Identification of Protein Tyrosine Phosphatases regulating FLT3 activity and of PTPs regulated by FLT3 ITD

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1 Summary

Acute Myeloid Leukemia (AML) is one of most commonly occurring forms of leukemia. About 90% of the affected are adults while children are affected more rarely. AML represents clonal growth of myeloid cells which over proliferate and do not terminally differentiate thereby resulting in accumulation of immature myeloid cells of different lineages. Receptor Tyrosine Kinases (RTK) are important players in controlling activities of a cell, and therefore crucial for their normal development. *Fms* like tyrosine kinase-3 (FLT3) is a member of the family of type III RTK and has been shown as a critical mediator for normal differentiation of monoctyes and B cells. Mutations in FLT3, like Tyrosine Kinase Domain (TKD) point mutations and Internal Tandem Duplication (ITD) result in constitutive activation of the receptor. FLT3 mutations are the second most common type of genetic lesion of AML. Signaling of wild type and mutated forms of FLT3 is known to a large extent. The FLT3 WT activates the RAS-MAPK and PI3K-AKT pathways and the FLT3 ITD activates in addition STAT5 which is required for transformation. However, details of FLT3 regulation still remain unclear. Protein Tyrosine Phosphatases (PTPs) are enzymes that cooperate with kinases and regulate the kinase activity by dephosphorylating the tyrosine residues on the RTKs. Therefore, they may play an important role in normal cellular functions. It was sought therefore to identify potential regulators of FLT3 by performing a lentiviral mediated shRNA-based screen for PTPs. PTP knockdown was done with the help of shRNA targeting PTPs in murine myeloid-derived 32D cells expressing FLT3 WT. A screen based on FL-stimulated ERK activation for 20 relevant PTPs was carried out to select phosphatases which could be implicated in FLT3 signaling. DEP-1 was identified as one of the PTPs from the screen potentially regulating FLT3. Signaling differences between DEP-1 knockdown cells and cells transduced with non targeting shRNA control were analyzed. It was observed that in the DEP-1 depleted cells FLT3 ligand (FL) induced stronger ERK activation and enhanced proliferation. Additionally, DEP-1 depletion in 32D cells expressing FLT3 WT resulted in weak STAT5 activation and hyperphosporylation of the tyrosine residues on the receptor. Specifically, hyperphosphorylation of the sites pY589, pY591 on FLT3 was observed upon DEP-1 depletion. These sites are involved primarily

in activation of STAT5. Transient depletion of DEP-1 in the human AML cell line THP-1 expressing FLT3 WT also caused increased receptor phosphorylation at specific tyrosine sites pY589, pY599 and weakly elevated activation of ERK. DEP-1 depleted 32D cells expressing FLT3 WT were capable of FL-induced colony formation in semi-solid medium. FL-dependence suggested this to be an FLT3-mediated effect. DEP-1 knockdown however, did not result in leukemia-like disease in mice. Taken together, this data indicates that DEP-1 is involved in negative regulation of FLT3 activity. Loss of DEP-1 may contribute to transformation in AML, but would presumably need co-operating mutations for transformation *in vivo.*

Another part of this thesis deals with the identification of PTPs which are possibly regulated by FLT3 ITD in AML. For this purpose, cDNA from human AML blasts expressing FLT3 WT or FLT3 ITD were compared for expression patterns of 92 human PTPs. The expression profile of 92 human PTPs was also analyzed for human AML-derived cell lines expressing FLT3 WT (EOL-1, THP-1 and RS4-11) and FLT3 ITD (MV4-11). 10 PTPs were selected for further analysis based on difference in expression between the FLT3 WT and FLT3 ITD expressing blasts, and overall high mRNA expression. The mRNA expression profile of the 10 selected PTPs was determined in a second set of human AML blasts expressing FLT3 WT or FLT3 ITD and in 32D cells with or without FLT3 expression. From the expression analysis data, the dual-specificity PTP DUSP6 was found to be upregulated by FLT3 ITD. The DUSP6 protein levels were also elevated in 32D cells expressing FLT3 ITD. FLT3 kinase inhibitors AG1295 and 1020 nearly blocked the DUSP6 expression in murine 32D cells and the human AML cell line MV4-11 expressing FLT3 ITD indicating that DUSP6 expression is dependent on constitutive FLT3 ITD receptor signaling activity. Pharmacological inhibitors of RAS-MAPK, PI3K-AKT and STAT5 pathways were used to study the mechanism of regulation of DUSP6 expression. U0126, a MEK inhibitor reduced DUSP6 expression. Wortmannin, PI3K inhibitor and STAT5 inhibitor had rather weak effects on lowering the DUSP6 expression. By analyzing a larger set of affymetrix data obtained in an independent study in Rotterdam, another dual-specificity PTP TNS1 was found to be regulated by FLT3 ITD. The TNS1 mRNA expression was downregulated by FLT3 ITD.

This could be recapitulated in the murine cell lines 32D and Ba/F3 cells: TNS1 expression was downregulated by FLT3 ITD. The observed changed expression of certain PTPs in presence of FLT3 ITD may indicate roles of these expression alterations for transformation in AML, a possibility which is currently explored.

2 Zusammenfassung

Die Akute Myeloische Leukämie (AML) ist eine der am häufigsten vorkommenden Formen der Leukämie. Etwas 90% der Erkrankten sind Erwachsene, während Kinder seltener von der Erkrankung betroffen sind. AML entsteht durch das klonale Wachstum myeloischer Zellen, welche stark proliferieren, aber nicht terminal differenzieren können. Dadurch akkumulieren sich unreife Zellen verschiedener myeloischer Differenzierungslinien. Rezeptortyrosinkinasen (RTK) sind wichtige Moleküle für die Regulation von Zellaktivitäten, und deshalb auch kritisch für die normale Zellentwicklung. *Fms* like tyrosine kinase-3 (FLT3) ist ein Mitglied der Klasse-III RTK Familie, und hat eine wichtige Funktion bei der normalen Differnzierung von Monozyten und B-Zellen. Mutationen im FLT3-kodierenden Gen, darunter Punktmutationen in der Tyrosinkinase-Domäne (TKD), oder interne Tandemduplikationen (ITD) vor allem in der Juxtamembran-Domäne, führen zur konstitutiven Aktivierung von FLT3. FLT3-Mutationen stellen den zweithäufigsten Typ genetischer Läsionen bei der AML dar. Über die Signaltransduktion von FLT3 und seiner mutierten Versionen ist schon viel bekannt. Wildtyp FLT3 aktiviert den RAS-MAPK- und den PI3K-AKT Signalweg, und FLT3 ITD aktiviert außerdem STAT5, was entscheidend für die Zelltransformation ist. Wie die FLT3 Aktivität im Detail reguliert wird, ist jedoch noch weitgehend unklar. Protein-Tyrosinphosphatasen (PTPs) sind Enzyme, die mit Tyrosinkinasen kooperieren, und die RTK Aktivität durch Dephosphorylierung regulieren können. Deshalb spielen sie wahrscheinlich eine wichtige Rolle für normale Zellfunktionen. Um potentielle Regulatoren der FLT3-Aktivität zu identifizieren, wurde ein shRNA-Screen für PTPs auf der Basis shRNA-kodierender lentiviraler Partikel durchgeführt. Der „Knockdown“ der Expression von PTPs wurde mittels shRNAs für 20 verschiedene PTP-Gene in murinen, myeloiden Zellen, sogenannten 32D-Zellen durchgeführt, welche Wildtyp FLT3 exprimierten. Die FLT3-Ligand (FL)-stimulierte Aktivität von ERK wurde als Messparameter für die Beeinflussung der FLT3-Aktivität benutzt. DEP-1 wurde in dem Screen als eine der potentiell die FLT3-Aktivität beeinflussenden PTPs identifiziert. Unterschiede in der FLT3-Signaltransduktion zwischen Zellen, die mit Kontroll-shRNA-exprimierenden Konstrukten transduziert worden waren, und solchen mit DEP-1 Knockdown wurden analysiert. Es wurde der Screeningbefund bestätigt, dass DEP-1-

Depletion die FL-stimulierte ERK-Aktivierung erhöht. Ebenso war die Proliferation der Zellen erhöht. Weiterhin resultierte die DEP-1-Depletion in einer schwachen STAT5-Aktivierung und einer Hyperphosphorylierung von FLT3. Bemerkenswerterweise wurde eine Hyperphosphorylierung der Phosphotyrosin-Reste pY589 und pY591 nach DEP-1-Depletion beobachtet. Diese Phosphorylierungsstellen sind an der Aktivierung von STAT5 beteiligt. Die transiente Depletion von DEP-1 in der humanen AML-Linie THP-1 verursachte auch eine erhöhte FLT3-Phosphorylierung, in diesem Fall an den Phosphotyrosinen 589 und 599, und führte auch zu einer schwach erhöhten Aktivierung von ERK. DEP-1-depletierte 32D Zellen, welche Wildtyp FLT3 exprimierten, waren in der Lage zur FL-induzierten Koloniebildung in halbflüssigem Medium. Die FL-Abhängigkeit ist dabei ein klares Indiz für die Rolle der FLT3-Aktivität in diesem Prozess. Der Knockdown von DEP-1 war jedoch nur partiell transformierend. So löste die Injektion der DEP-1-depletierten Zellen in syngene Mäuse keine myeloproliferative Erkrankung aus. Zusammengenommen zeigen diese Daten, dass DEP-1 die FLT3-Aktivität negativ reguliert. Der Verlust von DEP-1 könnte zur Transformation bei der AML beitragen, würde aber höchstwahrscheinlich kooperierende Mutationen erfordern, um zur Transformation *in vivo* zu führen.

Ein weiterer Teil dieser Dissertation beschäftigte sich mit der Identifikation von PTPs, die möglicherweise im Rahmen der AML durch FLT3 ITD reguliert werden. Zu diesem Zweck wurde cDNA aus humanen AML-Blasten gewonnen, die entweder Wildtyp FLT3 oder FLT3 ITD exprimierten. Das Expressionsmuster von 92 humanen PTPs wurde für diese Zellen sowie auch für humane Zelllinien, die entweder Wildtyp FLT3 (EOL-1, THP-1 und RS4-11) oder FLT3 ITD (MV4-11) exprimierten, analysiert. 10 PTPs wurden für eine weitergehende Analyse ausgewählt, basierend auf Expressionsdifferenzen zwischen Wildtyp FLT3- oder FLT3 ITD-exprimierenden Blasten, oder auf Grundlage generell sehr hoher mRNA-Expression. Das mRNA-Expressionsprofil dieser 10 PTPs wurde in einem zweiten Set von AML-Blasten analysiert, sowie in 32D Zellen, die entweder kein FLT3, oder die beiden unterschiedlichen Versionen von FLT3 exprimierten. Aus diesen Untersuchungen wurde klar, dass FLT3 ITD die dualspezifische PTP DUSP6 in der Expression stimulieren kann. Auch die DUSP6 Proteinspiegel waren

in 32D Zellen, die FLT3 ITD exprimierten, erhöht. Die FLT3 Kinase-Inhibitoren AG1295 und 1020 hemmten die DUSP6-Expression in den FLT3 ITD-exprimierenden murinen 32D-Zellen und humanen MV4-11-Zellen fast vollständig. Dies zeigte, dass die DUSP6-Expression in der Tat von der konstitutiven FLT3 ITD-Signalaktivität abhängig ist. Pharmakologische Inhibitoren des RAS-MAPK-Weges, des PI3K-AKT-Weges und von STAT5 wurden benutzt, um die Beteiligung dieser Signalwege an der DUSP6-Induktion zu prüfen. Während U0126, ein MEK-Inhibitor, die DUSP6-Expression stark verringerte, hatten Wortmannin und ein niedermolekularer STAT5-Inhibitor nur schwache Effekte. Die zusätzliche Analyse eines größeren Satzes von Affimetrix mRNA-Expressionsdaten, die in einer unabhängigen Studie in Rotterdam erhalten worden waren, zeigte, dass noch eine andere dualspezifische PTP, TNS1, offenbar durch FLT3 ITD reguliert wird. In diesem Fall war die Expression in Anwesenheit von FLT3 ITD verringert. Dieser Befund konnte in murinen 32D und Ba/F3-Zellen rekapituliert werden. Die beobachteten Veränderungen in der Expression bestimmter PTPs in Anwesenheit von FLT3 ITD könnten auf eine Rolle dieser Phänomene im Transformationsprozess hinweisen. Diese Möglichkeit wird gegenwärtig untersucht.

