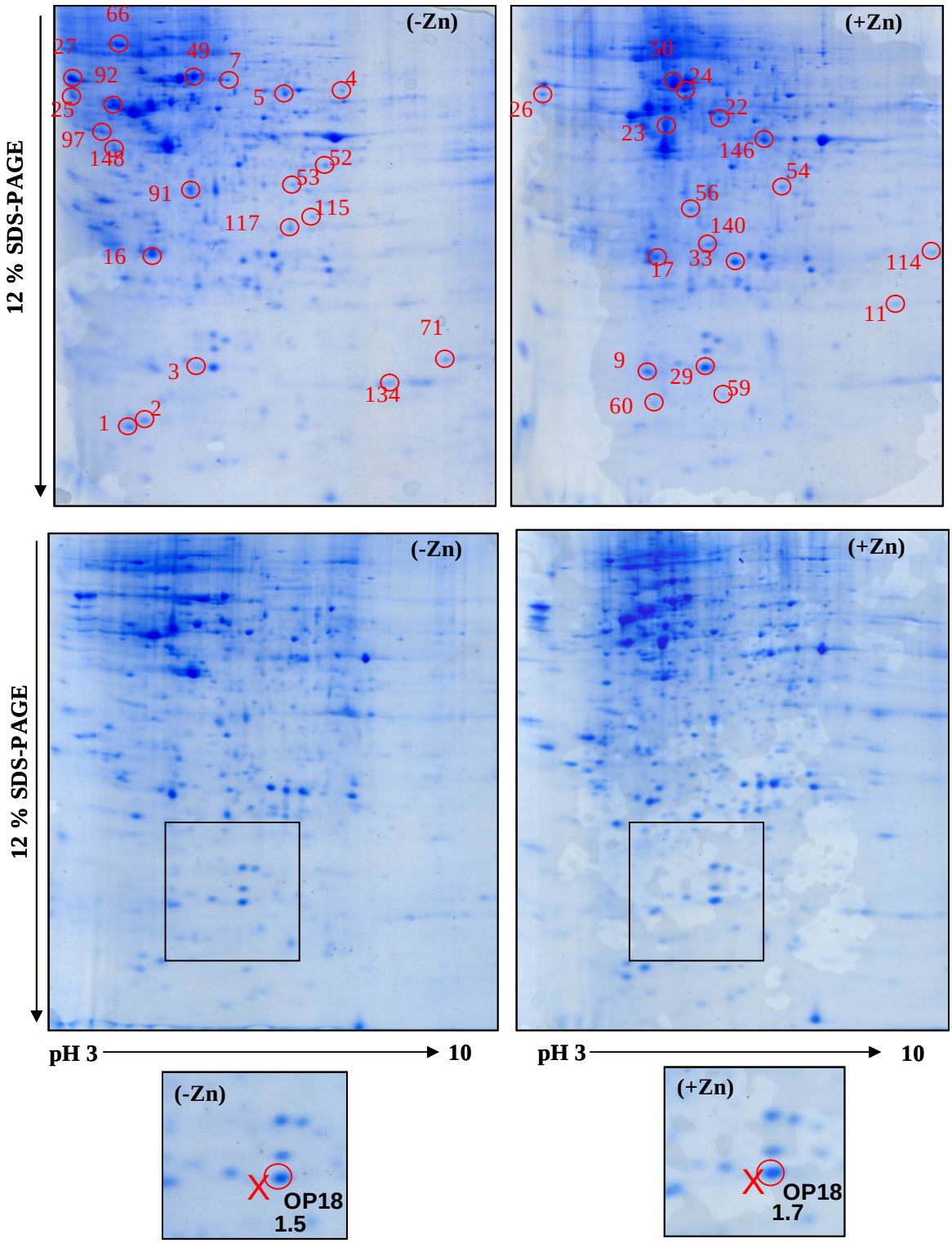
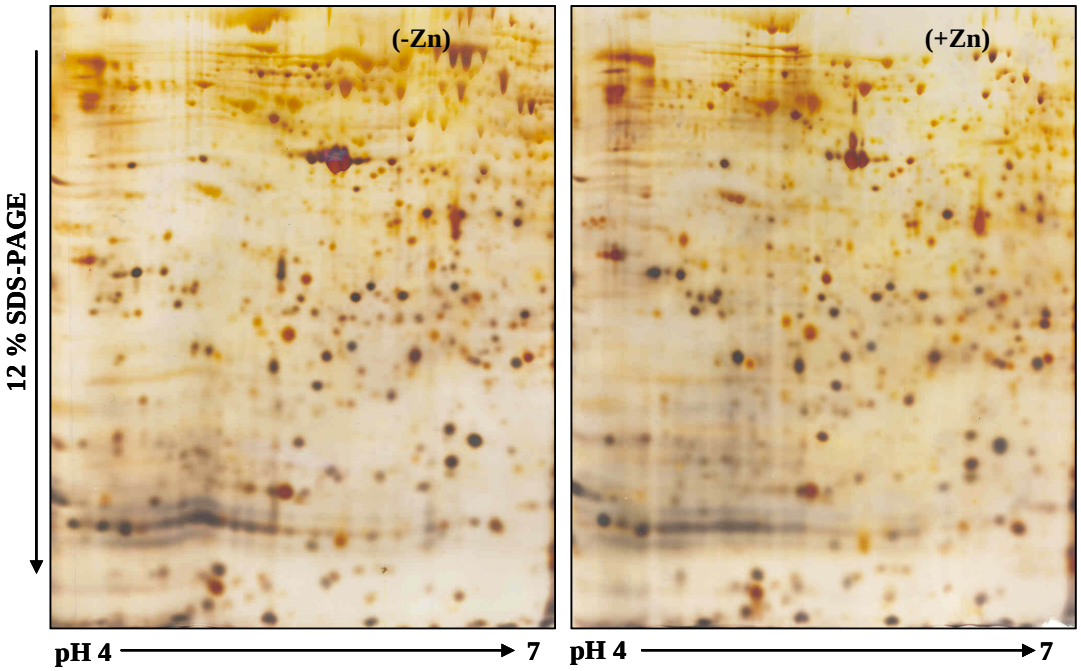


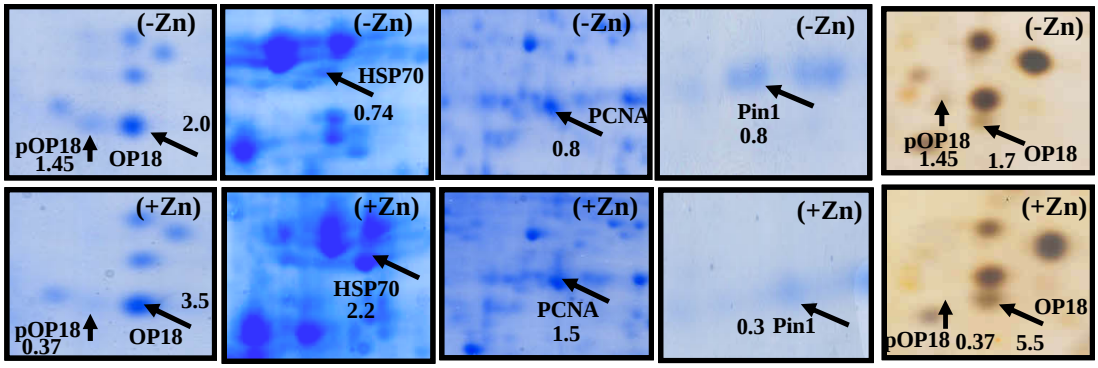
Figure 8: 2D gel electrophoresis showing PML-RARα regulated proteins. The 2D gel electrophoresis experiments were performed three times to obtain a reproducible protein pattern and were confirmed in all the experiments by MALDI-TOF MS and MS/MS analysis. **(a)** Coomassie-stained 2D gels from whole cell lysates of PR9 cells under

uninduced (-Zn, 0h) and induced (+Zn, 6h) conditions. The encircled spots represent proteins identified as identical in all the experiments and as differentially regulated by PML-RARα (top), U937 cells under similar conditions were used as Zinc control (bottom).

(b)

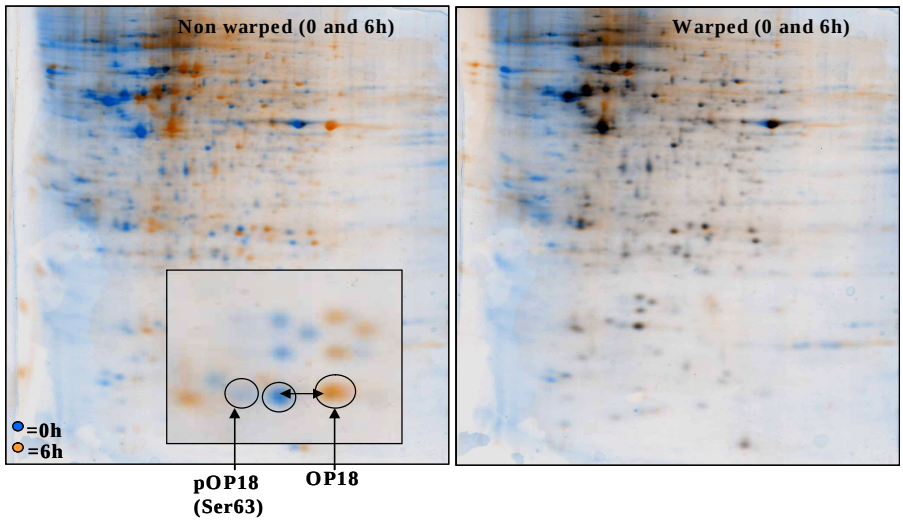


(c)

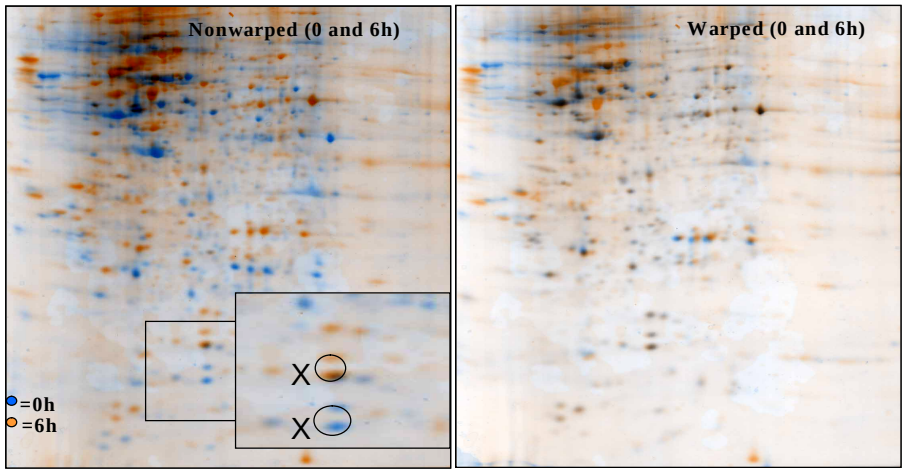


(b) Silver-stained 2-D gels under uninduced and induced conditions. **(c)** an enlarged view of some of the protein spots taken from the 2D gel image of Figure 8a and b; a comparison in expression values using ProteomWeaver software under uninduced and induced conditions are shown.

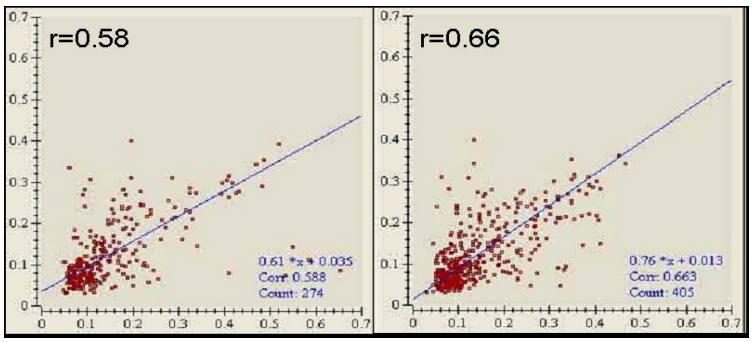
(d)



(e)



(f)



(d) PR and **(e)** U937; proteomWeaver based nonwarped and warped images for uninduced (0h) and induced (6h) conditions in a single gel for individual comparison of each spot. Warped image were generated automatically by overlaying two individual gels for correct matching of protein patterns. **(f),** Scatter plots to analyze gel similarities among a series of

gels. Different gels were matched automatically by the software with a reference gel (gel 1) and show a strong correlation (correlation coefficient r, 0.58 and 0.66).

3.3 Identification of proteins by mass spectrometry based approach: MS-MS analysis

All the proteins shown to alter in their expression by PML-RARα according to gel patterns were identified by MALDI-TOF mass spectrometry (MS). The peptide signature of the proteins identified in most cases was validated by MS-MS analysis using the Applied Biosystems AB4700 mass spectrometer. For example, one of the proteins identified was OP18. The MS spectra with m/z ratio for peptides generated are shown for illustration (Fig. 9a). The three monoisotopic peaks (shown as arrows in Fig. 9a) were subsequently selected for an automated MS-MS fragmentation run. For each precursor ion, a fragmented spectrum was generated which revealed the number and location of amino acids present in the peptide (Fig. 9b-d).

The identity of the precursor ions was analysed by comparison with tryptic peptides of OP18 (SwissProt accession P16949) generated in a theoretical digest with trypsin using ‘prospector.ucsf.edu’. The MS-MS 1388.67 (an OP18 tryptic peptide) produces C-terminus fragments (y-ion series) of 175.12, 272.17, 359.2, 472.29, 585.37, 698.46, 827.50 and 974.57, and N-terminus fragments (b-ion series) of 1214.64, 1117.59, 1030.56, 917.47, 804.39, 691.31, 562.26 and 414.19, which correspond to a sequence ASGQAFELILSPR of OP18 (Fig. 9c, compare with Fig. 9e). Likewise, the MS-MS 1488.74 (another OP18 tryptic peptide) corresponds to a sequence

KLTHKMEANKEN of OP18 (Fig. 9b) and the MS-MS 1052.56 corresponds to a sequence ASSDIQVKELEKR of OP18 (Fig. 9d). Using this procedure, we could clearly reveal the identity of OP18. The identity of all the proteins that were changed in expression by the fusion protein was confirmed this way (Fig.9f).