3 Introduction

3.1 Acute Myeloid Leukemia (AML)

AML is a clonal and malignant disease of hematopoietic cells occurring most frequently in adults and rarely in children. It arises as a result of somatic mutations occurring in the progenitor cells or more differentiated cells of hematopoietic lineage. The cell harboring mutations which lead to AML is referred as blast cell, which tends to accumulate in the bone marrow and circulates in the body finally reaching other tissues and organs like central nervous system, heart, skin (William's Hematology, 6th Edition, Ernest Beutler, Marshall A. Lichtman, Barry S. Coller, Thomas J. Kipps, Uri Seligsohn, McGraw-Hill, ISBN: 0-07-116293-3).

3.1.1 French-American-British (FAB) Classification

FAB classification was the first scheme proposed to classify AML (Bennett, Catovsky et al. 2008). It divides AML into subtypes (M0-M8) based on cytogenetics and appearance of cells under light microscopy. The table below lists the different types of AMLs based on FAB classification.

Type	Features
M0	minimally differentiated acute myeloblastic leukemia
M1	acute myeloblastic leukemia without maturation
M2	acute myeloblastic leukemia, with granulocytic maturation
M3	acute promyelocytic leukemia
M4	acute myelomonocytic leukemia
M4eo	myelomonocytic with bone marrow eosinophilia
M5	acute monoblastic leukemia (M5a), or acute monocytic leukemia (M5b)
M6	acute erythroid leukemia, including erythroleukemia (M6a), and pure erythroid leukemia (M6b)
M7	acute megakaryoblastic leukemia
M8	acute basophilic leukemia

3.1.2 Alterations occurring in AML

A number of factors including exposure to radiation, chronic exposure to chemicals like benzene and chemotherapeutic agents predispose to AML. However, genetic abnormalities including point mutations and chromosomal translocations, deletions, inversions are the important contributors in the development of AML (William's Hematology, 6th Edition, Ernest Beutler, Marshall A. Lichtman, Barry S. Coller, Thomas J. Kipps, Uri Seligsohn, McGraw-Hill, ISBN: 0-07-116293-3).

Mutations in proto-oncogenes like Ras, c-Kit, FLT3 result in cell proliferation, while mutations in genes like CEBP-α, AML1 lead to block in myeloid differentiation. Also, mutations in genes like TP53 and NPM1 can affect apoptosis and cell cycle regulation. Nucleophosmin (NPM1) is the most frequently mutated gene in AML (Renneville, Roumier et al. 2008). Point mutations in N- and K-isoforms Ras are present in 10-15% and 5% of AML patients respectively. Mutations in tyrosine kinases can make these proteins constitutive active, like c-Abl, FLT3, c-Kit, and PDGFR which are required for normal cell proliferation. Additionally, a number of cytogenetic abnormalities can result in leukemogenesis e.g., chromosomal translocation t(8:21) associated with AML M2 and results in a fusion product AML-ETO which acts as a repressor of tumor suppressor p14ARF. Another chromosomal translocation frequently associated with AML M3 is t(15:17) which results in a fusion product PML1/RAR-α, in which abnormal retinoic acid receptor is synthesized that can no longer aid in cell differentiation thereby making the cell to proliferate abnormally.

To explain the development of the disease, a “two-hit” model for leukemogenesis has been proposed (Gilliland and Griffin 2002), in which, class I mutations confers a proliferative or survival advantage, and class II mutations lead to a block in differentiation and apoptosis thereby maintaining the cell in an undifferentiated state. This is based on the fact that FLT3 mutations, which occur in about 30-40% cases in leukemia often occur in co-operation with gene rearrangements like t(15:17) and t(8:21). Also, in agreement with the two hit model is the rarity of occurrence (around 2%) of

mutations in both Ras and FLT3 in AML patients as reviewed by Gilliland and Griffin, 2002.

3.1.3 Gene Expression Profiling (GEP)

AML results from a number of mutations and genetic aberrations, which serve as important markers to diagnose the disease. They can be helpful in designing a therapeutic treatment accordingly. The cytogenetic and molecular analysis can be useful to identify the subtype of AML with different prognosis. Some aberrations like inv(16), t(8:21) and t(15:17) confer favorable prognosis, while others are associated with unfavorable prognosis. Also, some mutations in genes are associated with favorable or unfavorable response to treatment (Wouters, Lowenberg et al. 2009). In an adult AML study, 11q23 was considered as poor prognostic marker based on the complete remission and overall poor survival rate (Slovak, Kopecky et al. 2000), whereas, the Medical Research Council (MRC) AML 10 trial classified 11q23 in the intermediate risk marker (Grimwade, Walker et al. 2001). The most commonly occurring mutations in AML for instance, duplications in the gene FLT3 resulting in constitutive FLT3 ITD, and the gene encoding ectotropic integration site 1 (EVI1) confer poor prognosis and poor survival (Valk, Verhaak et al. 2004).

However, there are inconsistencies in defining a marker being favorable or unfavorable and hence there arises a need to refine the definition of AML types. DNA expression profiling represents a powerful tool to distinguish between lymphoid and myeloid leukemia and their subclasses. Gene expression profiling is a technology, where DNA microarray containing cDNA or oligonucleotide probes are used to measure mRNA transcripts of various genes. It is therefore, possible to identify cytogenetic abnormalities and new clusters of AML with characteristic gene expression signatures in a single assay with the help of GEP (Valk, Verhaak et al. 2004). It was shown that AML cases with favorable cytogenetics could be predicted with high accuracy with the help in GEP, whereas, additional molecular methods are needed for detection of other AML abnormalities. The information about expression patterns of various genes presents the

possibility to understand their role and elucidate mechanisms involved in disease formation.

Since, receptor tyrosine kinases (RTKs) and protein tyrosine phosphatases (PTPs) are involved in signaling for multiple cellular processes including growth, survival, migration, and adhesion. The role of RTKs has been studied extensively in AML in comparison to PTPs and therefore there is a need for identification of PTPs involved in AML. PTP expression studies have been performed to identify putative tumor suppressors and oncogenic PTPs. For example, the expression of PTPs was studied in melanoma cells, where PTP-κ was found to be downregulated (McArdle, Rafferty et al. 2001). The cDNA from the melanoma cells was used to perform qRT-PCR to analyze expression patterns of PTPs.

3.2 Receptor Tyrosine Kinases (RTK)

In a multicellular organism, cells constantly receive signals from other cells and the environment and thereby transmit the information from the extracellular environment into the cell. Often cell surface receptors of signaling molecules are required. One such class of signaling molecules are the polypeptide growth factors, which often stimulate cell proliferation and also induce cell differentiation and migration. The growth factors selectively interact with the respective receptors on the cell surface of the target cell. The receptors often posses tyrosine kinase activity, and are therefore called receptor tyrosine kinases (RTK). They are transmembrane glycoproteins which bind their cognate ligands and dimerize to get intracellularly phosphorylated at tyrosine residues. This enhances tyrosine kinase activity and creates docking sites for adaptor proteins which are phosphorylated in turn. The signal is transduced via a number of signaling proteins to activate transcription of genes required e.g., for cell proliferation or survival.

The RTKs can be classified into 20 subfamilies based on the kinase domain sequence (Reilly 2003). The insulin growth factor receptor (IGF-1R) belongs to the RTK class II which is the insulin receptor family. RTK class III comprises of c-Kit, FLT3, PDGFR, all

of which contain ligand binding extracellular region containing 5 IgG like domains. The fibroblast growth factor receptors (FGFR-1, FGFR-2, FGFR-3, FGFR-4) belongs to the RTK class IV and contains three extracellular IgG like ligand binding domain. Other RTK families include VEGF receptor family, RET and Trk family of receptors, which are important in neurogenesis.

Non-receptor type tyrosine kinases. Additionally, there are also tyrosine kinases which are non receptor-type (NRTKs). Janus kinases (JAKs) and Src kinases belong to NRTKs, being cytosolic and are important mediators of signal transduction by some surface receptors in the cell.

3.2.1 Fms-like tyrosine kinase (FLT3)

Expression of FLT3

FLT3 is a RTK which is expressed on the surface of hematopoietic progenitor cells. FLT3 plays important role in hematopoiesis. In murine hematopoiesis, FLT3 expression is restricted to the multipotent and the lymphoid progenitor stages at which cells are incapable of self-renewal, whereas, in human hematopoiesis, the expression of FLT3 is extended to early lymphoid, myeloid and granulocytic/macrophage progenitors (Kikushige, Yoshimoto et al. 2008). FLT3 belongs to class III family of RTK of which c-Kit, c-FMS, PDGFR-α/β are other important members. Human FLT3 gene is present on chromosome 13(13q12) and encodes a 993 amino acid protein of which 572-603 represents the juxtamembrane domain and 604-958 represents the tyrosine kinase domain (Rosnet, Schiff et al. 1993). The FLT3 protein is usually detectable in cells in two forms, which are, a 158-160 kDa membrane bound “mature” form which is complex glycosylated and a 130-143 kDa “immature” form which contains high mannose carbohydrate chains (Schmidt-Arras, Bohmer et al. 2005). In unstimulated state, when FLT3 is not bound to its ligand (FL), it is present as a monomeric protein on the plasma membrane. The interaction between FLT3 and FL activates signaling cascades driving transcription of genes involved in proliferation and differentiation of early hematopoietic

cells. Despite its widespread expression in early hematopoietic cells, FLT3 knockout mice show largely normal hematopoiesis. The only effects observed were reduction in pro B-cell and pre B-cell compartments (Mackarehtschian, Hardin et al. 1995).

Structure and function of FLT3

The FLT3 receptor comprises an extracellular domain consisting of 5 IgG-like domains, a single transmembrane region, one cytoplasmic juxtamembrane domain (JM) and one intracellular tyrosine kinase domain (TKD) interrupted by a kinase insert. Upon FL binding, the FLT3 receptor forms a homodimer on the plasma membrane. Receptor dimerization results in phosphorylation of tyrosine residues in the juxtamembrane and intracellular domains, and activation multiple signaling effectors in the cell involved in proliferation and differentiation. FL-stimulation of the receptor results in activation and recruitment of adaptor proteins Grb2 to Shc (see Figure 3-1). Activated Shc serves to couple FLT3 activation to RAS-MAPK pathway. FLT3 activation also causes association of phosphatidylinositol phosphatase SHIP to phosphorylated Shc. Activated FLT3 therefore signals through RAS-MAPK pathway activating the extracellular signal related kinase 1/2 (ERK1/2) to induce proliferation. Activation of FLT3 induces binding of p85 subunit of adaptor proteins Gab1 and Gab2 (Grb2 associated binder)-family proteins. They further associate and with SHP-2 and p85 and form an activated complex. The catalytic subunit p110 of PI3K is activated leading to activation of AKT (Schmidt-Arras, Schwable et al. 2004).

3.2.2 Aberrations in FLT3

About 70-100% AML and B-lineage acute lymphoblastic leukemia (ALL) cases express high levels of FLT3 (Gilliland and Griffin 2002), where it plays a role in survival and proliferation of blasts. Activating mutations of FLT3 are one of the most frequently occurring genetic lesions in AML after NPM1 which results in poor prognosis. The two main mutation classes of FLT3 which have been reported are, point mutations occurring in the activation loop of tyrosine kinase domain, and internal tandem duplications (ITD) occurring in the juxtamembrane domain (see Figure 3-2). The ITD mutations have been

observed in the non juxtamembrane domain of the receptor (Breitenbuecher, Schnittger et al. 2009).

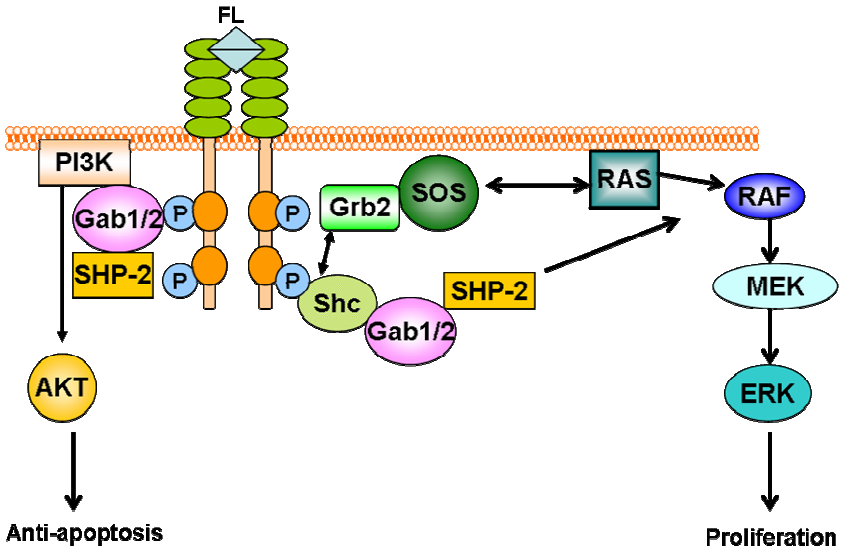


Figure 3-1. FLT3-mediated signaling. The association of FLT3 with its ligand FL induces receptor dimerization on the cell membrane and phosphorylation of tyrosine residues on the receptor. This in-turn activates PI3K and RAS pathways. The activated receptor interacts with Grb2 through Shc and activates Grb2 which binds SOS. The Grb2-SOS complex is recruited to the membrane associated RAS. This activates the MAPK pathway which includes RAF, MEK and ERK proteins. FL-stimulation of FLT3 activates the Gab1/2, and activates the PI3K-AKT pathway. Phosphorylated ERK and AKT result in activation of transcription factors involved in cell proliferation and anti-apoptosis.