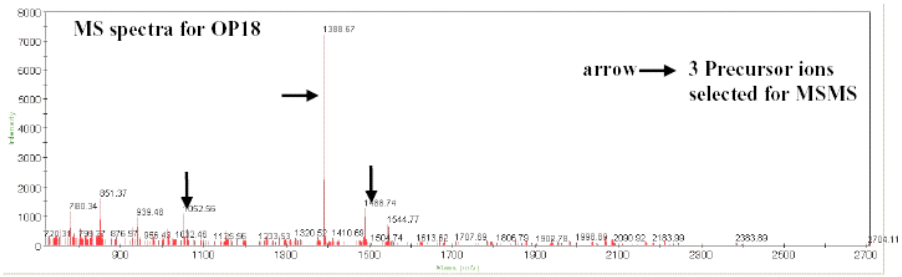
Table 2. Identified proteins that are differentially expressed by PML-RAR□-induction in PR9 cells: MALDI-TOF MS results quantified by proteom weaver and confirmed by MS-MS analysis

Protein KDa	Spot (-Zn)±SE	Score (+Zn) ±SE	Mr kDa	PI	Accession	Spot vol.	Spot vol.	Increase	Sequence coverage%
Proteins upregulated by PML-RARα									
RUFY2 Rabip4R	7	72	68.9	5.6	Q96P51	0.56±0.01	0.74±0.01	31.5	18
eIF 5A	9	63	16.6	5.08	IF5A_Hum	0.72±0.19	1.40±0.11	38.5	34
PEBP	11	66	20.4	7.18	1BD9A	0.17±0.05	0.36±0.06	56.6	37
RanBP1	17	70	23.3	5.19	S54290	0.99±0.16	1.75±0.18	61.5	27
Tubulin β-7 chain	22	114	50.1	4.78	S01713	0.29±0.02	0.58±0.12	100	25
Actin, β (fragment)	23	98	41.3	5.55	Q96HG5	0.21±0.01	2.9±0.06	128	47
HSP70	24	262	70.2	5.48	A45871	0.73±0.47	2.00±0.14	172	40
STMN (OP18)	29	132	20	5.77	A40936	1.90±0.07	2.95±0.38	55	25
HSP27	33	140	22.8	5.98	HHHU27	0.81±0.01	1.38±0.09	93.7	58
GRP75	49	153	74.0	5.87	AAA67526	2.43±0.47	4.45±0.45	116	32
ADH	53	62	36.7	6.34	AKA1_Hum	0.25±0.01	0.46±0.02	96	27
Annexin I	54	157	38.7	6.64	ANX1_Hum	0.34±0.02	0.50±0.02	68.7	44
IPYR	56	72	32.2	5.42	AAD24964	0.31±0.01	0.47±0.01	35.2	29
Ub protein ligase	59	69	17.2	6.13	JC4894	0.39±0.01	0.50±0.20	24	26
TBCA_Human	60	66	12.7	5.25	TBCA_Hum	0.48±0.02	0.65±0.01	27	40
Adenylate kinase	114	84	21.6	8.73	JC5893	0.13±0.01	0.60±0.001	117	59
PA28 α chain	140	101	28.8	5.78	A54859	0.44±0.01	0.87±0.06	113	33
PCNA	146	110	29.1	4.57	1AXCE1	0.78±0.01	1.70±0.13	50	36
ROK_Human	*	101	51.2	5.19	S43363	0.14	0.33		24
Prot. Endo.complex	*	67	26.5	4.74	S17521	0.20	0.42		19
H+- ATPase	*	101	56.5	5.26	A33370	0.68	1.10		31
CAF1	*	73	47.9	4.9	S36112	0.11	0.31		20
RNCC protein	*	90	27.2	5.09	CAB46078	0.57	0.79		34
Tropomyosin	*	177	29.2	4.75	A25530	0.70	1.40		37
PDI	*	208	56.7	5.98	JC5704	0.57	0.85		27
Proteins downregulated by PML-RARα									
Thioredoxin	1	93	11.6	4.82	THIO-Hum	1.30±0.17	0.11±0.01	-119	23
Galectin1	2	86	14.9	5.34	LEG1_Hum	1.10±0.10	0.30±0.03	-72.7	35
pOP18***	3	67	17.1	5.77	STN1-Hum	0.77±0.02	0.36±0.01	-53.2	31
hnRNPL	4	97	60.7	6.65	A33616	0.24±0.13	0.16±0.13	-33.3	21
CCT6	5	88	58.4	6.23	S48087	0.91±0.10	0.51±0.01	-43.9	18
Ly-GD1	16	75	23.0	5.1	A47742	3.29±0.01	1.70±0.01	-48.3	31
NAP1	25	85	45.6	4.36	S40510	3.35±0.40	0.63±0.07	-84.3	19
NASP	26	101	49.0	4.35	AAH03113	3.32±0.26	0.80±0.07	-69.1	28
CALR precursor	27	79	48.2	4.29	A37047	4.96±0.11	1.65±0.03	-68.7	19
DnaK mol.chap.	50	212	71.1	5.37	A27077	5.05±0.10	3.60±0.07	-32.6	34
ACP1	52	115	37.9	6.60	S58529	0.52±0.05	0.30±0.02	-44.2	42
EndP precursor	66	274	72.3	5.07	A35954	0.67±0.06	0.37±0.06	-51.7	26
Cofilin	71	66	18.7	8.22	S12632	0.65±0.01	0.05±0.02	-	31
Capping protein	91	140	33.1	5.45	G02639	1.14±0.20	0.85±0.16	-23.8	66
Prolyl hydrox.	92	224	57.1	4.76	CAA28775	1.6±0.00	0.80±0.00	-50.0	44
Rib. Prot. RS	97	82	32.9	4.79	A31233	0.67±0.30	0.90±0.03	-	37
Esterase10	115	93	31.5	6.54	Q9BVJ2	0.46±0.07	0.18±0.01	-32.2	23
Prot. α type	117	140	29.6	6.15	AAH2577	0.40±0.07	0.26±0.07	-46.7	31

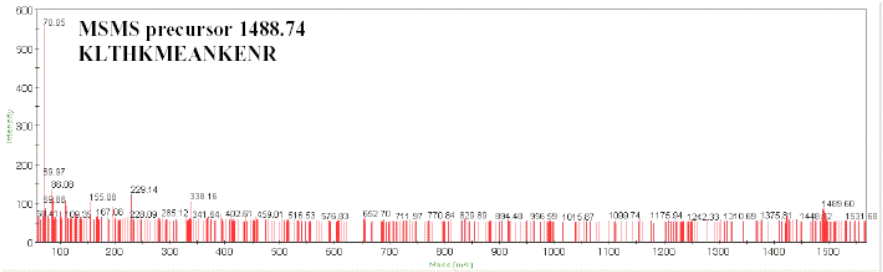
Pin1	134	94	17.9	7.85	1AK4B	1.02±0.11	0.27±0.05	-62.5	39
LBP	148	104	31.8	4.84	CAA43469	0.78±0.00	0.32±0.00	-58.9	43
ERLP 28	*	81	29.0	6.77	T09549	1.3	0.42	-67.8	28
RBP reg.subunit	*	69	20.1	6.33	014805	1.5	0.46	-69.3	34

*** Phosphor-isomer (Ser63) of OP18

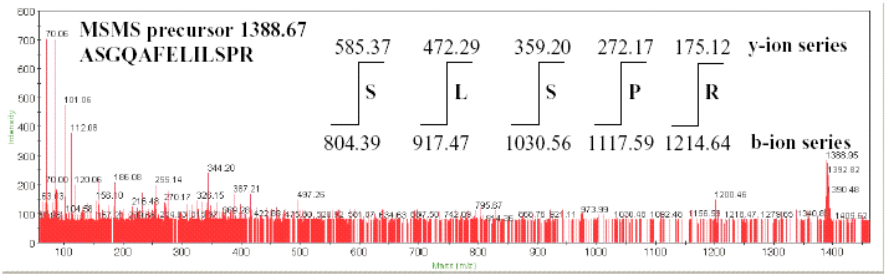
(a)



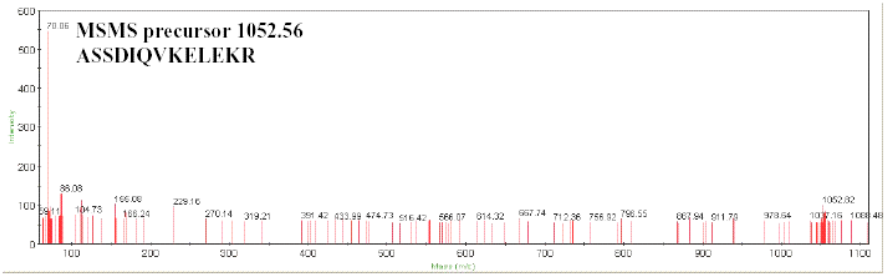
(b)



(c)



(d)

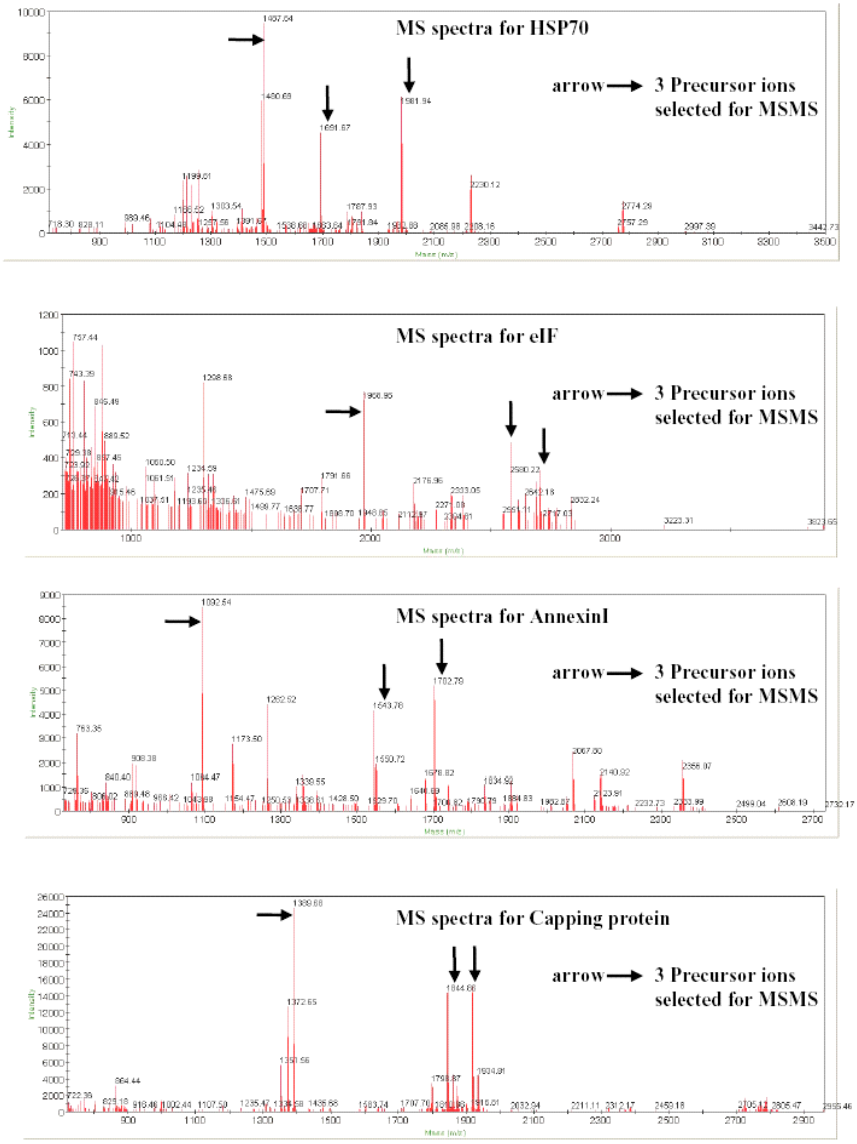


(e)

ASSDIQVKELEKR***ASGQAFELILSPR***SKESVPEFPLSPPKKK
DLSLEEIQKKLEAAEERRKSHEAEVLKQLAEKREHEKEVLQ
KAIEENNNFSKMAEE***KLTHKMEANKENR***EAQMAAKLERL
REKDKHIEEVRKNKESKDPADETEAD

Figure 9: Representative of MALDI-TOF MS and MS MS spectra: (a) the spectra was obtained from P/R9 cells for OP18 and **(b, c, d)** the MS MS spectra for OP18 peptide are detected from 3 precursor ion selected from MS spectra and **(e)** tryptic peptide of OP18 (P16949-OP18 sequence) generated in theoretical digest with trypsin using prospector.ucsf.edu

(f)



(f) the spectra was obtained from P/R9 cells for HSP70, eIF, Annexin I and Capping protein were shown with the molecular weight calculation (m/Z).

3.4 PML-RARα induction in PR9 cells is associated with changes in cell cycle associated proteins

When compared with control PR9 cells (uninduced), changes in proteins associated with cell cycle regulation such as increased levels of oncoprotein18 (OP18), proliferating cell nuclear antigen (PCNA), heat shock protein70 (HSP70) and glucose regulated protein75 (GRP75), and decreased expression of peptidyl-prolyl isomerase (Pin1) are seen in PML-RARα expressing PR9 cells (Fig. 10a-d upper panels and Table 2). OP18, a phosphorylation responsive microtubule destabilizing protein (Kuntziger et al., 2001; Sobel, 1991) and PCNA, an essential auxiliary protein for DNA replication and repair processes (Kontopidis et al., 2005) have defined roles in cell proliferation, and are therefore closely involved in cell cycle. HSP70 shows elevated levels in proliferating mammalian cells and a cell cycle dependent expression (Helmbrecht et al., 2000). Pin1, an important mitotic regulator is known to be overexpressed in malignant cells and its expression is also cell cycle-phase dependent (Xu and Manley, 2004). Thus PML-RARα induction brings about overlapping functions essential for cell cycle and mitosis regulation. As proof-of-principle, we validated our findings by performing immunoblots on OP18, HSP70, GRP75, Pin1 (Fig. 10a-10d bottom panels) and PCNA (Table 2). No significant change in the expression of OP18 or other analysed proteins was observed in the control U937 induced with zinc (Fig.10f bottom panel).

A notable observation seen in PML-RARα induced cells compared with control is a presence of more than one protein spot for OP18, one of which is increased (Fig. 8c Spot 29) and the other decreased by the fusion protein (Fig. 8c Spot 3). This observation was confirmed by 2D-western blotting (Fig. 10e). Presumably one of the spots corresponds to a phosphorylated form of OP18 (pOP18) having a lower isoelectric point (PI). Thus, PML-RARα may regulates OP18 at the level of a post-translational modification (phosphorylation). As can be seen, in fact this is the case. OP18 in other settings has been reported to be regulated by phosphorylation (Beretta et al., 1993; Leighton et al., 1993; Marklund et al., 1996). Based on the weight of these findings, we selected OP18 as a representative of the cell cycle network to characterize and understand further the role of PML-RARα in this cellular process.

3.5 Retinoic acid degrades PML-RARα expression and overcomes the increased OP18 expression

Some proteins that function in cellular defense (e.g; PA28), structural organization (e.g.; CapZ) or signal transduction (e.g; Ly-GD1), known to be upregulated by RA in NB4 cells, are downregulated by PML-RARα (Table2). Likewise, the proteins that function during the initiation and elongation stages of the protein synthesis mechanism (e.g.; eIFs), and the chaperones (e.g; HSP70), which are downregulated by RA in NB4 cells (Harris, Ozpolat, et al. 2004), are upregulated by PML-RARα (Table2). These data demonstrate that

effects on the levels of protein in PR9 cells upon zinc induction are specifically as a result of the fusion protein. Since, retinoic acid (RA) is known to degrade PML-RARα (Yoshida, Kitamura, et al. 1996) and induce differentiation, we first investigated the effect of RA on OP18 expression in PR9 cells. A western blot analysis with anti-OP18 antibody revealed increased OP18 expression upon PML-RARα induction (Fig. 11 upper panel, lane 2) compared with uninduced condition (lane1), consistent with our previous result. However, RA alone or in combination with zinc markedly decreases OP18 expression (Fig. 11 upper panel, lanes 3-5) below the basal level (lane 1). Reprobing the same blot with anti-RARα antibody revealed that there was no PML-RARα (normally induced by zinc treatment, Fig. 11, lane 2) in cells treated with RA (Fig 11, lane 3-5 bottom panel), which is consistent with reports showing degradation of PML-RARα with RA. RA in this cell system could induce differentiation as evidenced by CD11b expression, a marker of myeloid differentiation (data not shown). Thus enhanced OP18 expression in this cell system reflects a poor differentiation status. To ensure that the expression of PML-RARα does not fall to undetectable levels during the course of the experiment, we performed a western blot analysis for the fusion protein expression at different time points (data not shown). Furthermore, increased OP18 protein levels highly correlate with OP18 mRNA levels in PR9 cells (Fig 12) and clinically relevant settings from patients with acute myeloid leukemia.

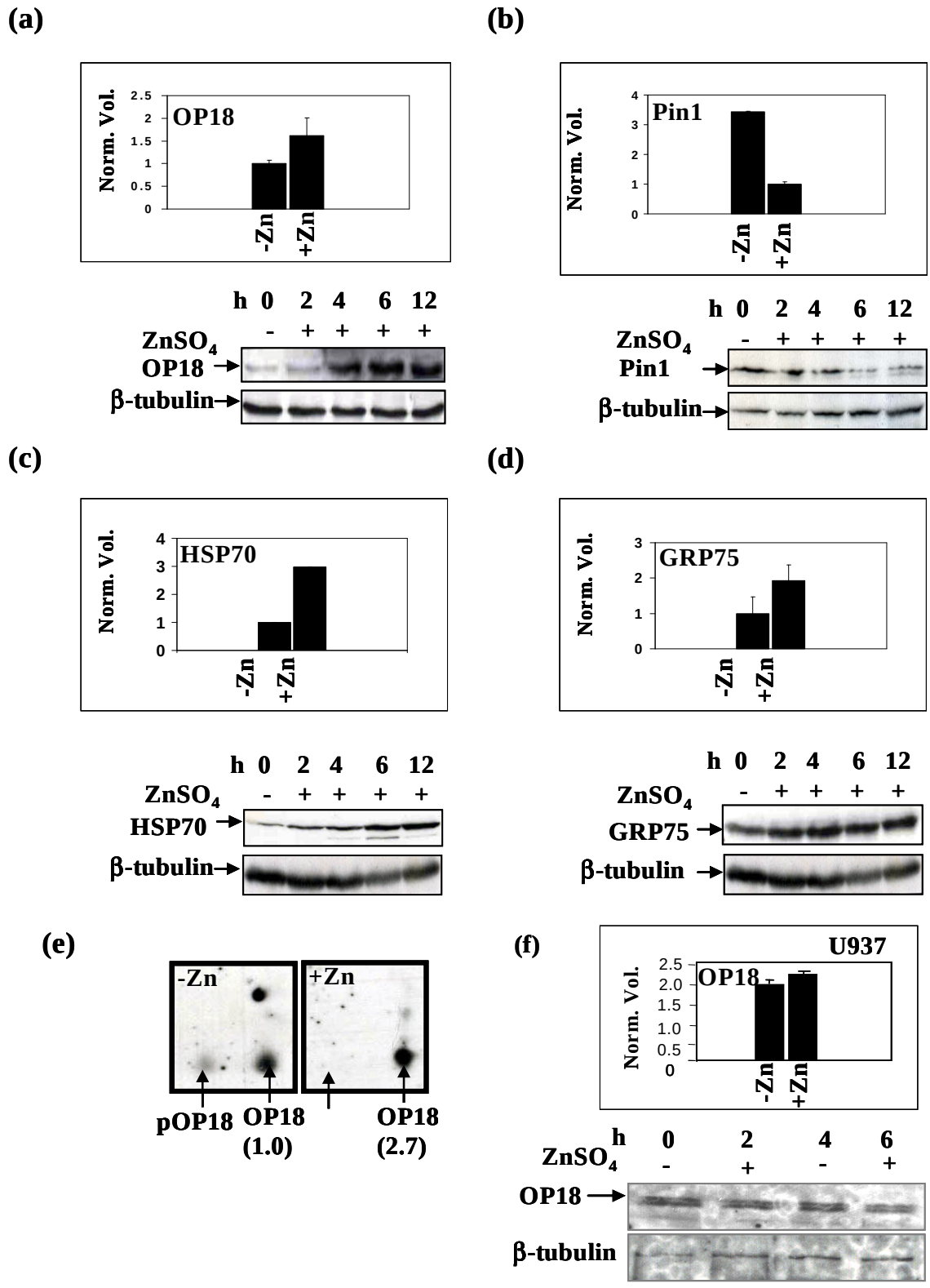


Figure 10: Comparison of 2-D gel analysis results and western blotting: (a, b, c, d) induction of PML-RARα with zinc (100μM $ZnSO_4$) increased expression of OP18, HSP70,

GRP75 and decreased expression of Pin1. Each histogram (a-d upper panel) represents normalized spot volume shown in table2. **(e)** 2D Western blot for OP18 from PR9 lysates after IEF at pH 3-10 and 2D gel electrophoresis, the proteins on the gel were transferred onto PVDF membrane and blotted for OP18 using anti-OP18 antibody (sc-20796 Santa Cruz). The numbers underneath denote densitometry values. **(f)** Zinc induction in U937 cells leads to no significant change in the expression of the protein (lower panel).The histogram represents normalized spot volume for OP18 expressed as mean standard deviations for OP18 from 2D gels (Upper panel)

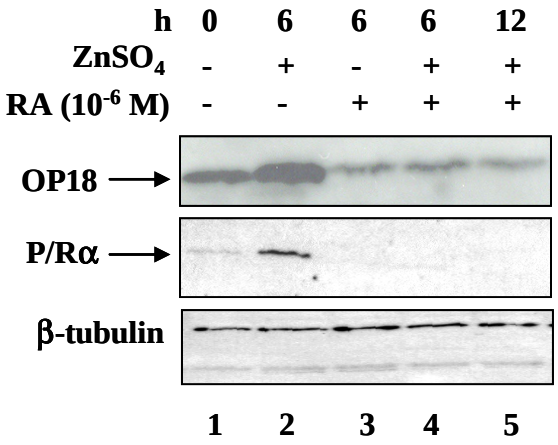


Figure 11: Retinoic acid overcomes expression of PML-RARα and OP18: Immunoblot for OP18 using anti-OP18 antibody (C-20, Santa Cruz) in PR9 cells before and after treatment with zinc sulfate and RA at different time points (Uper panel). The blot was stripped and reprobed with anti-RARα for the detection PML-RARα (Middel panel) (sc-551 Santa Cruz) and β-tubulin (lower panel).

3.6 Primary APL patient t(15;17) cells and PR9 cells express high levels of OP18 mRNA as compared to normal bone marrow

Using microarray (affymetrix), we examined the OP18 mRNA expression levels in clinically relevant settings from patients with myeloid leukemia as a first step towards deciphering its role in APL. The data revealed increased mRNA expression of OP18 PR9 cells after zinc induction and in patients with t(15;17) compared to normal bone marrow and other types of leukemia

(Fig.12 crossed bars). Interestingly, the expression of OP18 mRNA is elevated in blasts from patients with a complex aberrant karyotype, which have a prognosis similarly poor to the prognosis of APL before the advent of RA, suggesting its prognostic significance. The increased OP18 mRNA expression cannot be a mere reflection of the heterogeneity of leukemic samples since, increased OP18 expression is also observed in our settings using a common, clonal cell background, U937 (PR9) when PML-RARα is induced (Fig. 10e 2D western). To investigate the transcriptional effect of PML-RAR□on the *OP18* promoter, we performed a transient co-transfection assay in fibroblast 293T cells with *OP18* luciferase reporter, a kind gift from Dr. Herrera (Polzin, R. G et al., 2004) and either PML-RARαRARαor PML. The fusion protein PML-RARα (Fig. 12b, histogram 2-3) and not PML (histogram 5) or RARα alone (histogram 6) specifically activated the *OP18* luciferase reporter about 3-fold over the vector only lane (histogram 1) at the highest concentration used (Fig. 12b). Importantly, the fusion protein mediated activation of *OP18* was retinoic acid sensitive (Fig. 12b, histogram 4). Thus, PML-RARαis a potent activator of the *OP18* promoter.

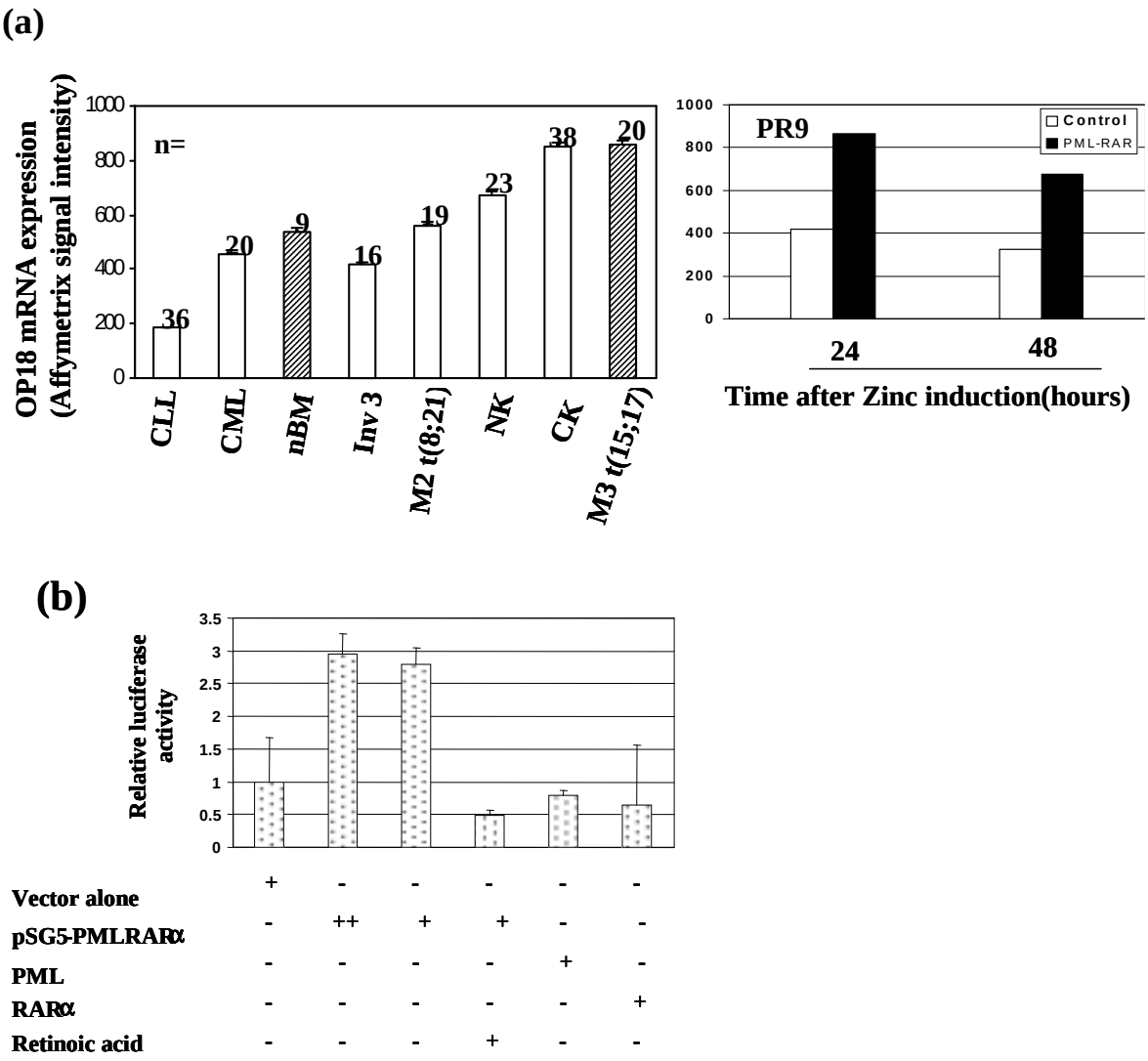


Figure 12: Microarray (affymetrix) data revealed increased mRNA expression of OP18 in patients with t(15;17). (a) Affymetrix data showing the mRNA expression values of OP18 in different patient samples, n on top of bars represent the number of patients evaluated in each subgroup. **(b)** PML-RARa is a potent activator of the OP18 promoter. OP18 luciferase activity in the presence of different amounts (0.2, 0.1 µg) of PML-RARa, RARa, or PML expression plasmids. Cells transfected with PML-RARa were also treated with 1µM RA 6h post-transfection

3.7 PML-RARα induces a G1 to S-phase transition

We have demonstrated that PML-RARα induction in PR9 cells leads to increased OP18 protein levels. OP18 has been shown to have a role in cell cycle and mitotic exit (Brattsand et al., 1994; Gavet et al., 1998; Lane and

Ley, 2005; Larsson et al., 1999; Marklund et al., 1994b; Misek et al., 2002). The observation that PML-RARα induces OP18 expression, led us to speculate about the role of PML-RARα in cell cycle and mitotic exit. So far PML-RARα has been shown to block differentiation. This is the first evidence showing its role in cell cycle and mitotic exit. We performed cell cycle, mitotic index and immunofluorescence experiments to understand the role of PML-RARα in the cellular processes mentioned above. Our results revealed that the proportion of cells in the S-phase significantly increased in induced condition as compared to the uninduced condition at 12h and 24h commitment period. On the other hand, the proportion of cells in the G2/M-phase was reduced in the induced condition as compared to uninduced condition (Fig. 13a). The experiments using synchronized cells revealed similar results. When PR9 cells were serum starved (0.5% FBS instead of normal 10%) for 24h, most of the cells became quiescent. Consequently, the proportion of cells in the G0/G1-phase increased dramatically (Fig. 13b). When the cells are induced for PML-RARα at 24h commitment period (synchronized condition, Fig. 13b right panel), the proportion of cells in the S-phase increased in the induced condition compare to uninduced condition, whereas the proportion of cells in the G2/M-phase drastically decreased in induced condition compared to uninduced condition. At this commitment period, the cells in PML-RARα induced condition had already exit the G2/M-phase to enter a new cycle compared to uninduced condition (Fig. 13b, compare 24h histograms). The

data from three independent experiments showing the P value are shown as histograms (Fig. 13c). U937 cells under similar conditions were used as Zinc control (data not shown). We observed no significant toxicity of PML-RARα in PR9 cells, consistent with a recent report by Lane *et al* (Lane & Ley 2005). Trypan-blue staining showed a significant increase in cell number under induced condition as compared to the uninduced condition observed over a period of three days (data not shown). These data indicate that even though the cells were arrested, PML-RARα gives them a survival advantage by allowing them to divide faster, exit mitosis and enter a new cell cycle.