Kinase domain mutations in the activation loop (TKD)

Missense point mutations have been observed most commonly at position Asp835 in the activation loop of FLT3 and have been reported in 7% AML, 3% myelodysplastic syndrome (MDS) and 3% of ALL patients (Gilliland and Griffin 2002). In the inactive form of FLT3 receptor, the activation loop is folded which blocks access of ATP and substrate to the kinase domain. As the receptor binds to the ligand, the loop unfolds and the tyrosine residues are available for phosphorylation. D835Y and other TKD mutations adopt an active configuration of the receptor resulting in constitutive active form in the absence of FL. This increases the intrinsic kinase activity of FLT3 resulting in cell proliferation. The biology of these point mutations closely resembles that of the wild type

FLT3 receptor. Much similarity between the two forms have been reported (Stirewalt and Radich 2003)

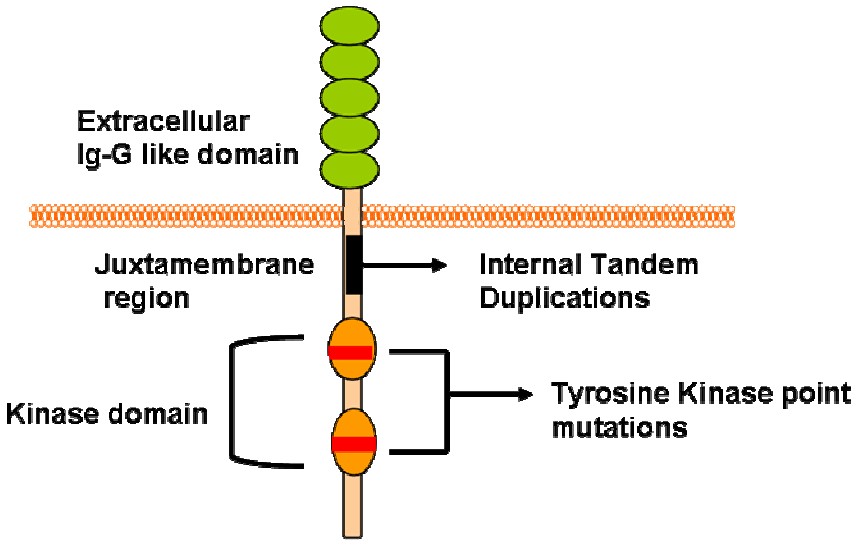


Figure 3-2. Structure of FLT3 and commonly occurring mutations. A schematic representation of FLT3 receptor which expresses primarily as a membrane bound from. It comprises an extracellular domain with 5-IgG like chains. A membrane spanning transmembrane region, juxtamambrane region and intracellular kinase domain with a kinase insert. The ITD mutation (shown in black) occurrs in the juxtamembrane region and point mutations (shown in red) occur in the kinase activation loop.

Internal Tandem Duplications (ITD)

ITD mutations constitute the most common mutations of FLT3 occurring in 15-35% of patients with AML and 5-10% of patients with MDS. They are formed as a result of duplications of sequence encoding the juxtamembrane region of FLT3. The duplications always in-frame and vary from 3-400 base pairs (Stirewalt and Radich 2003). More recently, ITD mutations have been reported in the tyrosine kinase domain (Breitenbuecher, Schnittger et al. 2009). The JM domain stabilizes the inactive conformation of FLT3 making the kinase domains inaccessible for phosphorylation (Griffith, Black et al. 2004). The association of FL with FLT3 WT results in dimerization of the receptor and consequent phosphorylation of specific tyrosine residues in the JM domain. This unfolds the inactive form of FLT3 thereby activating downstream activation. FLT3 ITD mutations result in loss of the autoinhibitory conformation of the

receptor which results in spontaneous activation and phosphorylation of other tyrosine residues in the cytoplasmic domain of FLT3. The FLT3 ITD mutation has been shown to activate STAT5 (Mizuki, Fenski et al. 2000), in addition to RAS-MAPK and PI3K-AKT pathway. This appears at least in part related to alterations in FLT3 ITD trafficking, notably accumulation of the receptor in the intracellular compartments in an immature form (Schmidt-Arras, Bohmer et al. 2009) (Choudhary, Olsen et al. 2009). It has been shown that FLT3 ITD confers factor independent growth and colony formation in 32D cells which are dependent on IL3 for growth. FLT3 WT sustains growth but no colony formation of 32D cells in presence of FL. Additionally, 32D cells stably transfected with FLT3 ITD and not FLT3 WT result in leukemia like disease when injected in syngenic C3H/HeJ mice.

3.2.3 Inhibitors of FLT3

FLT3 represents as an interesting target for chemotherapy in AML patients as it is expressed in most of the malignant blast cells. Moreover, FLT3 ITD confers a poor prognosis (Knapper 2007). Also, the signaling proteins involved in the FLT3 pathway have been implicated in tumorigenesis. Small molecule compounds were discovered for treatment of AML after the success of imatinib mesylate (Gleevec) in chronic myeloid leukemia (CML) therapy (Kantarjian, Sawyers et al. 2002). These compounds inhibit the tyrosine kinase activity of FLT3. Some of the compounds like CEP701, PKC412, and SU11248 have entered early phase clinical trials. All the FLT3 inhibitors are heterocyclic compounds which mimic the purine ring of adenosine in ATP and compete with ATP for binding to the kinase domain of the receptor. Initial observations with the PDGFR-family selective compound AG1296 showed that treatment resulted in inhibition of FLT3 activity in primary AML blasts harboring FLT3 ITD mutations (Levis, Tse et al. 2001) and also reduced STAT5 and ERK phosphorylation (Tse, Allebach et al. 2002). CEP701 and PKC412 are more potent but less selective FLT3 inhibitors with good oral bioavailability, and independent studies have shown that both are active against FLT3 WT and mutant forms of FLT3 (Knapper 2007). SU11248 (Sunitinib) is a hydrophobic broad spectrum tyrosine kinase inhibitor. The kinase inhibitor Sorafenib induces growth

arrest and apoptosis in AML blast with FLT3 ITD mutations and has been shown to be more potent in primary AML blast harboring FLT3 ITD mutations than FLT3 WT (Zhang, Konopleva et al. 2008). However, the use of small molecule inhibitors targeted against the kinase, can result in resistance of the AML blasts caused by secondary FLT3 mutations. One particular mutation G697R in the FLT3 resulted in resistance to many compounds like PKC412, SU11248, and compounds similar to CEP701 (Parcellsa, Ikedaa et al. 2006). Other mutations like N676K, A627E and F691L in the activation loop also confer resistance to FLT3 inhibitors (Cools, Mentens et al. 2004).

3.3 AML derived cell lines

A number cell models are used to study function of RTKs and growth factors in the development of hematopoietic cells and in cell transformation. A few of the cell lines used in this study are enlisted below.

32D cells represent an immortalized myeloblast line derived from bone marrow of C3H/HeJ mice. The most commonly used pool of 32D cells is the sub clone 3 (32Dcl3). They are cytokine dependent cells, and can proliferate in presence of IL3 only. Removal of IL3 results in apoptosis. 32D cells expressing FLT3 WT were created by transfection and show FTL3 phosphorylation in presence of FL. As mentioned above, transfection with FLT3 ITD results in IL3 independent growth, colony formation and myeloproliferative disease (MPD) upon injection in C3H/HeJ mice (Fenski, Serve et al. 2001). For the studies carried out in this thesis 32D cells with stable expression of murine FLT3 WT were used (Grundler, Thiede et al. 2003).

Ba/F3 cells are murine bone marrow-derived immortalized pro-B cells which are also cytokine dependent. When grown in presence of IL3, Ba/F3 cells are resistant to apoptosis and cytotoxic drugs. They can likewise be transfected with FLT3 or can be made IL3 independent when stably transduced with FLT3 ITD (Razumovskaya, Masson et al. 2009).

The THP-1 cell line was derived from the peripheral blood of a patient with acute monocytic leukemia. It is an immortalized human monocytic cell line which differentiates and becomes adherent when induced with phorbol esters or vitamin D3. THP-1 cells express large amounts of FLT3 WT receptor on the surface.

The EOL-1 cell line was derived from the blood of a patient with acute eosinophilic leukemia. EOL-1 expresses the fusion gene FIP1L1-PDGFRα. and FLT3 WT (Cools, Quentmeier et al. 2004).

The RS4-11 cell line was derived form the bone marrow of a relapse patient with AML characterized by a t(4:11) chromosomal aberration and an isochromosome for long arm of chromosome 7(7q). This cell line exhibits lymphoid and myeloid characteristics (Stong, Korsmeyer et al. 1985). It is a factor independent cell line expressing FLT3 WT.

The MV4-11 cell line was derived from a 10 year old patient with a biphenotypic B cell myelomonocytic leukemia characterized by the chromosomal aberration t(4:11). It is a model cell line frequently used for studying FLT3 ITD (Quentmeier, Reinhardt et al. 2003).

RS4-11 and MV4-11 cells are often used to compare FLT3 WT and FLT3 ITD signal transduction.

3.4 Protein Tyrosine Phosphatases (PTP)

PTPs are important enzymes that control cellular tyrosine phosphorylation. For example, they serve to counterbalance the effect of RTKs, by dephosphorylating the receptors and downstream signaling proteins, thereby maintaining a steady state of signaling in the cell (Östman and Böhmer 2001). PTPs have a characteristic active site with a conserved HCX_5R sequence motif. The conserved cysteine residue is required for catalysis. Protein tyrosine phosphatases are highly complex and diverse enzymes and around 100 genes

encoding for PTPs belong to the PTP superfamily. This superfamily comprises of classical PTPs and dual specificity phosphatases (Tonks 2006).

The classical protein tyrosine phosphatases (PTP) are enzymes with phospho-tyrosine-specific dephosphorylation activity. There are 38 PTP genes in mouse and rat and 37 PTP encoding genes in human genome. Classical PTPs are of two types; transmembrane or receptor like PTPs (RPTP) and non-transmembrane. Most of the RPTPs have two intracellular phosphatase domains, a membrane proximal D1 and membrane distal D2 domains. While the D1 domain harbors the catalytic activity, the D2 domain is presumably essential for maintaining the specificity and stability of the enzyme. For PTP-α, activity has also been demonstrated for the D2 domain. The non-transmembrane cytoplasmic PTPs e.g. SHP-1, SHP-2, PTP1B have regulatory sites in the vicinity of catalytic domain which regulate the activity and localization of the enzymes.

Dual specificity phosphatases are a diverse group of enzymes which exhibit activity against phosphoserine, phosphothreonine and phosphotyrosine residues in proteins. Some members of this family e.g., PTEN dephosphorylate preferentially non-protein substrates. A prominent sub-class of dual specificity phosphatases is the MAPK phosphatases (MKPs). MKPs have varied subcellular locations and substrate e.g., DUSP1/MKP1 is a nuclear phosphatase with specificity for p38 and JNK. DUSP6/MKP3 is cytosolic and has preferential activity towards ERK (Pulido and van Huijsduijnen 2008). Generally, the MKPs are capable of dephosphorylating pTyr and pThr in the activation loop of the different MAPKs. Other important members belonging to dual specificity phosphatases are PRLs, and slingshots (SSH). Some phosphatases which are termed as pseudoPTPs contain the conserved core features of PTP domain but do not exhibit activity due to replacement of the residues required for catalysis. Prototypical member of the psuedophosphatases is Styx, which lacks catalytic activity in which the catalytic cysteine is replaced by glycine, and Myotubularins (MTMs) which constitute the most prevalent group among the pseudoPTPs.