3.8 PML-RARα promotes mitotic exit

Since, it is not possible to differentiate the G2 and M-phase accumulation by propidium iodide staining; we substantiated the hypothesis that PML-RARα commits the cells to exit mitosis faster by mitotic index experiments.

When the cells are induced for PML-RARα, the proportions of mitotic cells were reduced to less than a half (mitotic index 11) as compared to the uninduced control cells (mitotic index 23, Fig. 14 compare DAPI panel). PML-RARα induced cells exhibit pronounced changes in the mitotic spindle structures (composed of tubulin polymers) characteristic of the depolymerised non-mitotic state (Fig. 14 compare tubulin panel). Furthermore, immunofluorescence staining of OP18 shows increased expression upon PML-RARα induction (Fig. 14 compare OP18 panel). Staining with MPM2, a marker for mitotic cells further supported our data (data not shown).

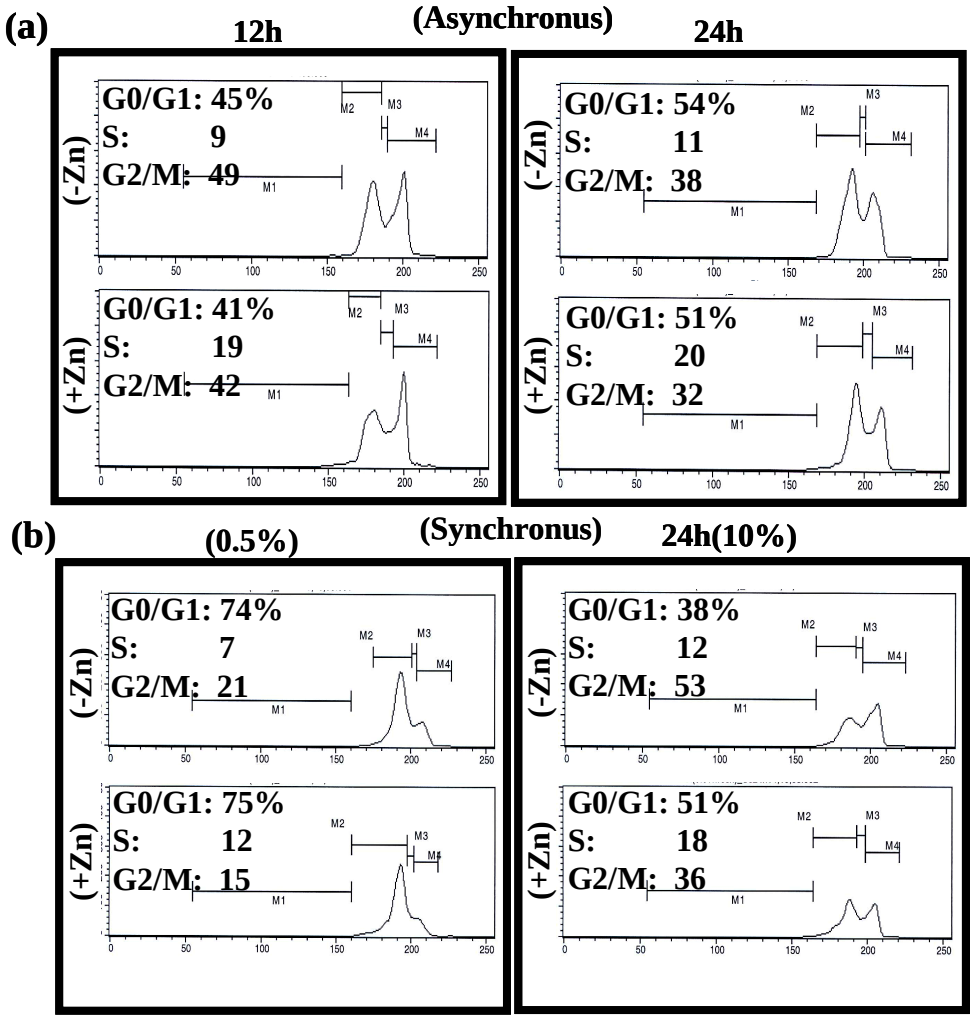
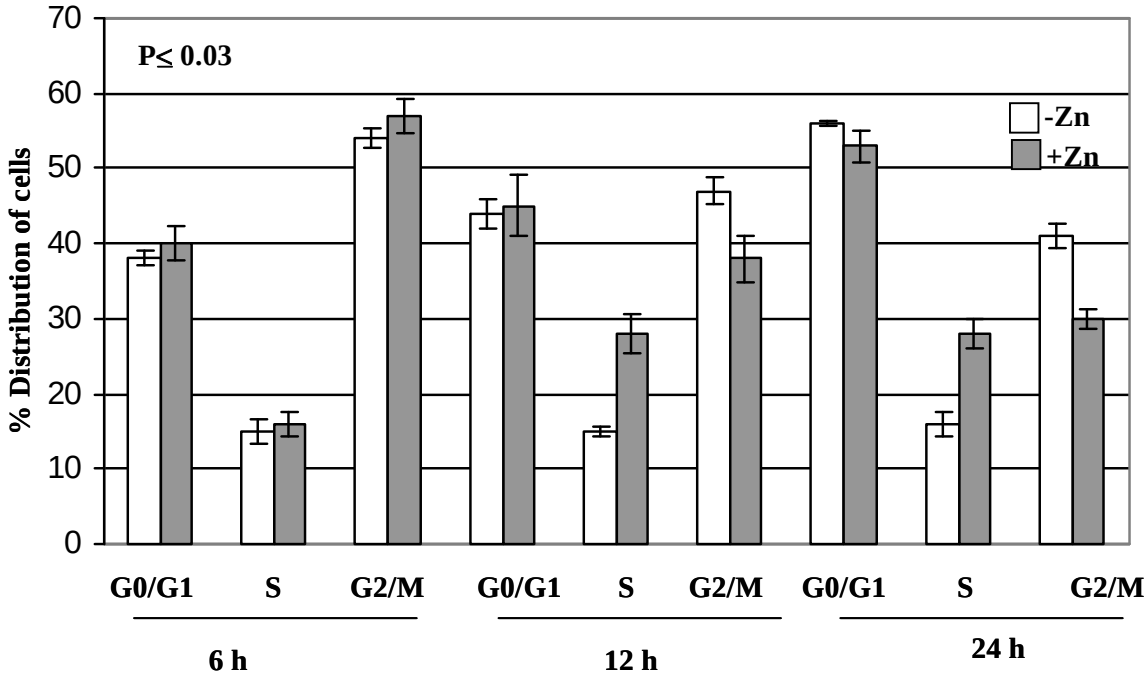


Figure 13: PML-RARα induces a G1 to S-phase transition: (a&b) Cell cycle distribution of PR9 cells before and after PML-RARα induction at the indicated time points under asynchronous and synchronous conditions. Cells were serum starved (0.5% FBS instead of normal 10%) for 24h and the following day supplied with media containing 10% FBS. PML-RARα was induced by addition of Zinc and the cell cycle phases were analysed at the indicated time points. Cells were washed with PBS and stained for DNA with propidium iodide (PI) and analysed for different phases by FACS analysis.

(c)



(c) Cell-cycle analysis was performed using propidium iodide staining. Median values are shown and error bars depict standard deviation from 3 independent experiments at the indicated time points. The P value was calculated by using student´s t- test by comparing the means of two different conditions in each experiment.

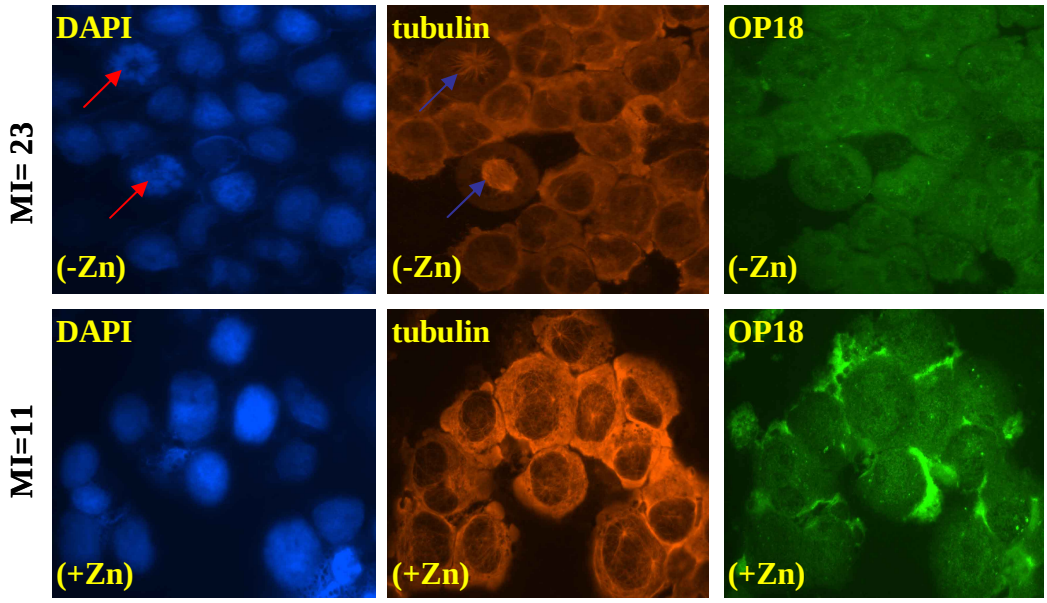


Figure 14: PML-RARα promotes mitotic exit for the cells to enter a new cell cycle. Indirect immunofluorescence staining for OP18 and α-tubulin using conjugated antibodies. Cells before and after PML-RARα induction were cytocentrifuged on glass slide cover slips, fixed with methanol/acetone, permeabilized with 0.3% TritonX , stained with respective antibodies (Alexa Fluor, Molecular Probes) and DAPI. The morphology of the cells was visualized under fluorescence microscope (100X). Mitotic index (MI) represents the quantification values of mitotic cells by counting 150-200 cells.

3.9 Induction of PML-RARα inhibits the expression of cell cycle inhibitors (p21) and increases the CDk2/CDk4 kinase activities

To elaborate on the role of PML-RARα in the cell cycle, we next investigated the effect of PML-RARα induction in PR9 cells upon cell cycle regulatory proteins. p21, a cell cycle inhibitor and CDK2/CDK4, a cyclin-dependent kinase system were an obvious choice in line with their role in G1 to S-phase transition. A decrease of the expression of p21 in PML-RARα induced condition was observed (Fig.15 a). As could be expected, PML-RARα decreases the expression of p21, a cyclin dependent kinase inhibitor (CDKIs) (Drdova and Vachtenheim, 2005; Liu and Lozano, 2005) which is involved in mediating growth arrest in the G1 phase of the cell cycle (Lowenheim et al., 2005). At the same time CDK2/CDK4 kinase activities were increased (Fig.15b). These observations suggest that decreased level of CDK inhibitor (p21) and the increased activities of CDKs (CDK2/CDK4) contribute to G1 to S-phase transition induced by PML-RARα.

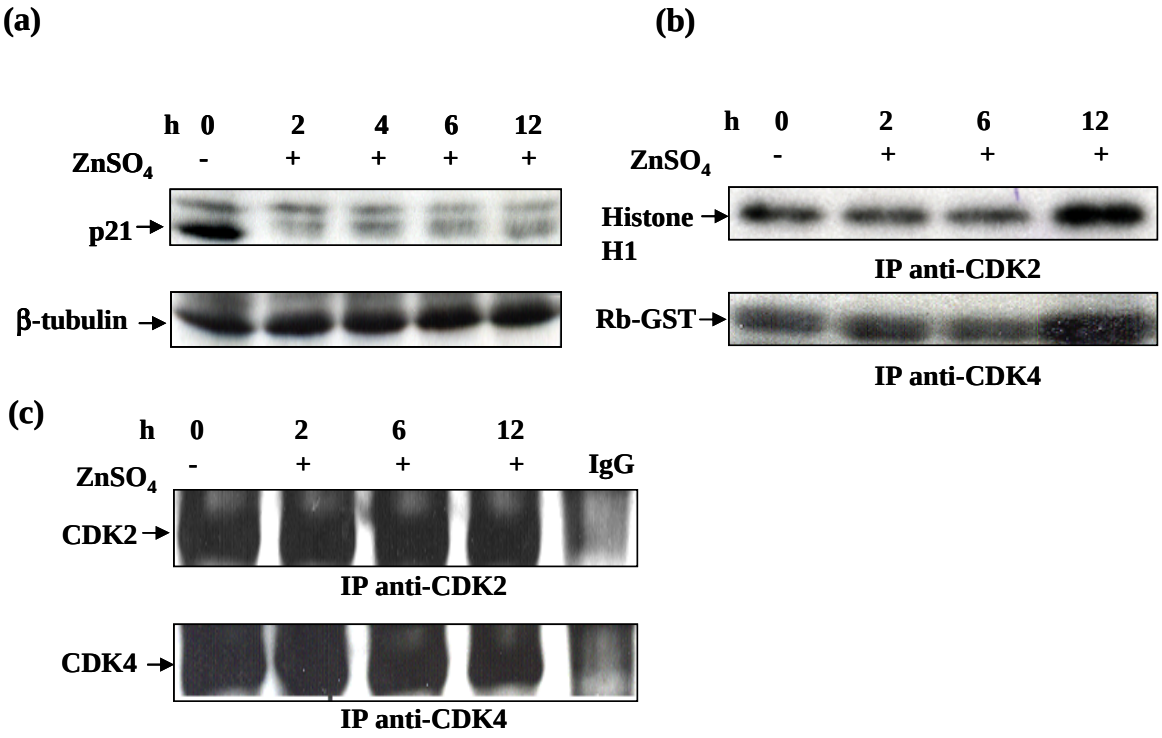


Figure 15: Induction of PML-RARα inhibits the expression of cell cycle inhibitors p21 and increases the CDK2/CDK4 kinase activities: (a) immunoblot analysis for p21 from whole cell lysates of PR9 cells before and after PML-RARα induction and **(b)** *In vitro* kinase assay for CDK2 and CDK4. Whole cell lysates were prepared from PR9 cells at the indicated time points and CDK2/CDK4 was immunoprecipitated using respective antibodies. Histone H1 and Rb-fusion protein were used as substrates for CDK2 and CDK4 respectively, in the *in vitro* kinase reaction. **(c)** an Immunoblot IP control for CDK2 and CDK4.