3.4.1 Mechanisms of PTP regulation

As PTPs have a critical role in many signaling pathways, there must be mechanism(s) by which PTPs are regulated. Various mechanisms have been identified (den Hertog, Östman et al. 2008). RPTPs posses extracellular domains and are presumably regulated by ligand interaction. Studies are ongoing in search of ligands for various PTPs. Heparan sulphate proteoglycans are proposed ligands for PTP sigma, and for LAR, the matrix proteins laminin and nidogen complex serve as ligands (Stoker 2005). RPTPs are also subject to regulation by dimerization which may either activate or inactivate the phosphatase. Unlike in the case of RTKs, ligand binding-induced dimerization of RPTPs results at least in some cases in inactivation by masking the active intracellular catalytic domain. For PTP-α, it has been shown that it exists as an active homodimer on the cell surface. Binding to a ligand leads to an inactive enzyme conformation. So far, physiological ligands for PTP- α have not yet been identified as reviewed in Stoker 2005. Another important mechanism by which the activity of phosphatases is regulated is by oxidation of the catalytic cysteine. Reactive oxygen species (ROS) such as hydrogen peroxide which can be generated through various cellular processes can oxidize the active cysteine in the catalytic domain of the PTP in vicinity to the ROS source. The oxidized form of the enzyme is rendered reversibly inactive by the process (Tonks 2005).

3.4.2 PTPs in leukemia

PTPs have been implicated in a number of physiological functions such as proliferation, migration, and survival because of regulatory effects on the RTKs and other tyrosine kinases. Several PTPs have now been recognized as either putative suppressors of tumors or as oncogenes as reviewed in (Östman, Hellberg et al. 2006). For example, SHP-1 (PTPN6) may function as a tumor suppressor in lymphomas where the SHP-1 promoter is often hypermethylated. Likewise, the CpG islands in the GLEPP1 (PTPRO) promoter are hypermethylated in hepatocellular carcinomas and lung cancers. Allelic loss of DEP-1 encoding gene *PTPRJ* occurs in colon, lung and breast cancers. Gain of function mutations in non transmembrane SHP2 resulting in excessive SHP-2 (PTPN11) activity can be the cause of Juvenile myelomonocytic leukemia (JMML). PRL3 (PTP4A3) is

shown to be upregulated in metastatic colon cancer. The cytoplasmic phosphatase PTP1B is involved in multiple signaling pathways where it has both positive and negative regulatory effects. For example, mice with PTP1B (PTPN1) depletion show significant delay in ErbB2 induced tumor progression (Julien, Dubé et al. 2007). In contrast, PTP1B behaves as a tumor suppressor in *PTP1B*$^{-/-}$ *Trp53*$^{-/-}$ mice (Dube, Bourdeau et al. 2005).

PTPs in FLT3 signaling: The SH2 domain PTPs SHP-1 and SHP-2 are often involved in RTK mediated intracellular signaling events in the cell (Neel, Gu et al. 2003). It has been proposed that SHP-1 expression is suppressed in FLT3 ITD transformed TF-1 cells and reduced expression of SHP-1 may contribute to elevated phosphorylation of the FLT3 ITD receptor in AML cells (Chen, Levis et al. 2005). SHP-1 can possibly dephosphorylate FLT3 when overexpressed (Schmidt-Arras, Bohmer et al. 2005). The role of SHP-2 in FLT3 mediated signaling has been investigated by shRNA mediated depletion. It was observed that SHP-2 in murine 32D cells contributes to the FL-dependent ERK activation and proliferation but that it is not indispensable for FLT3 ITD mediated transformation (Müller, Schönherr et al. 2008). Also, role of PTP PEST, and PTP1B in regulation of FLT3 was investigated by co-expressing the phosphatases with FLT3, which resulted in dephosphorylation of the receptor. This observation indicated a possible negative regulation by these PTPs (Schmidt-Arras, Bohmer et al. 2005). In the course of this thesis, further PTPs were implicated in FLT3 signaling comprising DEP-1, CD45 and DUSP6. They shall be briefly described in the following chapters.

3.4.3 Density Enhanced Phosphatase (DEP-1)/ PTPRJ/ CD148

DEP-1 is a receptor like PTP with a molecular mass of 180-220 kDa. It is expressed in many cell types like epithelial cells, endothelial cells and fibroblasts, in addition to hematopoietic cells (Autschbach, Palou et al. 2002). As shown in Figure 3-3, it comprises eight fibronectin type III repeats in the extracellular domain, a transmembrane domain and a single catalytic PTP intracellular domain (Ostman, Yang et al. 1994). As the name implies, DEP-1 expression was shown to be increased when cultures of same cell type achieve high density which was accompanied by an increase in phosphatase activity. This

is observation may be indicative of the role of DEP-1 in regulation of cell-cell contact, and cell density-dependent growth inhibition. In hematopoietic cells, DEP-1 has been shown to negatively regulate T-cell activation, and inhibit proliferation in macrophage cell lines (Baker, Majeti et al. 2001). Frequent deletion of a PTPRJ allele has been noted in human lung, colon and breast cancer, suggestive of a role as a tumor suppressor (Ruivenkamp, van Wezel et al. 2002). However, no tumor formation was observed in DEP-1 knockout mice (Trapasso, Drusco et al. 2006), which may suggest the need of additional mutations for induction of tumors in the mice.

3.4.4 Cluster of Differentiation 45 (CD45)/ PTPRC

CD45 is a receptor like PTP comprising an extracellular domain, a transmembrane region and an intracellular domain with two phosphatase domains; the enzymatically active D1 domain and an inactive D2 domain (see Figure 3-3). The CD45 isoforms comprise of proteins with molecular mass ranges from 180-220 kDa. CD45 is the most abundantly present as a surface glycoprotein and is expressed by all nucleated hematopoietic cells.

It plays an important role in the immune cells being involved in the development and activation of lymphocytes (Hermiston, Zikherman et al. 2009). The glycosylation of CD45 is highly regulated. Also, it exists in different isoforms due to alternate splicing of exon 4, 5, and 6 which encode for the extracellular regions A, B and C (CD45RABC). A non-glycosylated low molecular weight form also exists, which is designated as, CD45RO. The activity of CD45 is regulated by changes in isoform expression, glycosylation of the ectodomain, and negatively regulated by receptor dimerization. Development of autoimmune disease has been shown for mice expressing a gain of function mutation of CD45 due to impaired regulation by dimerization (Hendriks, Elson et al. 2008).

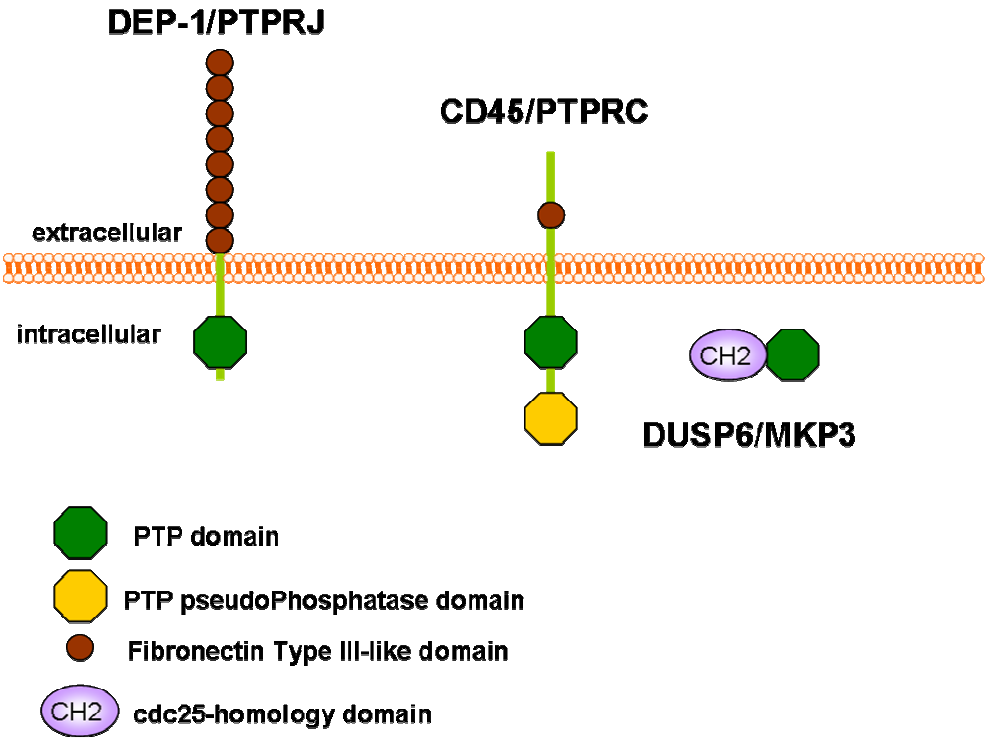


Figure 3-3. Schematic representation of PTPs; DEP-1, CD45, and DUSP6. Both, DEP-1 and CD45 are transmembrane PTPs, containing an extracellular fibronectin type III domain (shown in brown), transmembrane region and intracellular phosphatase domain; the active PTP domain (shown in green) and an inactive pseudoPTPase domain (shown in yellow). DUSP6 is a cytosolic PTPase comprising of an active PTPase domain and N-terminal CH2 domain (shown in pink) thought to play a role in substrate binding.

3.4.5 Dual specificity phosphatase 6 (DUSP6)/ MAP kinase phosphatase 3 (MKP3)

DUSP6 is a cytoplasmic dual specificity phosphatase (see Figure 3-3) which dephosphorylates ERK2, and is involved in the negative feedback regulation of RAS-MAPK pathway. DUSP6 is highly specific for ERK, and the specific interaction is mediated by the kinase interaction motif (KIM) domain in the N–terminal non-catalytic region of DUSP6. Physiologically, it is important in the cellular homeostasis in response to growth factors. DUSP6 null mice show an increase in the basal signaling of ERK1/2 in multiple tissues (Maillet, Purcell et al. 2008). DUSP6 mRNA and protein expression is induced by growth factor stimulation of cells via activation of ERK, this has been shown

in case of stimulation of PDGFR (Jurek, Amagasaki et al. 2009). Interference in this negative feedback loop may result in malignant growth, activating the RAS-MAPK pathway. Hypermethylation in the DUSP6 promoter results in DUSP6 downregulation and this phenomenon has been associated with a number of pancreatic cancers. A role of DUSP6 in lung cancer has been studied extensively, where downregulation of DUSP6 correlates with tumor progression (Pulido and van Huijsduijnen 2008). Over expression of DUSP6 reduces tumor growth in these cells. In ovarian cancers, loss of DUSP6 expression due to oxidative stress results in high ERK1/2 activation (Chan, Liu et al. 2008). In non-small lung cancer, DUSP6 is overexpressed, correlating with poor prognosis as reviewed by (Pulido and van Huijsduijnen 2008).

4 Aim of the project

I. The downstream signaling pathway of FLT3 WT is already known to an extent but the factors which regulate the activity of FLT3 remain to be understood. PTPs are physiological regulators of kinase activity and little is known about the role of PTPs in FLT3 signaling. To identify the PTPs involved in regulation of FLT3 signaling, gene expression of relevant PTPs was suppressed using shRNA. PTP depletion was to be done in a hematopoietic cell line; 32D myeloid cells stably expressing the FLT3 WT. By using lentiviral mediated delivery of shRNA targeting a panel of PTPs, stable cells with phosphatase knockdown should be created and the PTPs depleted lines screened to select a putative negative regulator of FLT3. The biological role of any found PTP regulating FLT3 signaling was to be studied.

II. Another aspect which was planned to undertake as a part of thesis was to perform expression analysis of PTPs in human AML blasts and cell lines expressing FLT3 WT and FLT3 ITD. PTPs showing differential expression patterns in the WT and FLT3 ITD should then be selected to study this expression regulation in murine cell lines; 32D cells and Ba/F3 cells expressing FLT3 WT and FLT3 ITD. To explain pathways of regulation, the effects of treatment with FLT3 ITD signaling pathway inhibitors was planned as further steps.