3.10 Knocking down OP18 expression by RNA interference in PR9 cells prevents PML-RARα induced mitotic exit and G1 to S-phase transition

PML-RARα was clearly able to promote mitotic exit and G1 to S-phase transition in PR9 cells, and a correlation between mitotic exit and OP18 expression was observed. Therefore, to assess specifically for the involvement of OP18 in the PML-RARα mediated cell cycle effects, we used RNA interference technology to suppress OP18 expression in PR9 cells. PR9 cells were transfected with two different small interfering RNAs (siRNA) and 18-

24h after transfection; cells were treated with zinc to induce PML-RARα. As shown as Fig.16, cells that had been transfected with siRNA against OP18 and induced for PML-RARα (Fig. 16a, left bottom panel) were unable to exit G2/M-phase compared to the control (Fig. 16a, left top panel) at 48h. To ensure specificity of the OP18 siRNA in its effects on cell cycle, a scrambled sequence was used as a non-silencing control. No significant change in cell cycle parameters was observed with non-silencing control (Fig. 16b). The data from three independent experiments with the P values are shown as histograms (Fig. 16c). These data indicate that siRNA against OP18 is able to block PML-RARα mediated mitotic exit. Most interestingly, we observed that siRNA against OP18 at early time points of 12h (Fig. 16a left panel) could not prevent PML-RARα. mediated mitotic exit effects. We reason that this is attributable to the long half-life of OP18 (approximately 14h), which is supported by the observation that the expression of OP18 is reduced completely only 48h after transfection of siRNA (Fig. 16d). Taken together, these observations clearly demonstrate that under our experimental settings mitotic exit in PR9 cells requires the presence of the PML-RARα. fusion protein and a consequent increase in OP18 expression. The transfection efficiency of siRNA transfected cells measured was greater than 80% using rhodamine-conjugated siRNA (Fig. 16e). The data from three independent experiments showing P value are showen as histogram.

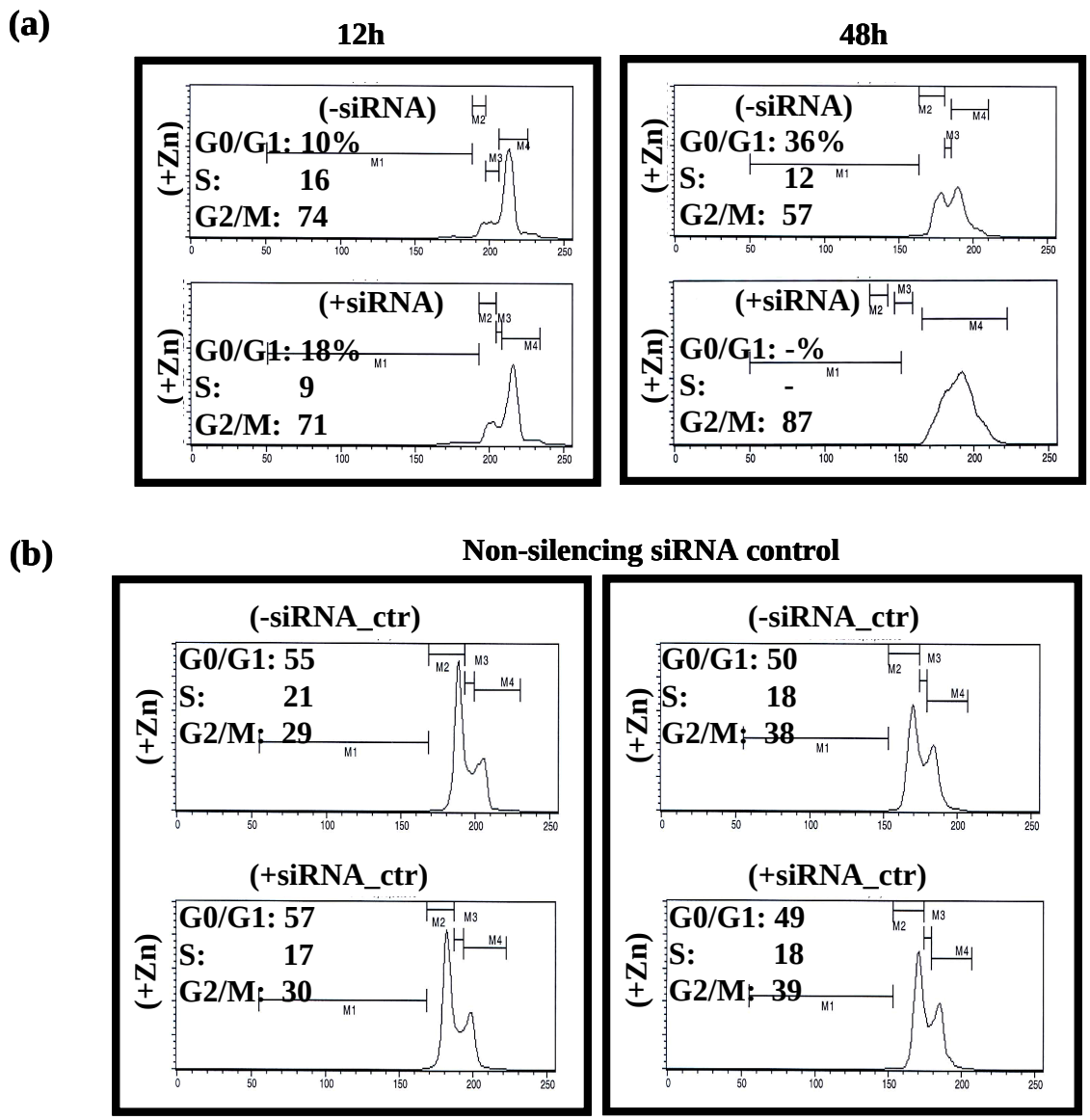
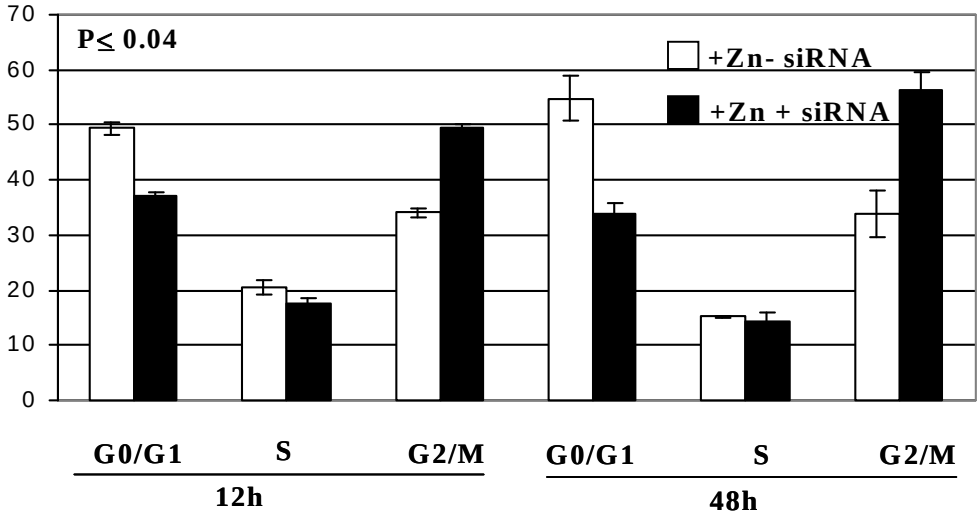
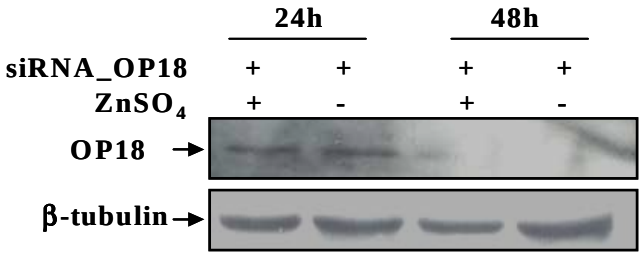


Figure 16: siRNA against OP18 overcomes the effects of PML-RARα. Cell-cycle analysis was performed using propidium iodide staining at 12h and 48h. **(a)** RNA interference experiment with cell cycle as a read-out. **(b)** Non-silencing siRNA does not overcome PML-RARα mediated cell cycle effects. PML-RARα was induced 18 hours post transfection and analyzed for cell cycle profile at different time points of PML-RARα induction.

(c)



(d)



(e)

(c) Cell-cycle analysis was performed using propidium iodide staining at 12h and 48h. Median values are shown and error bars depict standard deviation from 3 independent experiments. The P value was calculated by using student´s t- test by comparing the means of two different conditions in each experiment. **(d)** Immunoblot for OP18 after 48 hours of siRNA transfection Shown is a representative experiment of one siRNA. **(e)** Rhodamine-conjugated siRNA were used for transfection efficiency.

3.11 PML-RARα reduces OP18 (Ser63) phosphorylation levels in PR9 cells as well as t(15;17) carrying AML patient samples

The data presented thus far indicate that PML-RARα mediates its cell cycle effects at the least, through its effects on OP18 level. Previous reports have shown the role of OP18 phosphorylation in cell cycle and mitotic exit (Beretta et al., 1993; Leighton et al., 1993; Steinmetz et al., 2001). It has been shown that phosphorylation of OP18 on Ser63 which is in the α-helical region of OP18 disrupts its helical conformation thereby suppressing its binding to tubulin (Marklund et al., 1996). Most interestingly, this hypothesis favored our previous assumption that the effects of PML-RARα could be regulated at the level of posttranslational modification, which was based on the observation that the fusion protein led to a decrease in an isoform of OP18.

We have observed a decrease in phosphorylation of OP18 upon PML-RARα induction (Fig. 8a, spot3). To confirm this observation, we performed a normal and a 2D-western blotting using phosphospecific (Ser63) anti-OP18 antibody. Our results revealed a significant decrease in the level of OP18 phosphorylated on residue Ser63 by PML-RARα in PR9 cells both by normal (Fig. 17a) and a 2D-western analysis (Fig. 17b) consistent with 2D gel results. There was no significant effect on the level of Ser63 phosphorylation in U937 cells used as zinc control (Fig. 17a, bottom panel). A similar result was obtained from AML M3 (APL) patients compared to normal bone marrow (nBM) and other AML samples (Fig. 17c). Furthermore, the induction of

PML-RARα led to a dramatic increase in the expression of okadaic acid sensitive phosphatase PP2A and also a slight increase in the expression of phosphatase PP2BA (Fig. 17d). PML-RARα could no longer induce PP2A when the cells were treated with 0.5 µM okadaic acid prior to the treatment of zinc to induce PML-RARα (Fig. 17d, compare lane 1 and 2 right panel). Under these conditions (0.5 µM okadaic acid and PML-RARα), we however, observed increased level of Ser63 phosphorylation of OP18 compared with no okadaic acid condition (Fig. 17e, compare lane 2 and 3). Taken together, these data suggest that reduced phosphorylation level of OP18 by PML-RARα involves the Ser/Thr phosphatase pathway. Thus, PML-RARα not only initiates its effects (in this case cell cycle) at the protein level of OP18 but also at the level of post-translational modification of OP18 levels (in this case phosphorylation).

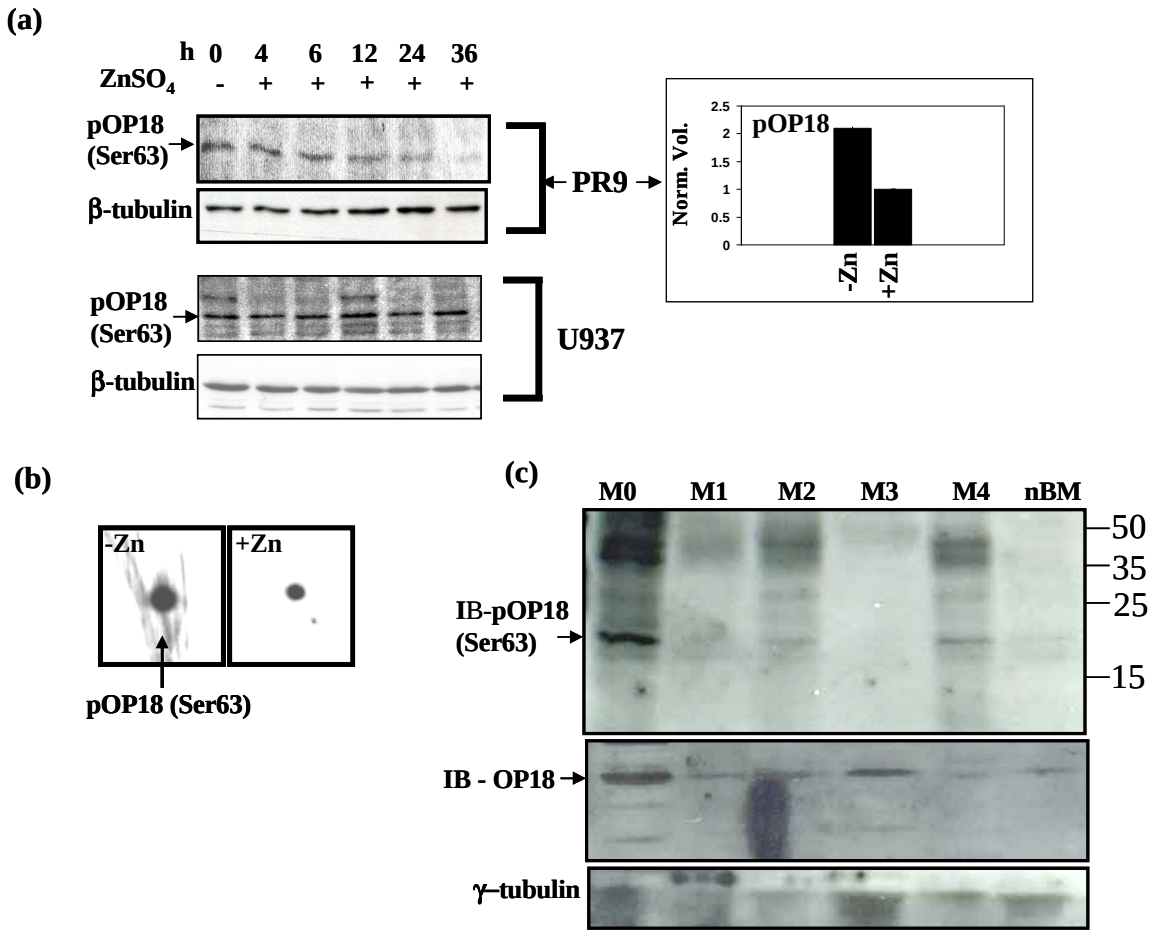
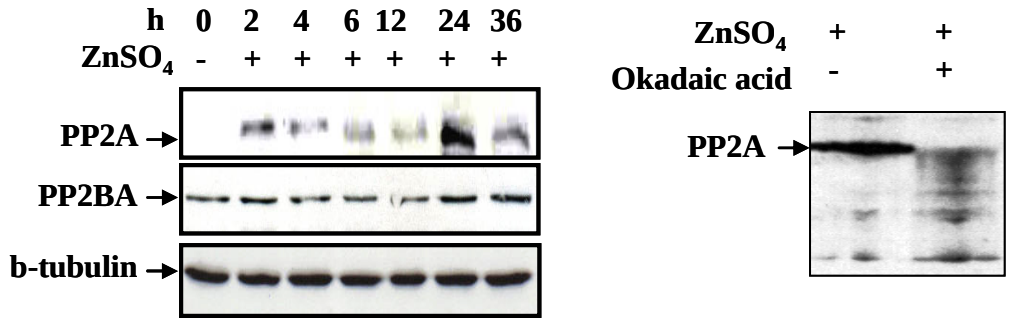
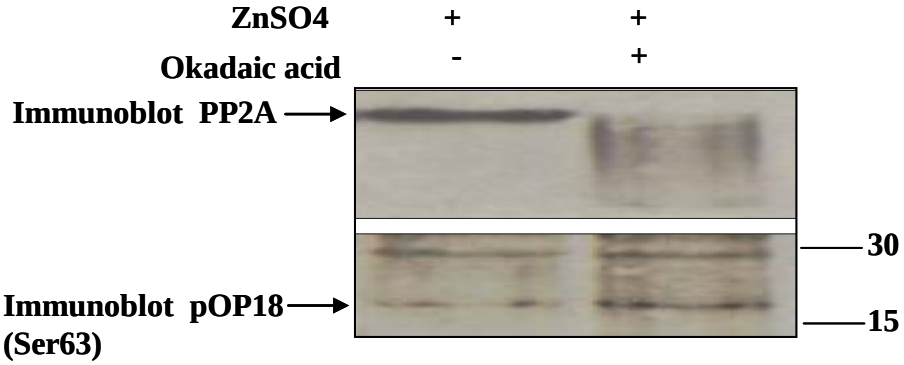