5 Materials and Methods

5.1 Antibodies and reagents

Primary Antibodies

Antibody	Type	Dilution	Source	Cat. No.
Anti-mupY591	Rabbit polyclonal	1:1000	Cell Signaling, Frankfurt, Germany	3474
Anti-muDEP-1	Goat polyclonal	1:1000	R&D systems, Wiesbaden, Germany	AF1934
Anti-Phospho ERK	Mouse monoclonal	1:1000	Cell Signaling, Frankfurt, Germany	9106S
Anti-ERK	Mouse monoclonal	1:1000	BD Transduction Lab, Hiedelberg, Germany	M12320
Anti-Phospho AKT	Rabbit polyclonal	1:5000	Cell Signaling, Frankfurt, Germany	4058S
Anti-AKT	Rabbit polyclonal	1:1000	Cell Signaling, Frankfurt, Germany	9272
Anti-PhosphoSTAT5	Rabbit monoclonal	1:1000	Epitomics	1208-1
Anti-STAT5	Rabbit monoclonal	1:1000	Cell Signaling, Frankfurt, Germany	9310
Anti-β-actin	Mouse monoclonal	1:5000	Sigma Aldrich, Diesenhofen, Germany	A5441
Anti-vinculin	Mouse monoclonal	1:1000	Biozol Diagnostica, Eching, Gemany	BZL0310 6
Anti-muDUSP6	Rabbit polyclonal	1:1000 (1X TBST+1% nonfat milk)	Kindly provided by Dr. Johan Lennartson, Sweden	

Anti-huFLT3 (S-18)	Rabbit polyclonal	1:1000	Santa Cruz, Heidelberg, Germany	sc-480
Anti-huFLT3 (C-20)	Rabbit polyclonal	1:1000	Santa Cruz, Heidelberg, Germany	sc-479
Anti-muFLT3	Goat polyclonal	1:1000	R&D systems, Wiesbaden, Germany	AF768
Anti-SHP-1 (C-19)	Rabbit polyclonal	1:1000	Santa Cruz, Heidelberg, Germany	sc-287
Anti-SHP-2 (N-16)	Rabbit polyclonal	1:1000	Santa Cruz, Heidelberg, Germany	sc-424

Site specific antibodies for phospho-huFLT3 were kindly provided by Prof. Lars Rönnstrand, Malmö, Sweden. All the primary dilutions were prepared in 1X TBST (see chapter 5.7.3) pH7.6 with 1% BSA and 0.02% sodium azide and stored in 4°C. The membranes were incubated at 4°C with primary antibodies overnight for detection of FLT3, DEP-1 and all phosphoproteins for best results. For other proteins at least 1hr incubation with primary antibody was required.

Secondary Antibodies

Secondary antibodies were prepared fresh in 1 X TBST pH7.6 with 1% BSA buffer and the membranes were incubated 1hr with the secondary antibodies.

Antibody	**Type**	**Dilution**	**Source**	**Cat. No.**
Anti-Goat IgG-HRP	Donkey polyclonal	1:10,000	Santa Cruz	sc-2056
Anti-Mouse IgG-HRP	Goat polyclonal	1:10,000	Medac GmbH, Wedel, Germany	074-1806
Anti-Rabbit-IgG-HRP	Goat polyclonal	1:10,000	Medac GmbH, Wedel, Germany	074-1506

Chemicals

Reagents	Source
FLT3 ligand	PeproTech Ltd., London, UK (AF-300-19)
IL3	PeproTech Ltd., London, UK (Cat.No. 213-13)
G410 sulphate	PAA, Coelbe, Germany (P25-011)
PEI	Exgen Fermentas 500
AG1295	Axxora Deutschland GmbH (ALX.270-035)
1020	Kindly provided by Prof. Siavosh Mahboobi, Regensburg, Germany
Wortmannin	Axxora Deutschland GmbH (ALX.350-020)
U0126	Tocris, UK (Cat. No.1144)
STAT5 inhibitor	Calbiochem, UK (Product. No. 573108)

All the chemicals and reagents used for buffer preparation and solutions were purchased from Roth GmbH and Sigma-Aldrich, except otherwise mentioned.

Plasmids and oligonucleotides

Mission® shRNA lentiviral vectors: pLKO.1-puro Vectors were purchased from Sigma-Aldrich. The packaging plasmids required for the lentivirus production pMDL, pVSV, pRSV were kindly provided by Dr. Carol Stocking, Hamburg (Dull, Zufferey et al. 1998).

Target PTP	Source/Reference	Clone ID	Sequence
pLKO.1-puro non-targeting control	Sigma Aldrich-SHC002		CCGGCAACAAGATGAAGAGCACCAACTCG AGTTGGTGCTCTTCATCTTGTTGTTTTT
shRNA DEP-1/PTPRJ (A2)	Sigma Aldrich-TRCN0000029955	NM_008982.2-470s1c1	CCGGCCGAATCCTATATTTGACATTCTCG AGAATGTCAAATATAGGATTCGGTTTTT
shRNA DEP-1/PTPRJ (A3)	Sigma Aldrich-TRCN0000029956	NM_008982.2-2688s1c1	CCGGCCTCGGATACTTATGTCACATCTCG AGATGTGACATAAGTATCCGAGGTTTTT
shRNA CD45/PTPRC (B6)	Sigma Aldrich-TRCN0000029931	NM_011210.1-3188s1c1	CCGGCCTTGTTCAATCTCTTGGAAACTCG AGTTTCCAAGAGATTGAACAAGGTTTTT
shRNA CD45/PTPRC (B7)	Sigma Aldrich-TRCN0000029932	NM_011210.1-960s1c1	CCGGGCCTAATAATGTGACCAGTTTCTCG AGAAACTGGTCACATTATTAGGCTTTTT
DEP-1 siRNA (sense)	Qiagen-VC300A2		5'-UACUGUGUCUUGGAAUCUAdGdC-3'
DEP-1 siRNA (antisense)	Qiagen-VC300B2		5'-UAGAUUCCAAGACACAGUAdGdG-3'

List of qRT-PCR primers for murine (mu) and human (hu) genes used:

Gene	Source	Cat. No.
mu-DEP-1/PTPRJ	Qiagen	QT00169785
mu-CD45/PTPRC	Qiagen	QT00139405
mu-OSTPTP/PTPRV	Qiagen	QT01070930
mu-PTP-alpha/PTPRA	Qiagen	QT00141610
mu-GLEPP1/PTPRO	Qiagen	QT00134540
mu-SHP-1/PTPN6	Qiagen	QT00155967
mu-SHP-2/PTPN11	Qiagen	QT00103362
mu-PTP1B/PTPN1	Qiagen	QT00166418
mu-DUSP1/MKP1	Qiagen	QT00288638
mu-DUSP2/PAC1	Qiagen	QT00248283
mu-DUSP4/MKP2	Qiagen	QT00140357
mu-DUSP6/MKP3	Qiagen	QT00101997
mu-DUSP8	Qiagen	QT00118650
mu-LMW/ACP1	Qiagen	QT00135135
mu-PTP4A1/PRL	Qiagen	QT00156464
mu-MTMR5/SBF1	Qiagen	QT01549450
mu-PTPN2/TCPTP	Qiagen	QT01063573
mu-TNS1	Qiagen	QT01077643
mu-ACTB	Qiagen	QT01136772
mu-PSMB3	Qiagen	QT00103649
mu-CANX	Qiagen	QT00152579
hu-DUSP1/MKP1	Qiagen	QT00036638
hu-DUSP2/PAC1	SABiosciences	PPH00049A
hu-DUSP4/MKP2	SABiosciences	PPH00058A
hu-DUSP6/MKP3	Qiagen	QT00209986
hu-DUSP8	Qiagen	QT00009212
hu-LMW/ACP1	Qiagen	QT00022820
hu-PTP4A1/PRL	Qiagen	QT00028203
hu-MTMR5/SBF1	SABiosciences	PPH11198E
hu-PTPN2/TCPTP	SABiosciences	PPH21327A
hu-DEP-1/PTPRJ	Qiagen	QT00043876
hu-ACTB	Qiagen	QT00095431
hu-PSMB3	Qiagen	QT00014483
hu-CANX	Qiagen	QT00092995

5.2 Cell culture

All the cells used were kept in a humidified 37°C incubator with 5% CO_2 and maintained by changing medium every 3-4 days. *Cell freezing*- no less than 1 X 10^6 cells were frozen in polystyrene containers in complete medium containing 10% serum and 10% DMSO. The cryovials were stored in -80°C for couple of days before placing in -150°C. For retrieving the cells, the freezings were thawed and centrifuged to remove the medium

containing DMSO; the cells were suspended in fresh medium and incubated. The requirements for different cell lines were as follows:

5.2.1 32D cells

32D cells is a myeloid cell derived from mouse and the most commonly used pool of 32D cells which is the sub clone 3, was used for the study. This IL3 dependent cell line was bought from The German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. 32D cells were maintained in 1640 RPMI with HEPES (Biochrome Berlin, Germany) supplemented with 10% heat inactivated FCS (BioWest, Berlin, Germany), 1 mM sodium pyruvate (PAA, Cölbe, Germany), and 2.5 ng/ml murine recombinant IL3. 32D cells expressing muFLT3 WT or ITD receptor and GFP from a bicistronic mRNA were kindly provided by Dr. Justus Duyster and Dr. Grundler, Münich, Germany (Grundler, Miething et al. 2005).

5.2.2 Ba/F3 cells

Ba/F3 cells are derived from mouse bone marrow and are growth factor dependent. The cells were cultured in 1640 RPMI with HEPES (Biochrome) supplemented with 10% heat inactivated FCS, 1 mM sodium pyruvate, 2.5 ng/ml murine recombinant IL3. Parental Ba/F3 cells without FLT3 expression and FLT3 WT or ITD expressing cell lines were kindly provided by Prof. Lars Rönnstrand, Malmö, Sweden (Razumovskaya, Masson et al. 2009).

5.2.3 Human derived AML cells lines: MV4-11, THP1, EOL1, RS4-11 cells

These are factor independent cells and were maintained in 1640 RPMI with glutamine (PAA) supplemented with 10% heat inactivated (hi) FCS.

5.2.4 HEK293T cells

HEK 293 cells are adherent cells originally derived from Human Embryonic Kidney cells which are transformed with adenovirus. These cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) from GIBCO, Invitrogen, Karlsruhe, Germany,

supplemented with 10% FCS. The cells were trypsinized with Trypsin –EDTA solution (PAA).

5.3 Generation of PU.1-luciferase reporter cell line

The plasmid DNA pXP2-PU.1-*bsr* was generated by digesting the Blasticidine S-resistance gene (*bsr*) along with the SV40 and EM-7 promoters from pcDNA6/TR (Invitrogen, Karlsruhe, Germany) by using NgoMIV restriction enzyme, and by ligating into the pXP2PU.1 vector back bone (plasmid were kindly provided by Frank Rosenbauer, Germany) which was linearized with BstZII171 restriction enzyme. Standard cloning protocol followed from the Molecular cloning Manual (Sambrook and Russell, CSHL Press) and all the enzymes required for cloning were purchased from New England Biolabs (NEB, Frankfurt, Germany) or Jena Biosciences (Germany). Stable cell pool of 32D FLT3 WT-pXP2PU.1 was generated by electroporating the above generated plasmid respectively using a BioRad-Gene Pulser^{TM} (U.S) electroporator. In summary, actively growing cells were plated in the density 2 X 10^5 cells/ml 48 hr prior to transfection. For each transfection 6 X 10^6 cells were suspended in 300µl medium containing 10% FBS and transferred in an electroporation cuvette. 20 µg DNA was added to the cuvette and electroporation was performed at 300 Volts, 960 Faradays. The cells were plated in the density of 2.5 X 10^5 cells in medium containing IL3 and selected with blasticidine in the final concentration of 15 µg/ml (stock 1 mg/ml) after 24 hr. Single cell clones giving the maximum luciferase reporter activity were selected by limited dilutions and used in transcription based screening of PTP depletion lines.

5.4 Lentivirus production

The shRNA constructs targeting murine PTP and non targeting shRNA constructs were purchased from Sigma-Aldrich (MISSION® shRNA lentivirus mediated transduction system). The viruses were produced in HEK293T cells by the following protocol. The cells were seeded at the density of 1.5 X 10^6 cells/10 ml in 94 X 16 mm cell culture dishes in DMEM/F12 with 10% FCS and penicillin/streptomycin one day prior to transfection. At the following day, the medium was carefully discarded and replaced with

serum free DMEM/F12 medium. The cells were transfected with the DNA cocktail, which was prepared in serum free DMEM/F12 medium containing the plasmid pLKO.1vectors (10 µg) and the helper plasmids pRev (5 µg), pEnv-VSV-G (2 µg) and pMDLg (10 µg) (kindly provided by Dr. Carol Stocking, Hamburg, Germany). The transfection reagent used was Polyetheylemine (PEI). The PEI was added to the DNA in the ratio of 2.5:1 and vortexed briefly. The DNA-PEI mixture was incubated at room temperature for 25-30 minutes. The mixture was then added drop wise on the cells. The plate was incubated for 8 hr in 37° C with 5% CO_2, after which the medium was replaced by DMEM/F12 with 10% FCS. After 24hr incubation, the medium containing virus was collected and filtered sterile by using a 0.2 micron filter. Fresh medium was added to the cells and incubated. A second harvest was done after 24 hr, and stored in a separate tube. A total of three virus harvests were prepared and pooled for concentrating the virus. The pooled virus harvest was transferred to the Amicon concentration tubes and centrifuged at 3000 g at room temperature for 5 minutes till the final volume of 1 ml was achieved. The concentrated virus obtained was used directly for infection. Aliquots of the virus were made and stored at -80° C for later use.