Figure 17: PML/RARα dephosphorylate OP18 at Ser63. (a), Comparison of expression of pOP18 defined by 2-dimensional gel analysis (**a**, upper right panel) and immunobloting for phospho-OP18 (Ser63) using phospho-Ser63 specific antibody (sc-12949, Santa Cruz) before and after PML-RARα induction in PR9 cells and U937 cells as zinc control (**a,** left upper and lower panel). **(b),** 2-D immunoblotting for phospho-OP18 (Ser63) in PR9 cells after six hours of induction. **(c),** Immunoblot analysis in AML patient samples for phospho-OP18 (Ser63) and OP18. nBM stands for normal bone marrow, and M0, M1, M2, M3 and M4 represent different AML FAB subtypes.

(d)



(e)



PML-RARα decreases the expression of phosphatase PP2A and PP2BA. **(d),** Immunoblot for PP2A and PP2BA before and after PML-RARα induction in PR9 cells (left panel), and in the presence and absence of okadaic acid (right panel). **(e),** Immunoblot for PP2A and phospho-OP18 (Ser63) after treatment with zinc sulfate and okadaic acid. 0.5 μM okadaic acid was used to inhibit the phosphatases 1-2 h before zinc sulfate treatment prior to the induction of PML-RARα.

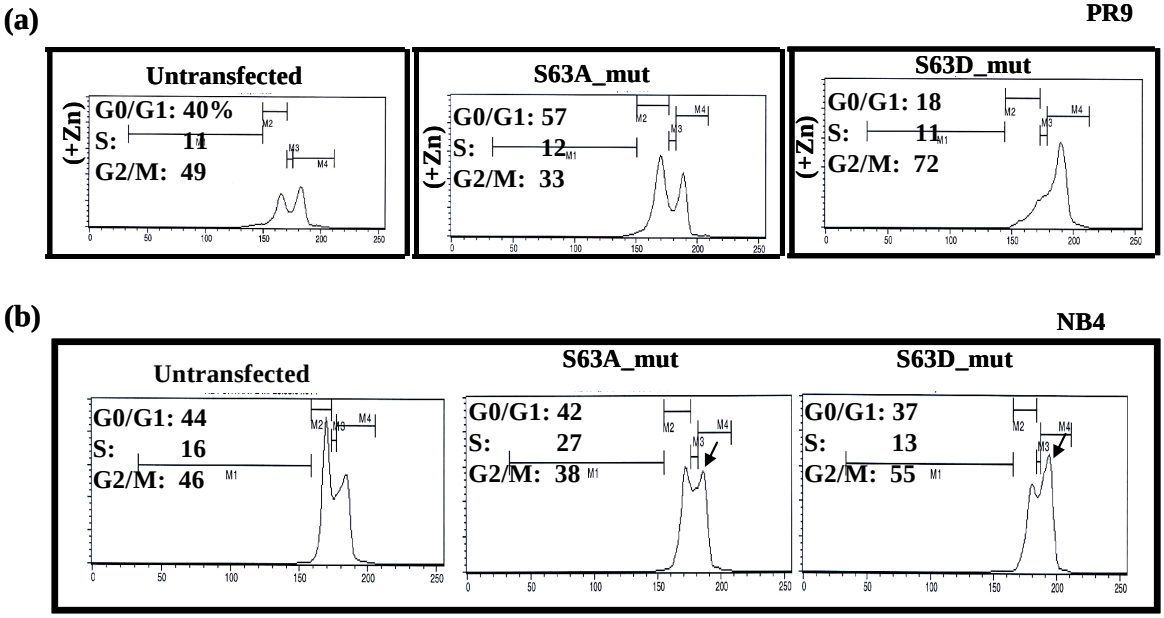
3.12 Reduced phosphorylation of OP18 by the PML-RARα fusion protein at a single Ser63 residue is sufficient for its mitotic exit effects

To further characterize the role of OP18 (Ser63) dephosphorylation in APL, we generated a phosphorylation-deficient mutant Serine to Alanine (S63→A63; S63A) and a constitutively phosphorylated mutant Serine to Aspartic acid (S63→D63; S63D). Overexpression of these mutants in PR9

cells had profound but opposite effects on cell cycle and mitotic exit events. Overexpression of phosphorylation-deficient mutant (S63A) could mimic the effects of PML-RARα on mitotic exit whereas the constitutive-phosphorylation mutant S63D resulted in a mitotic block at 12h commitment periods. Comparing the data from untransfected PR9 cells (Fig.18a left panel) with S63A transfected cells (Middle panel) clearly demonstrates that the phosphorylation-deficient mutant mimics the effects of PML-RARα (leads to mitotic exit). In contrast, results from PR9 cells transfected with a constitutively phosphorylated mutant (S63D) revealed the opposite. The proportion of cells in the G2/M-phase was significantly higher (Fig.18a right panel) in S63D transfected cells at 12h commitment period. Similar results were obtained at 24h commitment period. The data from three independent experiments with P value are shown as histograms (Fig.18d). Thus, constitutively active mutant of OP18 in PR9 results in a mitotic block in contrast to phosphorylation deficient mutant, which leads to a mitotic exit. In other words, presence of the constitutive phosphorylation of OP18 is able to prevent PML-RARα mediated mitotic exit. These data clearly indicate that dephosphorylation of OP18 at Ser63 is necessary and sufficient for PML-RARα mediated mitotic exit since the constitutively active mutant is able to overcome PML-RARα effects. Mitotic index measurements showed similar results using S63A-mut compared to S63D-mut upon induction of PML-RARα (data not shown).

To rule out cell line specificity and overcome the potential disadvantages of the otherwise simplistic model system, in its feasibility to match the clinical situation, we confirmed our data in APL cell line NB4 (Fig. 18b) as well as APL patient samples (Fig.18c). In a similar manner as described for PR9 cells, NB4 cells were transfected with the mutants and cell cycle parameters analyzed. As it is shown in Fig.18b, NB4 cells transfected with S63A-mut exhibit an increased proportion of cells in the S-phase and a decreased proportion of cells in the G2/M-phase (Fig.18b middle panel) compared with untransfected control (Fig. 18b left panel). In contrast, NB4 cells transfected with S63D-mut exhibit an increased proportion of cells in the G2/M-phase (Fig.18b right panel) compared with untransfected control (Fig. 18b left panel). These effects are similar to those observed for PML-RARα in PR9 cells. This is not surprising given the fact that untransfected NB4 cells (which have PML-RARα translocation) would be expected to have less proportion of cells in the G2/M-phase as proposed by our hypothesis. Our results demonstrate a proof-of-principle. Our observations in this study point to the fact that cell cycle and mitotic exit effects are specific for the fusion protein studied, irrespective of the cell line models used. To extend our hypothesis from APL cell line model systems to the primary APL cells, we took advantage of the nucleofection technology (AMAXA) to transfect S63A-mut and S63D-mut in patients with newly diagnosed disease. The cell cycle profile was evaluated 12h post-transfection. We observed that untransfected

and S63A-mut transfected primary APL cells show a very few proportion of cells in the G2/M-phase (18c, left and middle panel). Contrary to this, S63D-mut transfected primary APL cells show a greater proportion of cells in the G2/M-phase (Fig.18c, right panle) giving a further support to our hypothesis. To rule out the possibility that the cell cycle alterations might be due to zinc effects, we show that zinc induction has no significant effect on the cell cycle in U937 cells used as control under our experimental settings compared with PR9 cells (data not shown). Taken together, our data demonstrates that OP18 is an important downstream target of PML-RARα and indicates that phosphorylation at Ser63 is not sufficient to explain PML-RARα effects on mitotic exit but decreased level of OP18 phosphorylation at Ser63 plays an important role in the fusion protein mediated effects on cell cycle since, the constitutively active Ser63 OP18 mutant is able to prevent the effects.



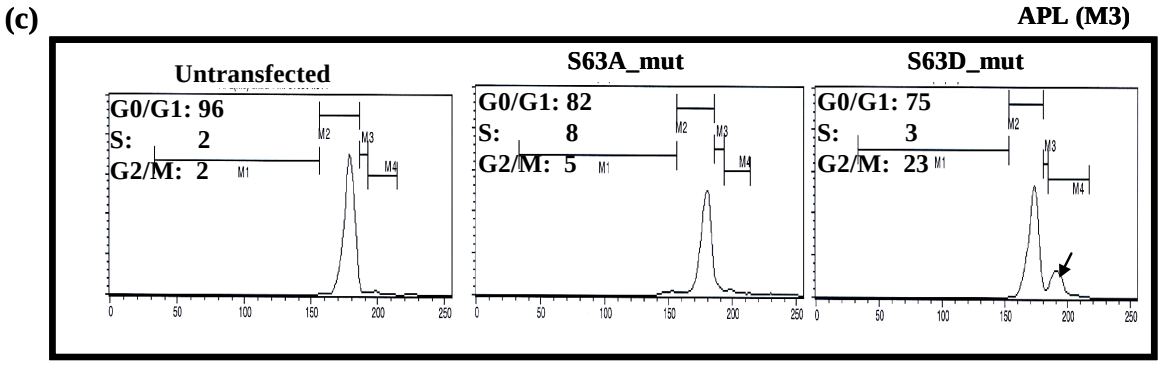
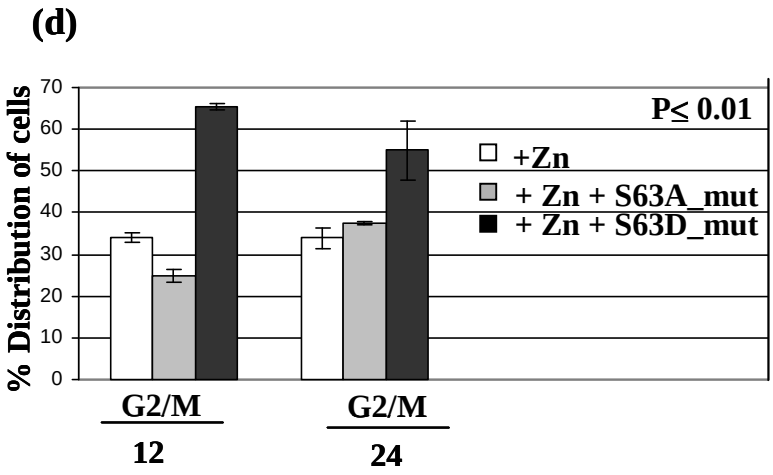


Figure 18: Dephosphorylation of OP18 at Ser63 is important for PML-RARα mediated cell cycle effects in PR9 cells, in NB4 cells and APL patients. (a) Cell-cycle analysis was performed using propidium iodide staining after 12 hour in PR9 cells overexpressing a phosphorylation-deficient mutant (S63A-mut) and a constitutively active mutant (S63D-mut) of OP18. Cells were transfected with the mutants (generated as described in Methods) using effectene (Qiagen), induced for PML-RARα with Zinc after 12 hours analysed for cell cycle phases**. (b)** Cell cycle profile in NB4 cells overexpressing S63A and S63D OP18 mutants**. (c)** Cell cycle profile in fresh APL patient samples overexpressing S63A and S63D OP18 mutants. Fresh samples were transfected with the mutants using Nucleofector Kit (AMAXA) and then analyzed for their cell cycle phases 14 hours post transfection.



(d) Cell-cycle analysis was performed using propidium iodide staining in PR9 cells at 12h and 24h. Median values are shown and error bars depict standard deviation from 3

independent experiments. The P value was calculated by using student´s t- test by comparing the means of three different conditions in each experiment.

In summary, we conclude that PML-RARα brings about overlapping functions essential for cell cycle and mitosis regulation. The regulation of OP18 expression and phosphorylation by PML-RARα (Fig.17 and 18) play a major role, albeit other mechanisms cannot be ruled out in the effects of the fusion protein on cell cycle.

4. Discussion

Recent advance in proteomics offer opportunities to rapidly identify new biomarkers or pattern of markers for the early detection and diagnosis of cancer and to monitor the therapeutic efficacy and toxicity of treatment used to improve long term survival of patients. The characterization of leukemogenesis associated genetic alterations, such as the combined presence of activating mutation of genes together with altered function of transcription factors, and the documented impact of these mutations upon prognosis of AML as well as APL, suggests AML or APL as a primary candidate for proof-of principle studies with new high throughput protein analysis techniques.