5.5 Phosphatase knockdown

5.5.1 Stable depletion: Lentivirus mediated transduction

32D cells expressing muFLT3 WT were suspended in complete medium and seeded in 12 well-plates at the density 0.3 X 10^6 cells per well. 100 µl of column concentrated pseudoviral particles were added to the cells in addition to polybrene and penicillin/ streptomycin (final concentration 1X). The plate was then centrifuged in a Heraeus Multifuge 1S centrifuge (ThermoScientific) for one hour at 500 g at 25° C and placed for six hours in the incubator at 37°C with 5% CO_2. Viral infection was done three times after which the cells were suspended in fresh medium to remove polybrene. The selection was done after 48 hr with 2 µg/ml puromycinfor 7 days after which the cells were used for desired experiments.

5.5.2 siRNA mediated transient knockdown

Human THP-1 cells were transfected with DEP-1 siRNA duplex oligonucleotides or control siRNA (Qiagen, Hilden Germany). The transfection was performed by using AMAXA® Nucleofector® Technology and Transfection solutions (Lonza, Cologne, Germany). 1.5 X 10^6 cells were used from a well growing culture and centrifuged at 900 rpm for 5 minutes. The medium was discarded and the cell pellet was suspended in 100 µl AMAXA/LONZA nucleofectin transfection solution V. The cell solution was added to 2.1 µg siRNA solution (8 µl of 20 µM) and transferred to electroporation cuvette. Electroporation was performed using the program V-01 in the nucleofector and 500 µl RPMI medium with 10% hi FCS. The transfected cells were plated in a 6well-plate containing 500 µl RPMI with 10% hi FCS and penicillin/streptomycin and incubated. After 24 hr, fresh complete medium was added to the cells and incubated for 48 hr. The cells were starved in RPMI with 0.5% hi FCS for 4 hr prior to stimulation with FL (50 ng/ml) for varying time points. Cells were lysed and analyzed for knockdown and signaling proteins.

Flow cytometry and cell sorting: - 32D cells were sorted on the bases of similar GFP level in order to get similar FLT3 receptor levels with the use of an ARIA FACS cell sorter (Becton Dickinson, Heidelberg, Germany).

5.6 Luciferase based reporter assay

32D muFLT3 WT expressing cells with PU.1 luciferase reporter were washed and suspended in 1640 RPMI Cells were counted and distributed equally in culture flasks. The cells were incubated for 3 hr at 37°C after which they were induced with FL (20 ng/ml) and IL3 (5 ng/ml). Aliquots of equal volumes were taken after 2 hr till 6 hr and the cells were lysed in Passive Lysis Buffer®, Promega (Cat.no.E1941). All other chemicals required for the assay were purchased from Promega. Lysis was performed according to manufacturer's instructions. The lysates were plated in flat bottom 96 well-plate and 100 µl of luciferase assay buffer was injected in the wells to measure the

luciferase reporter activity (according to the protocol described in Dyer et al 2000) in stimulated and non stimulated cells.

Luciferase firefly buffer
25 mM Glycyl-Glycine
15 mM K2HPO4 (pH 8.0)
15 mM MgSO4
4 mM EGTA
0.05% NaN3
1 mM DTT, 2 mM ATP, 75 µM Luciferin and 0.1 mM CoA were added prior to use.

5.7 Western blotting

5.7.1 Preparation of the lysates

The cells were harvested and washed with 1 X PBS pH 7.4 and lysed in RIPA lysis buffer. One million cells were lysed in 30 µl lysis buffer. The cells were placed on ice for 10 minutes for lysis and the lysates were cleared by centrifugation at 13000 rpm for 15 minutes in a cooling centrifuge. The supernatant was transferred to a fresh tube and was then subjected to protein determination using the BCA protein detection kit from ThermoScientific Pierce (Bonn, Germany). Once the protein amounts were determined, equal amounts of the protein (30 µg) was used for loading the acrylamide gel for electrophoresis. The lysates were stored at -80°C in case of later use.

RIPA lysis buffer
50 mM Tris pH 7.5
1% NP-40
0.25% Deoxycholate
1 mM EDTA
150 mM NaCl

Protease inhibitors were added freshly: 1 mM PMSF, 1 µg/ml Aprotinin, 1 µg/ml Leupeptin, 1 mM Sodium Orthovanadate (freshly boiled), and 2 µg/ml PepstatinA.

5.7.2 Preparation and electrophoresis of the samples

Equal amounts of lysates were taken and the volume of all the samples was adjusted similar. To this 6X gel loading buffer was added and the samples were boiled for 5 minutes at 95°C. The samples were loaded on 10% acrylamide gels for signaling experiments and 8% acrylamide gels for DEP1 and FLT3 protein blots. The gels were run with 1X SDS running buffer at constant current till the dye front reached the end of the gel.

5.7.3 Transfer of proteins to the PVDF membranes

Transfer of protein from gel to membrane was done by the semi-dry method. After the electrophoresis, the gel was carefully removed from the plates and rinsed with water. Meanwhile, the whatman filter papers were soaked in cathode buffer and anode buffer separately. The polyvinylidene fluoride (PVDF) membrane (Millipore, Eschborn, Germany) was first wetted with methanol and equilibrated in cathode buffer. The gel was placed in cathode buffer for a minimum of 5 minutes. A sandwich of filter papers, membrane and gel was prepared and arranged in the following order:

Bottom (Anode)

Filter papers soaked in Anode buffer

Membrane

Gel

Filter papers soaked in Cathode buffer

Top (cathode)

The air bubble between the gel and membrane were removed by rolling a falcon tube and excess buffer was blotted away. The semi dry transfer apparatus (Millipore) was arranged in a way that the current passes from the cathode (black) to the anode (red). The transfer was done at a constant voltage of 17 Volts for 1 hr and 10 minutes. At the end of transfer, the membrane was rinsed in water to get rid of the buffer. For a quick check before blocking the membrane, Ponceau S solution was poured over the membrane and kept for

1 minute. The Ponceau was kept back for reuse, and the membrane was washed with water briefly and analyzed for traces of any air bubbles during transfer. The membrane was blocked with 1X TBST with 1% BSA for one hour. The membrane was then incubated in primary antibody in a specific dilution (see chapter primary antibodies) for 1-2 hr for pan proteins antibodies and overnight for phosphoproteins antibodies. Following incubation with primary antibody, the membrane was washed thrice for 10 minutes with 1X TBST and incubated with secondary antibody for 1hr. For developing, the membrane was washed thrice for 10 minutes with 1X TBST to remove any traces of secondary antibody. Visualization of bands was done after adding Western lightening, Chemiluminiscent Reagent (Perkin Elmer Life Sciences; Boston, USA) on the membrane, and incubating for 1 minute. Pictures were taken at various exposures times in the Fujifilm LAS4000. The images were quantified by densitometric analysis using MultiGuage software.

To reprobe the membrane for other proteins, the membrane was placed in stripping buffer for 30 minutes at 56°C. The membrane was washed with water briefly and thrice with 1X TBST to remove traces of β-mercaptoethanol after which it was probed with another primary antibody.

6X Sample buffer:
30% β-Mercaptoethanol
40% Glycerol
6% SDS
Bromophenol blue

5X SDS running buffer
2 M Glycine
247 mM Tris
0.5% SDS
1X solution was used for gel electrophoresis

Cathode buffer

0 322 mM Tris

40 mM Aminocproic acid

20% Methanol

0.005% SDS

Anode buffer

300 mM Tris

20% Methanol

10X TBST

100 mM Tris

1.5 M NaCl

0.5% Tween-20

1X solution was used from 10X stock

Strip buffer

100 mM Tris

2% SDS

0.8% β-mercaptoethanol

pH was adjusted to 6.8

5.8 Cell signaling and inhibitor treatments

For cell signaling experiments, the cells were washed with RPMI medium without serum and IL3 and counted. Nearly 5 X 10^6 cells were used per experiment. The cells were then starved in incomplete medium for 4 hr and then stimulated at 37°C for different time points with FLT3 ligand FL (50 ng/ml). Usually 1 X 10^6 cells were used for stimulation per time point. The cells were kept on ice after stimulation and centrifuged for 3 minutes at 3000 rpm in a pre-cooled centrifuge (4°C). The cell pellet was lysed in RIPA buffer

with freshly added protease inhibitors and placed on ice for 10 minutes after which the lysates were subjected to SDS-PAGE and western blotting.

For FLT3 immunoprecipitation, approximately 28 X 10^6 cells were used. The cells were washed and resuspended in RPMI medium without serum and incubated at 37°C for 4 hr. After starvation, the cells were centrifuged and suspended in 4 ml of RPMI medium without serum and stimulated with FL (100 ng/ml) for the time points 2.5, 5 and 10 minutes. The cells were placed on ice after stimulation and centrifuged in a pre-cooled centrifuge (4°C) at 2000 rpm for 4 minutes after which the cells were washed with ice cold PBS. The cells were lysed in IP lysis buffer supplemented with protease inhibitors on ice for 20 minutes and centrifuged at 14000 rpm at 4°C for 10 minutes. FLT3 immunoprecipitations were performed by incubating lysates with 0.5 µg of S-18 anti-FLT3 antibody at 4°C overnight, followed by incubation with Protein A- or Protein G–Sepharose beads (Sigma Aldrich). For each immunoprecipitation 1 mg Protein A-Sepharose was used. The immunoprecipitates were incubated at 4°C for 1 hr and centrifuged 1000 rpm at 4°C for 1 minute. They were washed three times with 750 µl IP lysis buffer and suspended in 15 µl of 2X sample buffer before subjecting to SDS-PAGE and western blotting.

For inhibitor treatments, 32D FLT3 ITD cells and MV4-11 cells were used. Approximately, 12 X 10^6 cells were washed and starved overnight in 10% hi FCS containing medium and serum free RPMI medium respectively. Inhibitors were added after starvation to the cells in desired concentration. For the control, cells were treated with DMSO, which was solvent for all of the inhibitors used. The cells were taken out at specific time points and washed before proceeding with preparation of cell lysates (1 X 10^6 cells) and RNA (5 X 10^6 cells).

Buffer for IP
1% NP-40
20 mM HEPES pH 7.4
0.15 M NaCl

2 mM EDTA
1 mM EGTA

Protease inhibitors were added freshly: PSMSF (1:100), Aprotinin (1:100), Leupeptin (1:1000), PepstatinA (1:1000), Sodium orthovanadate (1:1000).

5.9 Cell proliferation

5.9.1 Green Fluorescent Protein-based proliferation assay

As the 32D cells are stably transfected with IRES containing bidirectional vector with genes *flt3* and *gfp*, there is similar expression of muFLT3 WT and GFP. By monitoring the amounts of GFP is reflective of the cell proliferation and therefore can be used as a method to assay cell growth. The cells were washed twice with 1640 RPMI medium and suspended in 10% heat inactivated FCS and 1X penicillin/streptomycin. Cells were counted and diluted to get desired concentration. Cells were then plated in Micro-Assay-opaque 96 well-plates with transparent bottoms (REF. 655098, Greiner, Frickhausen, Germany) in the concentration of 20,000 cells per well per 200 µl. The cytokines FL and IL3 were added in the concentration 20 ng/ml and 2 ng/ml, respectively. The plate was then placed in a humid chamber and incubated at 37°C with 5% CO_2. For the measurement, the plate was taken out and read in the TECAN infinite 200® plate reader (Crailsheim, Germany) at 485-520 nm. The experiment was performed in triplicates, average of three values and standard error was plotted on the graph.

5.9.2 MTT assay

32D were washed twice with 1640 RPMI medium without serum and suspended in 0.5% heat inactivated FCS and 1X penicillin/streptomycin. Cells were counted, diluted in desired cell concentration and plated in clear flat bottom 96 well-plates in the density of 20,000 cells per well per 100 µl. Cytokines FL and IL3 were added to the wells in the final concentration of 20 ng/ml and 2 ng/ml, respectively. The plate was kept in a humid chamber and placed in 37°C incubator with 5% CO_2. MTT (Methylthiazolyldiphenyl-tetrazolium bromide) was added to each of the wells in the concentration of 50 µg per

100 µl (stock 5 mg/ml) and the plate was incubated again for 4 hr. After 4 hr of incubation, 100 µl of solubilization buffer was added to all the wells and the plate was incubated overnight at 37°C. The plate was then read at 570 nm in a multiwell plate reader (Anthos Hill®). The experiment was performed in triplicates, average of three values and standard error was plotted in the graphs.