The mechanism by which PML-RARα fusion protein causes an APL phenotype remains largely unknown. Although the role of Neutrophil elastase in facilitating the leukemogenic potential of PML-RARα has been reported (Lane and Ley, 2003; Lane and Ley, 2005), other factors within a cell that collaborate with PML-RARα to initiate the disease have not been described. The identification of proteins and/or protein pathways regulated by PML-RARα on a global level is critical to gain insights into the mechanism of how the cells become blocked at a particular differentiation step. Microarray analysis has been used to investigate thousands of RNA expression levels and to identify patterns associated with biological characteristics (Beer et al., 2002; Bhattacharjee et al., 2001; Carbone, 1997; Garber et al., 2001). However, mRNA expression is not correlated with protein expression.

Therefore, such analysis can not detect important post translational modifications of proteins (Phosphorylation, Glycosylation or Sumoylation), all of which are important processes in determining protein function (Garrels et al., 1997; Wilkins et al., 1996).

In this study, we have applied a mass spectrometry-based proteomic analysis approach to identify both direct target proteins and the downstream effectors of the PML-RARα fusion protein. We hypothesized that identification of the target proteins of the PML-RARα fusion protein may be a useful tool to elucidate the molecular mechanisms of the fusion protein in leukemogenesis and a fruitful line of inquiry for developing novel therapies for the treatment of leukemia. To address this question, we used an inducible *in-vitro* APL cell line model system, the PR9 cell line. The rationale was that expression of the fusion protein in this model system is not toxic and apoptogenic, and is comparable to that of APL blasts (Grignani, Ferrucci, et al. 1993). The expression of PML-RARα in other systems has been reported to be toxic and lead to apoptosis (Ferrucci et al., 1997; Lane and Ley, 2003; Walter et al., 2004), which surprisingly would not favor leukemogenesis associated with the fusion protein.

Our data suggested that PML-RARα potentiates proteins involved in cell cycle and mitosis regulation. Based on this we have unraveled a novel role for the fusion protein PML-RARα in mitotic exit and G1 to S-phase transition. Furthermore, we demonstrate that increased expression and

decreased Ser63 phosphorylation of OP18 is important for cell cycle effects of PML-RARα. We show that significant differences exist in the PML-RARα-mediated effects on the proteome. We show that PML-RARα induction brings about overlapping functions essential for cell cycle and mitosis regulation. For example, increased levels of oncoprotein18 (OP18), heat shock protein70 (HSP70) and glucose regulated protein75 (GRP75), and decreased expression of peptidyl-prolyl isomerase (Pin1) are seen in PML-RARα expressing PR9 cells. This observation is important as this formed the basis of our hypothesis that PML-RARα could have a positive effect on cell cycle progression so as to favor leukemogenesis since these proteins have known roles in cell cycle and mitosis regulation. OP18 is highly expressed in leukemic blasts (Melhem, Zhu et al. 1991; Roos, Brattsand et al. 1993). Although the exact role of increased OP18 expression in various cancers remains unclear, evidence of the role of OP18 in various cellular processes (Grignani et al., 1993b; Melhem et al., 1991; Roos et al., 1993) led us to further characterize its role in APL. Based on the observation that OP18 (Fig. 8a, Spot 29) and its phosphoisoform (Fig. 8a, Spot 3) were increased and decreased in their expression by PML-RARα, respectively. The role of OP18 as a mitotic regulator that a function via its ability to modulate microtubule stability has also been reported (Mistry & Atweh 2002). The fact that in cells PCNA and p21 can participate in quaternary complexes with CDK4/cyclin D1(Xiong, Zhang, et al. 1992), and that it is required absolutely for cell proliferation suggests its contribution to

the coordination of cell cycle progression and DNA replication. Experimental evidence positions heat shock protein family (HSP70 and GRP75) as cancer-relevant survival proteins. These so called chaperones show a correlation of their expression with increased cell proliferation and poor differentiation (Rohde, Daugaard, et al. 2005, Helmbrecht, Zeise, et al. 2000). HSP70 overexpression has been shown to shorten G2 cell cycle arrest in doxorubicin-mediated cytotoxicity, thereby showing a one-to-one link with the cell cycle.

It has been shown that pin1 expression is increased in varies types of cancers and it has been reported to be involved in cell cycle progression (Bao et al., 2004; Wulf et al., 2004). Moreover, Pin1 shows a variable behaviour during different phases of cell cycle, for example during G1 phase of the cell cycle its expression is enhanced while as it decrease in expression during S phase (Zhu et al., 2004). Here we demonstrated that up on PML-RARα induction the expression of Pin1 goes down and correspondingly the proportion of cells in the S phase increase. Microarray (affymetrix) data revealed increased mRNA expression of OP18 in patients with t(15;17) compared to normal bone marrow and other types of leukemia. Interestingly, the expression of OP18 mRNA is elevated in blasts from patients with a complex aberrant karyotype, which have a prognosis similarly poor to the prognosis of APL before the advent of retinoic acid.

Identifying retinoid target genes is an important step in developing a mechanistic understanding of RA effects in APL. Retinoic acid is known to

degrade PML-RARα (Yoshida et al., 1996). Proteomic analysis data previously demonstrated an increased OP18 protein expression in cells expressing the PML-RARα fusion protein, which was downregulated by treatment of cells with RA (Pitha-Rowe et al., 2003). This reinforced the identification of OP18 as a PML-RARα target and suggested that regulation of OP18 may be one major axis of the fusion protein function. OP18 is regulated by both transcriptional and post-transcriptional mechanisms. The OP18 gene is a direct target of the E2F family of transcription factors (Polager & Ginsberg 2003) and activation of OP18 by PML-RARα is very unlikely to be a direct transcriptional effect. We also show that PML-RARα is a potent activator of the *OP18* promoter. Since, the *OP18* promoter contains RARE sequence; it is possible that PML-RARα might bind to the *OP18* promoter directly to regulate its transcription. However, this needs further experimental evidence. The mechanism by which the PML-RARα fusion protein regulates OP18 remains to be determined. One possible hypothesis would be regulation at the level of post-translational modification, which in fact is the case. We demonstrate that PML-RARα regulates OP18 at the level of phosphorylation. So far PML-RARα has been shown to block differentiation. This is the first evidence showing its direct role in cell cycle and mitotic exit. Using three independent methods (propidium iodide staining, mitotic index measurements and MPM2 staining), we have demonstrated that PML-RARα induction in PR9 cells leads to mitotic exit and G1 to S-phase transition. Our data indicate

that PML-RARα gives cells a proliferative advantage by allowing them to divide faster, exit mitosis and enter a new cell cycle. The enhanced proliferative potential and survival role of PML-RARα has been proposed to occur by direct action on extremely different molecular targets: transcriptional regulation of RAR targets genes and regulation of p53 stability. p53, a tumor suppressor and its target p21, a cell cycle inhibitor are both relevant to cell cycle regulation. The modulation of p53 (Insinga, Pelicci, et al. 2005) and p21 (Casini & Pelicci 1999) functions and the induction of cyclin A1 mRNA levels in leukemic cell lines by PML-RARα (Muller, Yang, et al. 2000) suggested a possible role of the fusion protein in cell cycle regulation. This is a reasonable assumption given the fact that in murine models of PML-RARα the effects of fusion protein on the differentiation program cannot be explained solely by their ability to block differentiation (Minucci, Monestiroli, et al. 2002; Westervelt & Ley 1999). Thus, the positive effect of the fusion protein PML-RARα on the cell cycle would favour leukemogenesis. MPM2 specifically recognizes a subset of mitosis specific phosphoproteins and widely used as a marker for mitotic cells (Kishi et al., 2001) or a marker of M phase in the cell cycle (El-Khodor et al., 2003). However, at 24 and 48h of induction, we could not detect any protein bands, indicating an exit from mitosis. Consistent with these data, we also observed that PML-RARα decreases the expression of p21. It has been shown that p21 is one of the cyclin dependent kinase inhibitor (CDKIs) (Drdova and Vachtenheim, 2005;

Liu and Lozano, 2005) which is involved in mediating growth arrest in the G1 phase of the cell cycle (Lowenheim et al., 2005). In addition CDK2/CDK4 kinase activities were increased at the same time. Together these data explain why blasts carrying the translocation t(15; 17) are unable to differentiate after mitosis, since the fusion protein commits them to exit this phase faster and enter a new cell cycle. PML-RARα might thus function to coordinate mitotic exit and differentiation block resulting in an enhanced proliferative potential of the APL blasts. Despite this, we do not yet know whether the commitment by the fusion protein to promote mitotic exit is directly linked to a block in differentiation. Further study will be required to address this question. We further demonstrate that under our experimental settings mitotic exit in PR9 cells requires the presence of the PML-RARα fusion protein, and an increase in OP18 expression represents an important downstream step in PML-RARα mediated mitotic exit since siRNA against OP18 was able to block the PML-RARα effects. In addition, the effects of PML-RARα on mitotic spindle structures (microtubule dynamics and mitotic exit) can be attributed to increased OP18 expression. Overexpression of OP18 dramatically shifts the equilibrium between free tubulin dimers and microtubules in the direction of free tubulin dimers, thereby causing microtubule destabilization (Larsson, Marklund, et al. 1997). Furthermore, changes in OP18 expression are observed due to the activation of p53, which results in a cell cycle arrest at G2/M (Ahn, Murphy, et al. 1999, Johnsen, Aurelio, et al. 2000) and by

overexpression of p21 (Steinman, Yaroslavskiy, et al. 2000). Interestingly, both p53 and p21 are known targets of PML–RARα (Casini & Pelicci 1999, Insinga, Pelicci, et al. 2005). Thus, OP18 is an important regulatory protein and may represent an important target for leukemogenic proteins that regulate cell cycle.

The regulation of protein function by posttranslational modification is likely to be protein-specific. In addition Protein phosphorilation-dephosphorylation is the most common form of posttranslational modification used to regulate cell function. For the regulation at post-translational modification level, sumoylation of PML (Zhu, Zhou, et al. 2005) and acetylation of p53 by PML-RARα (Insinga, Pelicci, et al. 2005) has been recently reported. It has been reported that during cell cycle progression, OP18 is invariably regulated by phosphorylation on four distinct Serin residues in intact cells, namely, Ser16, -25, -38, and Ser63 (Leighton, Curmi, et al,Beretta, Dubois, et al. 1995,Andersen, Ashford, et al. 1997). *In-vitro* and *in-vivo* studies have demonstrated that OP18 becomes highly phosphorylated during mitosis thereby turning off its microtubule destabilizing activity (Marklund, Larsson, et al. 1996). Consistent with the previous report that, phosphorylation of OP18 regulates its biological actions and reduce its affinity for tubulin (Beretta et al., 1993; Leighton et al., 1993; Marklund et al., 1996; Steinmetz et al., 2001; Zada et al., 2003) and OP18 enhance its action on microtubule dynamic, which allows for progression of mitosis (Curmi et

al., 1999; Wittmann et al., 2004). Our results from 1D and 2D western blot analysis using a phospho-specific Ser63 anti-OP18 antibody revealed a decrease in the expression of Ser63 phospho-isomer of OP18 implying that PML-RARα could regulate OP18 at the level of phosphorylation. To further understand the importance of this observation, the cell cycle as a read out for the effects of PML-RARα was a reasonable assumption given the fact that the overall activities of PML-RARα may not rely only on its ability to affect myeloid differentiation. Moreover, the role of PML-RARα in cell cycle control remains unclear and has not been thoroughly investigated so far. This novel assumption supports the hypothesis that the dominant negative action of PML-RARα on PML could result in a shortening of the transition through the cell cycle. Pml-/- cells are reported to have an increased proliferative potential in view of a faster transition through the G1-phase of the cell cycle (Pandolfi, 2001). Our results from cell cycle analysis are in agreement with the proposed dominant negative function of PML-RARα on PML effects on cell cycle. Thus, indirect positive effect of PML-RARα on cell cycle would favor leukemogenesis.

Our results demonstrate the importance of decreased phosphorylation of OP18 at Ser63 in PML-RARα mediated effects on cell cycle. Since a constitutively phosphorylated Ser63 mutant of OP18 prevented the cell cycle effects of PML-RARα compared to a phosphorylation-deficient mutant. We

concluded that reduced phosphorylation of OP18 by the PML-RARα fusion protein at a single Ser63 residue is sufficient and important for its effects on cell cycle. This reduced phosphorylation might be due to increased activities of the Ser/Thr phosphatases PP2A and PP2BA. It has already been reported that, the activity of Serin/threonine protein phosphatase (PP2A, PP2BA) are potential targets for novel therapeutic with applications in many diseases, including cancer (Klumpp and Krieglstein, 2002) and the expressions level in leukemic blasts is low (Yamamoto et al., 1999). The fact that okadaic acid prevented both PML-RARα mediated increased PP2A phosphatase expression as well as decreased Ser63 phosphorylation level of OP18, led us to conclude that decreased phosphorylation occurs via this phosphatase pathway. In fact it has been suggested that protein phosphatases are responsible for dephosphorylating OP18 as cells enter a new cell cycle (Mistry et al., 1998). Decreased phosphorylation of OP18 on Ser63 would consequently favor microtubule destabilization and a mitotic exit induced by PML-RARα as proposed by our previous results. Although the role of other phosphorylation sites has also been proposed in mitosis regulation we demonstrate that reduced phosphorylation of OP18 by the PML-RARα fusion protein at a single Ser63 residue is sufficient for its mitotic exit effects since the constitutively phosphorylated mutant blocks the effects of PML-RARα (results in mitotic block) compared to a phosphorylation-deficient mutant (results in mitotic exit). The role of other phosphorylation sites in PML-RARα mediated mitotic

exit remains to be determined. Decreased phosphorylation of OP18 on Ser63 would consequently affect cell cycle in a manner as proposed by our results in APL patient cells, PR9 and NB4 cells. Decreased phosphorylation of OP18 during mitotic exit induced by the PML-RARα fusion protein could in turn, contribute to an increased non-phosphorylated form of OP18 (Fig. 8a, spot 29), in support with a model proposed by Mistry *et.al*, (Mistry & Atweh 2001). Therefor, our data indicated that PML-RARα mediates its cell cycle effects not only at the level of OP18 expression, but at the level of a post-translational modification as well.

In summary, on the basis of our findings we propose a model (Fig.19)) wherein, in the presence of PML-RARα, decreased phosphorylation of OP18 at Ser63 via the PP2A phosphatase pathway would ensue a cell (blast) to exit mitosis thereby increasing a nonphosphorylated pool of OP18 since, a constitutively-phosphorylated mutant or siRNA against OP18 results in a mitotic block. Our data provides a conceptual advance in scientific understanding of a human disease by suggesting that targeting proteins of the mitotic apparatus may represent a therapeutic option for treatment of APL and other AML subtypes.

Thus, proteomic study coupled with functional analysis has clearly provided a framework towards understanding of the systems biology of APL and might serve as a useful tool for elucidating multiple pathways/cascades in the biology of other diseases.

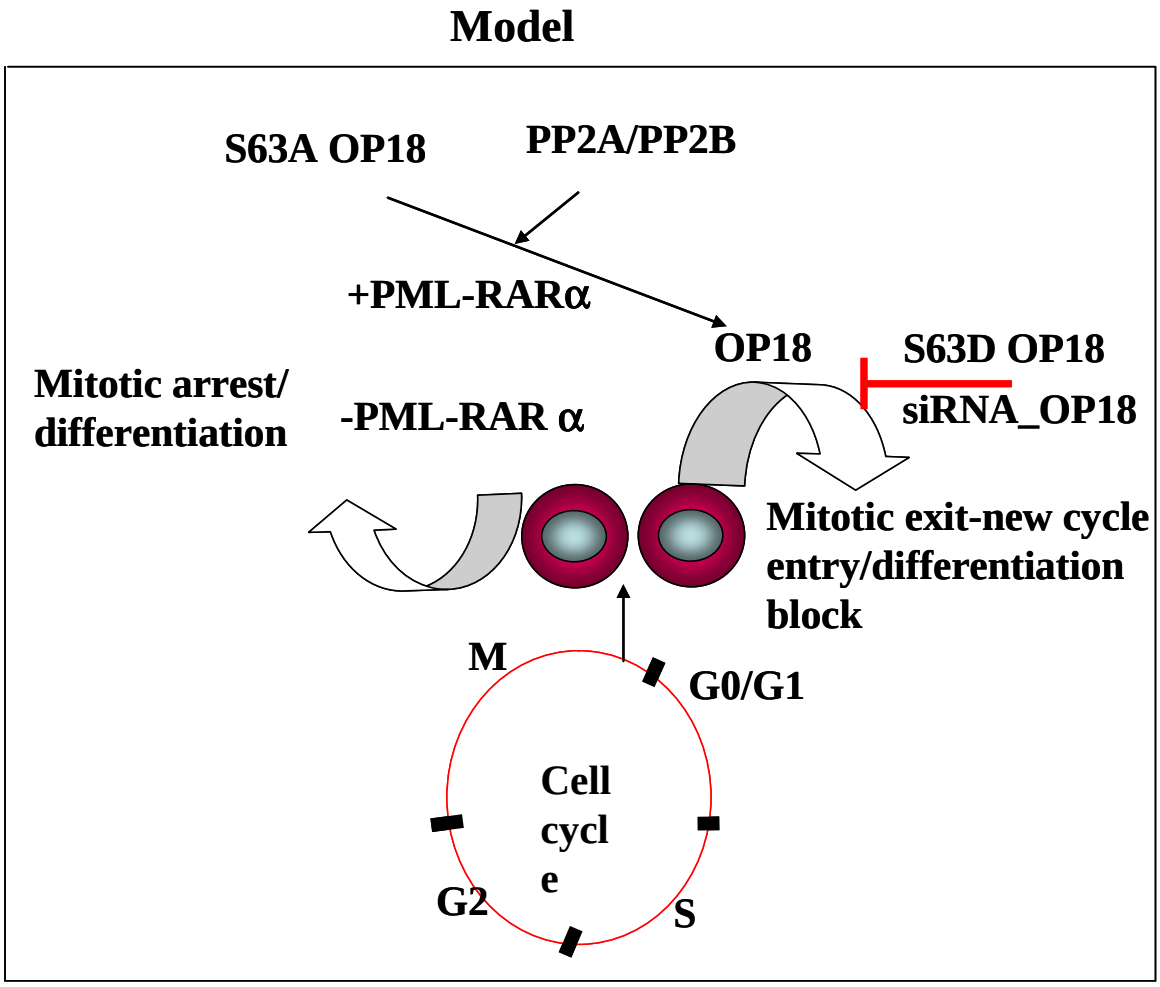


Figure 19: Model: our model shows that, how PML-RARα promotes cell cycle to exit mitosis by increasing expression of OP18 and down regulation of pOP18 at Ser63 by PP2A phosphatase thereby inhibiting differentiation. .

5. Summary

We applied mass spectrometry based approach to explore the proteins differentially regulated by PML-RARα a translocation characteristic of acute promyelocytic leukemia (APL). Differentialy expressed proteins, a number of which are related to cell cycle function, including OP18, HSP70, GRP75 and Pin1 were identified by mass spectrometry. Further analysis of the OP18 pathway indicated that mRNA expression of OP18 was higher in APL patients and the increased OP18 protein expression upon PML-RARα induction was overcome by retinoic acid treatment. PML-RARα induced cell cycle progression and led to mitotic exit. RNA interference experiments revealed that siRNA against OP18 overcomes PML-RARα effects on cell cycle progression. In addition to increased OP18 expression by PML-RARα, 2D gel electrophoresis revealed an isomer of OP18, subsequently confirmed as Ser63 phosphomer to be downregulated by PML-RARα. Based on these findings, point mutation experiments indicated that decreased phosphorylation of Ser63 in OP18 is important for PML-RARα mediated cell cycle and mitotic index effects since constitutive phosphorylated mutant (Ser63-asp) of OP18 overcame the PML-RARα effects in U937-PR cells, NB4 and APL patients. In summary, our results demonstrate that the effect of PML-RARα on cell cycle progression and mitotic exit is via two mechanisms: increasing the expression of OP18 and decreasing the phosphorylation of OP18 at Ser63.

6. Zussamenfasung

Diese Arbeit nutzt einen auf der Massenspektrometrie fußenden Ansatz, um die durch das Fusionsprotein PML-RARα, welches für die akute Promyelozytenleukämie (APL) charakteristisch ist, differentiell regulierte Proteine zu erforschen. Differentiell ausgedrückte Proteine, eine Zahl, von der mit Zelle Zyklusfunktion zusammenhängen, einschließlich OP18 HSP70, GRP75 und Pin1 wurden durch Massenspektrometrie gekennzeichnet. Die weiterführende Analyse des OP18-Pfades ergab, dass die mRNA-Expression von OP18 in APL-Patienten erhöht ist und dass die durch PML-RARα erhöhte Proteinexpression von OP18 durch Retinolsäure gesenkt wird. PML-RARα induziert eine Progression durch den Zellzyklus und führt zu einem Austritt der Zellen aus der Mitose. Experimente mit RNA-Interferenz ergaben, dass siRNA gegen OP18 die Effekte von PML-RARα auf den Zellzyklus antagonisiert. Zusätzlich zu erhöhter Expression von OP18 durch PML-RARα zeigte sich in zweidimensionaler Gelektrophorese ein Isomer von OP18, welches in Folge als das Ser63 Phosphomer bestätigt werden konnte, als supprimiert. Auf diesen Daten basierend zeigten Experimente mit Punktmutanten, dass die verminderte Phosphorylierung von Ser63 erheblich für den durch PML-RARα vermittelten Austritt aus dem Zellzyklus und die Effekte auf den mitotischen Index ist, weil eine konstitutiv phosphorylierte Mutante (Ser63Asp) von OP18 die Effekte von PML-RARα in U937-PR9-Zellen, NB4-Zellen und APL-Patientenproben überwinden konnte.

Zusammengefaßt demonstrieren unsere Ergebnisse, dass der Effekt von PML-RARα auf die Progression der Zelle durch den Zellzyklus und auf den Austritt aus der Mitose durch zwei Mechanismen vollzogen wird: die Erhöhung der Proteinexpression von OP18 und die Verminderung der Phosphorylierung von OP18 an Ser63.

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9. Lebenslauf

NAME: MULU GELETU HEYE

DATE OF BIRTH: 13 AUGUST 1968

PLACE OF BIRTH: ADDIS ABABA, ETHIOPIA

NATIONALITY: ETHIOPIAN

MARITAL STATES: MARRIED

SEX: FEMALE

ADDRESS: Wieland str. 31

06114, Halle (Saale), Germany

Telephone: +49-345-4704239

+49-1626900311

Email: geletu.mulu@medizin.uni-halle.de, mgeletu2003@yahoo.com,

Education

2002-2006: PhD student, under supervision of Prof. med Dr. Wolfgang Hiddemann, Director Med III, worked on the topic "Proteomic analysis of acute promyelocytic leukemia: PML-RARα promotes mitotic exit by increased expression and decreased phosphorylation of OP18 at serine 63" at Ludwig Maximilians University (LMU), GSF-Hematology and Department of Internal Medicine III, University Hospital Grosshadern, Munich, Germany

1987-1992: MSc in Biology (Biochemistry), Saint Petersburg state University, Russia Thesis title: Composition and content of lipids in different cerebrum sections of rat brain in normal condition and with cresol cramps

1992: Certificate in Massage from Saint Petersburg Institute of Culture and Physical Training Department

Work Experience

Jan 2006-untill date working as post doctoral fellow working on "Target proteins of C/EBPα-p30 in AML: C/EBPα-p30 enhances Sumoylation of C/EBPα-p42 via up regulation of Ubc9" **at State Centre for Cell and Gene Therapy, Internal medicine IV, Martin Luther University, Halle (Saale) 06120, Germany**.

1995-2001: Senior Research Assistant and Radiation officer. Armauer Hansen Research Institute, Addis Ababa, Ethiopia

1993-1995: Physiotherapy Technician, Alpha Physiotherapy Special Clinic Addis Ababa, Ethiopia

1994–1995 laboratory Assistant (on office practice basis) Ethiopian Authorities for standardization, Addis Ababa, Ethiopia

Techniques dealt with during Masters and PhD thesis

During my study time I employed different biochemical and molecular biology techniques and also proteomics and gene knock out technology.

Cell and Molecular Biological Technique: Plasmid and DNA isolation, Genomic DNA isolation from bacteria, Gene cloning, Bacterial transformation, Restriction digestion, Polymerase chain reaction, real time PCR, Purification of DNA fragments from Agarose gel, Ligation, Electrophoresis, Luciferase Assay, *In vitro* kinase assay, *In vitro* and *In Vivo* sumoylation assay, cell culture, transfection, $CD34^{+}$ hematopoietic cells isolation, Flow cytometric analysis

Biochemistry: Co- immunoprecipitation, Western blotting, ECL, His Purification, Enzyme-linked immunosorbent assay (ELISA)

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

Immunological Technique: Leukocyte preparation, Antigen preparation, Leukocyte cultures, isolation of mononuclear cells using Ficol-Hypaque

Publications

1. **Mulu Geletu***, Abdul A Peer Zada*, John A Pulikkan*, Carsten Müller-Tidow, Venkateshwar A Reddy, Maximilian Christopeit, Wolfgang D. Hiddemann, Hermann M. Behre, Daniel G Tenen, Gerhard Behre. Proteomic analysis of acute promyelocytic leukemia: PML-RARα leads to decreased phosphorylation of OP18 at Serine 63 (Proteomics 2006, July 10).

2. Balkhi MY, Trivedi AK, **Geletu M**, Christopeit M, Bohlander SK, Behre HM, Behre G. Proteomics of acute myeloid leukaemia: cytogenetic risk groups differ specifically in their proteome, interactome and post-translational protein modifications. (Oncogene 2006, may 26)

3. **Mulu Geletu**, Mumtaz Yaseen, Abdul A Peer Zada Arun Trivedi, Jhon A. Pullikan, Maximilian Christopeit, Herman M. Behre, Gerhard Behre. Target

proteins of C/EBPα-p30 in AML: C/EBPα-p30 enhances Sumoylation of C/EBPα-p42 via up regulation of Ubc9 (Manuscript Submitted)

4. Abdul A Peer Zada, John A Pulikkan, Deepak Bararia, **Mulu Geletu**, Arun K Trivedi, Mumtaz Y Balkhi, Wolfgang D Hiddeman, Daniel G Tenen, Herman M Behre, Gerhard Behre. Proteomic discovery of MAX as a novel interacting partner of C/EBPα: a Myc/Max/Mad link (Leukemia 2006 September)

5. **Mulu Geletu***, Abebe Habte*, Joseph Okao Olobo, Dawit Kidane, Yohannes Negesse, Mohammed Ahmed Yassin, Bereda Kifle, Getahun Abate, Morten Harboe, Abraham Aseffa. T-Cell Mediated Immune Responses in Patients with Tuberculous Lymphadenitis from Butajira, Southern Ethiopia (Ethiopian Medical J.; 2004)

6. Olobo J, **Geletu M**, Demissie A, Eguale T, Hiwot K, Aderaye G, Britton S. Circulating TNF-alpha, TGF-beta, and IL-10 in tuberculosis patients and healthy contacts. (Scand J Immunol. 2001 Jan; 53(1):85-91.)

7. A. Demissie; P. Ravn; J. Olobo; T.M Doherty; T. Eguale; **M. Geletu**; W. Hailu; P. Andersen, S. Britton. T-cell recognition of Mycobacterium tuberculosis Culture Filtrate Fraction in Tuberculosis Patients and Their Household Contacts. (Infection and immunity, Nov. 1999, 67: 5967-5971))

Fellowships Awarded:

*Awarded with prestigious German fellowship, Germen academic exchange services (**DAAD)** for four years from October 2001 to September 2005.

*Awarded with Junior Research fellowship for master thesis by Russian Government, September 1987-June 1992.

Course and conference attended

1. Poster presentation "Target proteins of C/EBPα-p30 in AML: C/EBPα-p30 enhances Sumoylation of C/EBPα-p42 via up regulation of Ubc9" at the Annual Meeting of American Society of Hematology (ASH) on December 10th 2006 in Orlando, USA.

2. Attended Advanced Course (September20-24, 2004) given at the University of Southern Denmark On the topics of "Mass Spectrometry in protein chemistry and Proteomic".

3. poster presentation (Proteomic pathway discovery of C/EBPα- p30 target proteins in acute myeloid leukemia: the C/EBPα-p30 AML mutant interferes with nuclear mRNA processing) at the Joint Annual meeting of Germany Austria and Switzerland Society of Oncology on October 3rd 2004 in Innsbruck, Austria

4. Oral and poster presentation (Proteomic analysis of acute promyelocyte leukemia reveals that PML-RARα induces cell cycle progression and mitotic exit by increased expression and decreased Ser63 phosphorylation of OP18) at the Annual Meeting of American Society of Hematology (ASH) on December 6th 2003 in San Diego, USA.

5. Participated the Baltic Summer School course on the topic of Current Trend in Immunology and Signal Transduction held in Kiel, Germany from September 17- 28, 2000.

6. Attended the National Training Course on the safe uses of ionizing radiation from 23-27 November 1998 and awarded Certificate of participation from the National Radiation Protection Authority.

7. Attended a 20 hours lecture course (June 15 to July 9, 1998) given at Armauer Hansen Research Institute on selected topics in Molecular Biology.

8. Participated and poster presented on an immunology workshop and Fourth International conference given at the Federation of African Immunological Societies, Yaounde, Cameroon from February 12-18, 2000. Awarded Certificate.

10. 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