Solubilization buffer
10% SDS
10 mM HCl

5.10 Apoptosis assay

32D cells were washed twice with RPMI 1640 to get rid of cytokines in the culture medium. Approximately, 1 X 10^6 cells /ml were seeded in two 12 well-plates. One of the plates was irradiated with 5 Gray units of γ radiation using the Gammacell 40, MDS Nordion radiator in Fritz Lipmann Institute, Jena and the other was not irradiated and used as a zero hour control. After the irradiation, 10% heat inactivated FCS, 1X penicillin/streptomycin, and cytokines FL and IL3 in the concentration of 50 ng/ml and 1 ng/ml respectively were added to the wells. The plate was then incubated for 24, 48, and 96 hr. The cells from non irradiated plate were stained to access the viability by using AnnexinV PE and 7-AAD (BD Pharmingen, Heidelberg, Germany) following manufacturer's instructions. Similarly, the cells after 24, 48, and 96 hr of irradiation were also stained. The cells were analyzed in FACS Canto cytometer (Becton Dickinson, Heidelberg, Germany) using FlowJo software (BD Biosciences, Palo Alto, CA).

5.11 Transformation assay

5.11.1 Methylcellulose assay

32D cells were washed twice with IMDM (Iscove's Modified Dulbecco's Medium, PAA). The cells were suspended in IMDM medium and counted. The assay mix for three wells was prepared in UV crosslinked sterile tubes as follows:

2 ml 2.3% Methylcellulose

46 µl Penicillin/streptomycin

920 µl heat inactivated FCS

1.17 µl IMDM medium

Due to the semi solid nature of methylcellulose and the difficulty to pipette it, disposable plastic pipettes were used.

To the assay mix cell suspension was added in the final concentration of 3000 cells. The cytokines FL and IL3 were added in the concentration of 20 ng/ml and 2.5 ng/ml respectively. The preparation was mixed carefully so as to avoid air bubbles, and 1.2 ml was added to each well of a 12 well-plate. The plate was placed in a humid chamber and incubated at 37°C with 5% CO_2. The plates were taken out 6 day for taking pictures. In some cases, the plates were incubated longer for development of colonies. The pictures were taken using Zeiss AxioCam HRC digital camera attached to a Zeiss Axiovert 25 inverted microscope.

5.11.2 Animal Experiments

Eight to ten-week old male C3H/HeJ mice, syngenic to 32D cells, were used to assess the *in vivo* development of leukemia-like disease (Müller, Schönherr et al. 2008). The cells were washed with 1X PBS and 2 X 10^6 cells were injected into the lateral tail vein of the animal. The experimental protocols were reviewed and approved by the local Committee on Animal Experimentation (Registration Number: 02-00/09).

5.12 Expression Analysis

5.12.1 RNA and cDNA preparation

Approximately 2 X 10^6 cells were harvested from a well growing culture and centrifuged at 3000 rpm for 5 minutes to pellet the cells. The supernatant was discarded and cell pellet was used for RNA preparation using the RNeasy, Qiagen (Hilden, Germany) RNA preparation kit. To digest the DNA in the cells, DNAse treatment was done during the

RNA preparation by using Qiagen DNAse Kit according to the manufacturer's instructions.

Human AML samples: For the phosphatase expression analysis study in human AML the samples characterized for FLT3 WT or FLT3 ITD expression were kindly provided by Prof. Thomas Fischer, Dr. Florian Heidel (University Hospital, Magdeburg), and Dr. Sebastian Scholl (Department of Hematology, University Hospital, Jena). For most of the samples, already isolated RNA from patient blasts was received but for some samples, the frozen blast cells in DMSO containing RPMI medium were obtained. The blasts were thawed and transferred into a fresh tube. The cells were centrifuged at 3000 rpm for 5 minutes at room temperature to pellet the cells and the supernatant was discarded. The RNA was then extracted as described before.

RNA was quantified and checked for quality based on 260:280 ratios and 260:230 ratios. 1 µg RNA was used directly for preparation for cDNA. For most of the experiments, cDNA was prepared using the Fermentas cDNA synthesis kit (Germany) according to manufacturer's instructions.

For the human AML cell lines, the RNA was prepared from three independently growing cultures of MV4-11, THP-1, EOL-1 and RS4-11 cells using the Qiagen RNA preparation kit. For preparation of CDNA from human AML samples and cell lines, a BioRad cDNA synthesis kit (Münich, Germany) was used. The cDNA sample obtained after the preparation was diluted and a final amount of 40 ng of cDNA was used for the real time PCR. The quality of RNA from human AML blasts was monitored by using the Agilent 6000 Nano chip Kit. The assay is based on the electrophoretic mobility of the two subunits of RNA and was done only for RNA samples analyzed by real time PCR at Merck Serono, Geneva.

5.12.2 Real time PCR

The primers for amplification of most of the genes were purchased from Qiagen (QuantiTect® Primer Assay) except for huDUSP2, huDUSP4, huSBF1, huPTPN2,

huPTPN22 and huPTPRR, the primers for which were bought from SABiosciences (Germany). For the RTPCR, Maxima™ SYBR green was purchased from Fermentas (Cat. No.K0221) and the reaction mix were prepared according to manufacturer's instructions. SYBR green master mix was used in the final concentration of 1X (stock 2X), and the final concentration of primer used was 1X (stock 10X). The RT reaction for the AML patient samples and AML cell lines was carried out using FastStart Universal SYBR Green Master mix from Roche (Cat. No. 04913850001, U.S.) in the Applied Biosystems 7900HT Fast Real-Time PCR system (Merck Serono facility, Geneva, Switzerland). For all other reactions an Eppendorf Realplex^4 Mastercycler was used.

The program used for all RT PCR reactions was the same. 95°C for 15 minutes to activate the hotstart *Taq* polymerase; this was followed by 40 cycles of:
Denaturation 94°C for 15 sec
Annealing 55°C for 30 sec
Extension72°C for 30 sec
The Threshold Cycle (Ct) was determined after the completion of RTPCR and calculations for the relative expression of a gene were done.

For AML samples and AML lines: The samples were grouped on the basis of FLT3 WT or ITD expression. Three housekeeping genes (HKG) namely; PSMB3, CANX, and β-actin were used and delta Ct was calculated by taking the difference of mean Ct of gene under study and the average Ct of three HKG. The relative expression of the gene was depicted as a percentage of:
$100/2^{(\text{delta Ct})}$

Subsequently, all the relative percentage expression values were pooled from all the samples of a group (FLT3 WT or FLT3 ITD) in order to get an average expression and standard error. An example of the calculation sheet is shown in Figure 5-1.

For knockdown of gene expression: The difference in the Ct values (delta Ct) of the shControl cells and PTP knockdown cells for a gene of interest was calculated. Similarly

delta Ct was calculated for the HKG (β-actin) in the control and experimental line. The difference of the resulting values was used for calculating the percentage expression as described.

				Average of all values	Average of HKG 21.014		Average gene - Average House keeping	
				22.933				
human gene name	Well	Position	Sample Name	Ct	Average ct	ST dev CT	Delta ct	% HKG (3 genes)
ACTIN	1	A01	blast A2	**18.791**	18.710	0.114	**-2.304**	**493.700**
ACTIN	2	A02	blast A2	**18.630**				
CANX	3	A03	blast A2	**21.527**	21.617	0.128	**0.603**	**65.836**
CANX	4	A04	blast A2	**21.707**				
PSMB3	5	A05	blast A2	**22.770**	22.715	0.079	**1.701**	**30.766**
PSMB3	6	A06	blast A2	**22.659**				
hDUSP1	9	A09	blast A2	**20.575**	20.527	0.067	**-0.487**	**140.131**
hDUSP1	10	A10	blast A2	**20.480**				
hDUSP4	11	A11	blast A2	**25.391**	25.339	0.073	**4.325**	**4.989**
hDUSP4	12	A12	blast A2	**25.287**				

Figure 5-1. Representative example of the calculations for percentage expression of the PTPs relative to three HKG.

6 Results

6.1 Identification of Phosphatases controlling FLT3 signaling

As outlined in the introduction, FLT3 has important signaling functions in hematopoiesis, as a result of which myeloid cells proliferate and undergo differentiation. Largely, the regulation of FLT3 signaling is unknown, and efforts were made therefore in this project to elucidate phosphatases which control the activity of the receptor. A scheme showing the experimental strategy for identification of PTPs regulating FLT3 signaling is shown in Figure 6-1.

6.1.1 PTP knockdown in 32D cells

To identify the PTP involved in the regulation of the signaling of FLT3, a set of 20 PTPs were selected on the basis of previously reported expression data obtained in 32D cells expressing muFLT3 (Mizuki, Schwable et al. 2003) and additional data kindly provided by Prof. Hubert Serve, Germany. Other factors like expression levels in hematopoietic cells and the proposed role as oncogenes or tumor suppressors were also taken into consideration while making the selection of the phosphatases, e.g.; SHP-1 and HePTP are abundantly expressed in the hematopoietic cells, and SHP-2, PTP1B, and TCPTP are ubiquitously expressed in most tissues. A complete list of all PTPs included is shown in Table 1.

To identify the PTPs involved in the signaling of FLT3 kinase, the selected PTPs were depleted by shRNA in myeloid-derived murine 32D cells expressing muFLT3 WT receptor. At the time of these experiments, four to five non validated shRNA targets were available for every PTP. Lentiviruses containing shRNA expression vectors for different PTPs were prepared and used for stable transduction. Following selection with 2 µg/ml puromycin, the generated cell pools stably expressing shRNAs were used for subsequent experiments.

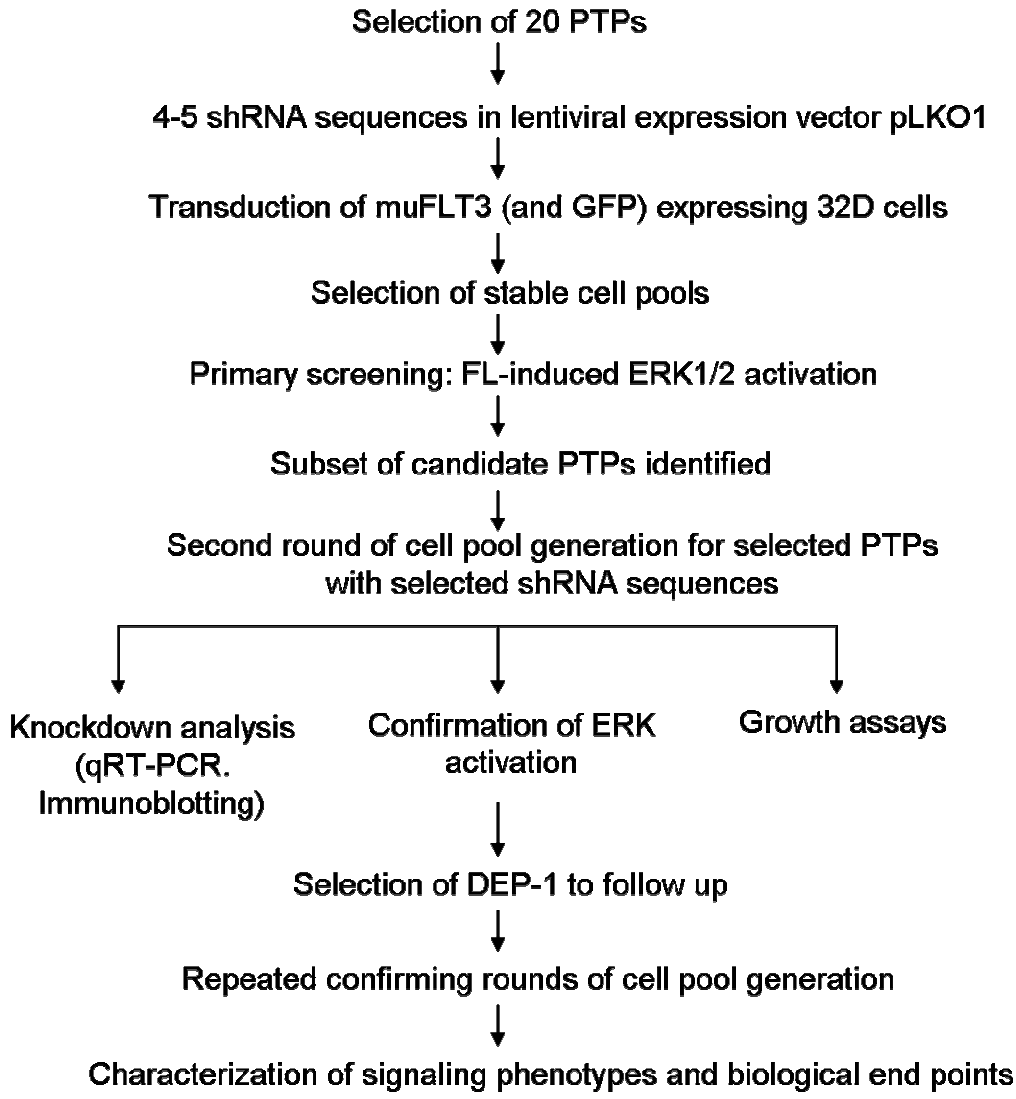


Figure 6-1. Experimental scheme for screening of PTPs involved in regulation of FLT3 signaling.

While, the screening readouts (see below) were analyzed without prior assessing of the achieved knockdown level of the PTP, we analyzed some selected cell pools to obtain an impression of knockdown efficiency. Immunoblotting was performed for a few PTPs to detect reduction in protein levels (see Figure 6-2). Cell lines with different shRNA targets of SHP-1 and SHP-2 knockdowns were used as an example to analyze knockdown of SHP-1 and SHP-2 at protein level. SHP-1 and SHP-2 protein levels were reduced differently by lentiviral mediated shRNA transduction. The lines G6 and F5 showed

marked reduction in SHP-1 and SHP-2, respectively as compared to other lines. The respective protein level in the control cell line with non targeting shRNA was used as a reference for monitoring the protein reduction in knockdown lines.

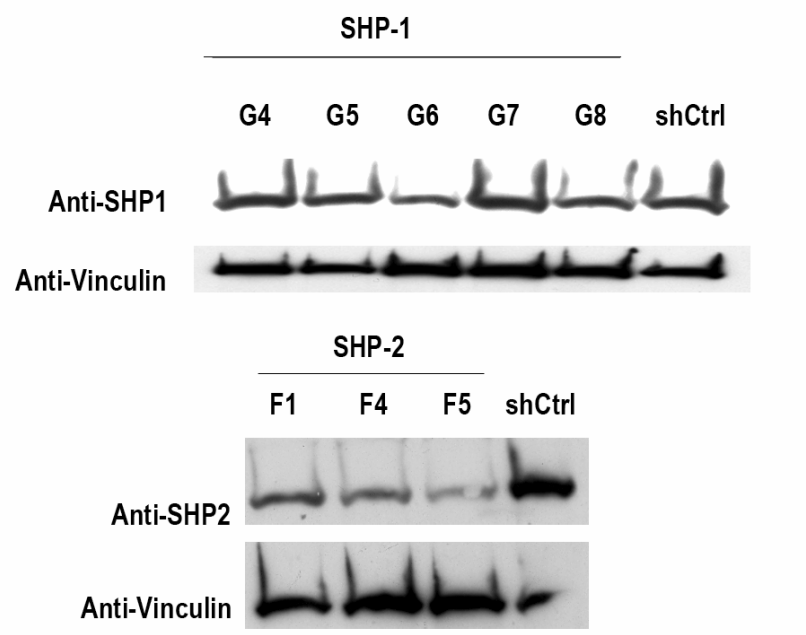


Figure 6-2. Suppression of SHP-1 and SHP-2 at protein level. 32D muFLT3 WT expressing cells transduced with shRNA for SHP-1 knockdown (G4, G5, G6, G7, and G8) and SHP-2 (F1, F4, and F5) were used for analyzing the extent of knockdown. 32D cells with non targeting control shRNA (shCtrl) were used as a control. Equal numbers of cells were lysed in RIPA buffer and run on 10% acrylamide gel. The lysates were subjected to western blotting and probed with antibodies recognizing SHP-1 and SHP-2 respectively. Vinculin was used as a loading control.

These experiments reveal that the knockdown strategy was working in principle, but only a fraction of the targeting sequencing of shRNA were efficient. The extent of knockdown varied among the different PTPs, and different shRNA targets.

6.1.2 Screening of PTP depleted lines

For analysis of effect on FLT3 signaling, different screening strategies were tested, a transcriptional reporter activity and activation of MAPK ERK 1 and 2.

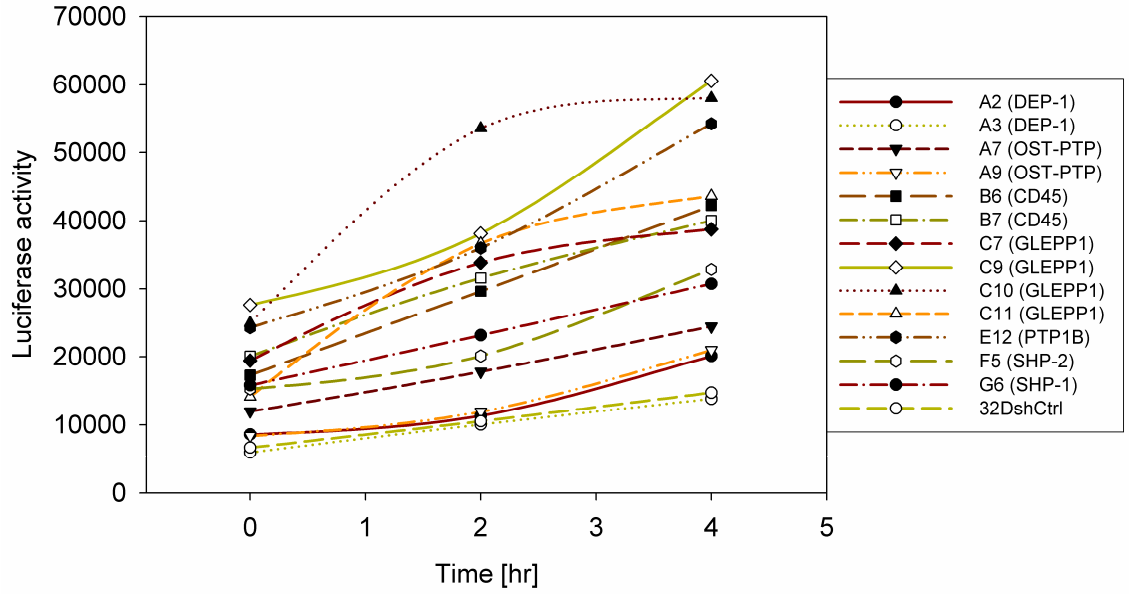
Luciferase reporter-based activity

In order to monitor the effect of PTP depletion in the cell pools using this assay, 32D cells with muFLT3 WT were first stably transfected with a PU.1 luciferase reporter construct to create 32D muFLT3 WT -PU.1 lines. PU.1 is a transcription factor involved in differentiation of monocytes (DeKoter and Singh 2000) and is therefore a downstream effector of FLT3 which is important for monocyte and B-cell differentiation. Activation of the receptor FLT3 by FL results in transcription of PU.1 (Mizuki, Schwable et al. 2003). A reporter construct harboring PU.1 promoter (Rosenbauer, Owens et al. 2005) controlling expression of the firefly luciferase gene (see 5.3) will therefore mediate luciferase expression upon FLT3 activation.

Depletion of putative regulatory PTPs which may regulate FLT3 negatively would result in enhanced PU.1 activity and henceforth higher luciferase activity. Therefore, the cells after stable PTP depletion were stimulated with FL (20 ng/ml) or IL3 (5 ng/ml) for 2 hr and 4 hr after which the luciferase activity was measured. The cells incubated in absence of cytokine showed basal PU.1 transcription whereas the cells treated with IL3 showed increased activity of luciferase as IL3 is a very strong stimulus for PU.1 expression (Mizuki, Schwable et al. 2003). Stimulation with FL resulted in differences in reporter activity in various lines (see Figure 6-3). The elevation of luciferase activity for different targets of GLEPP1 (C9, C10, and C11) was observed to be the highest. There was around 6-fold induction of luciferase activity when compared to the shControl. A 5-fold induction was observed in CD45 knockdowns (B7 and B6 targets).

However, this assay based on transcriptional regulation proved to be cumbersome and not sufficiently reproducible to be used as a robust screening method. Therefore, it was not further employed to screen the lines generated after PTP depletion, instead, probing for phosphorylated proteins such as ERK following stimulation was considered and then established as the method of choice for screening.

A)



B)

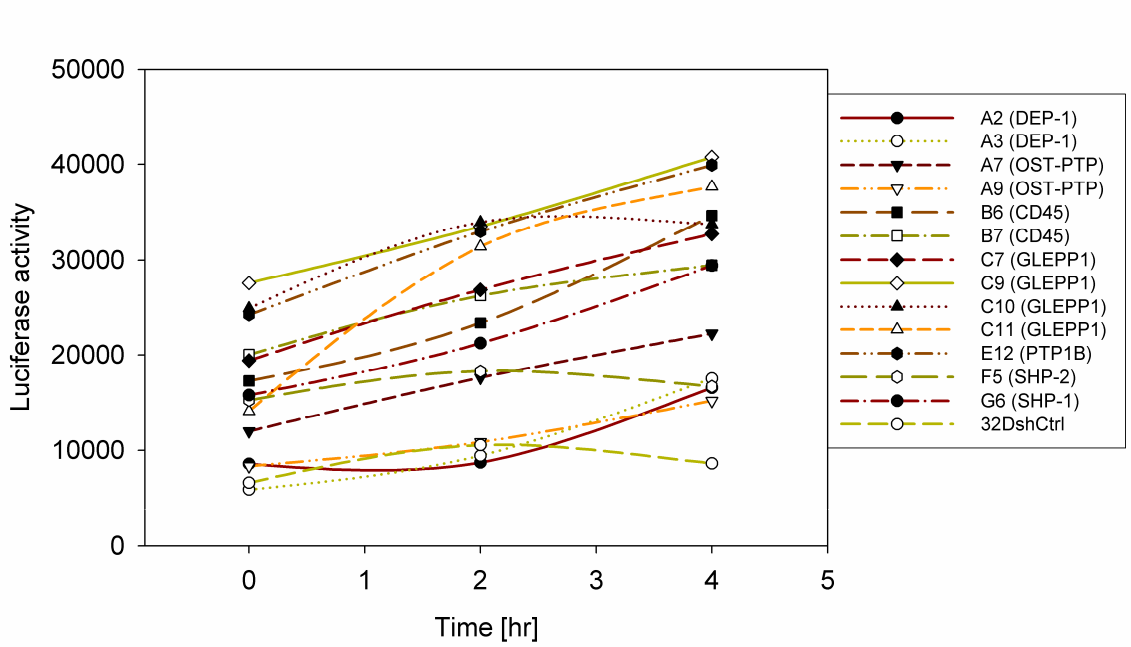


Figure 6-3. Comparison of PU.1-luciferase activity of various PTP knockdown lines. Equal number of cells were washed and starved in serum and IL3 deficient medium for 4 hr. The cells were then stimulated with A) FL or B) IL3 in the concentrations 20 ng/ml and 5 ng/ml respectively and incubated. Equal volume aliquots of cells were harvested after 2 hr and 4 hr and lysed with Passive Lysis Buffer from Promega after which the luciferase activity of the lysates was measured.

Phosphorylated ERK-based screen

32D cells expressing FLT3 WT were infected with lentiviruses expressing shRNAs targeting the selected 20 PTPs. Following the selection of stable knockdowns, the cell pools were screened for the effect on FLT3 signaling by specific FL stimulation. The effect on activated ERK was assayed, while pan ERK blots were used to assess comparable loading. This served as a robust screening strategy for PTP depletion, as activation of RAS-MAPK pathway is directly downstream of FLT3 signaling and depletion of a possibly regulating PTP can influence the pathway.

Example from the screen are shown in Figure 6-4 where cell lines harboring non-targeting shRNA and target for PTP1B (E12), GLEPP1 (C10, C11, C9, and C7), DEP-1 (A2, A3) and CD45 (B6, B7) were compared to the control cell lines after stimulation. The phospho-ERK levels were increased after the cells were stimulated with FL for 10 minutes. The strongest activation though occurs in GLEPP1-C9, the GLEPP1-C10 also shows increased activation when compared with control cell lines. ERK activation was also observed in shDEP-1 (A2, A3) and shCD45 (B6, B7) cell lines. In contrast, targeting of PTP1B did not cause an increase, rather mild decrease of phospho-ERK activity than compared with control cell lines.

After performing the phospho-ERK screen, the different targets were compared and scored qualitatively. The results of the screen were tabulated as shown in Table 1, where the scores were assigned for different targets based on the comparison of phospho-ERK in PTP depleted lines and control line infected with lentiviral particles encoding non targeting shRNA. Phosphatases with the scores of 2 and 3 were considered as positive hits and further followed for validation of knockdown. Among 4-5 shRNA targets of each PTP, only a few showed increase of phospho-ERK levels upon stimulation by FLT3 ligand. Some of the shRNAs resulted in impaired growth e.g., the G10 shRNA for MEG-1 and therefore were not studied further. Candidate PTPs negatively regulating FLT3 that were selected from the screen were, GLEPP1, DEP-1, CD45, OST-PTP, PTP-α and SHP-1. Downregulation of these PTP enhanced the signaling activity of FLT3 in

response to specific stimulation; therefore these PTPs may play a role in negative regulation of FLT3.

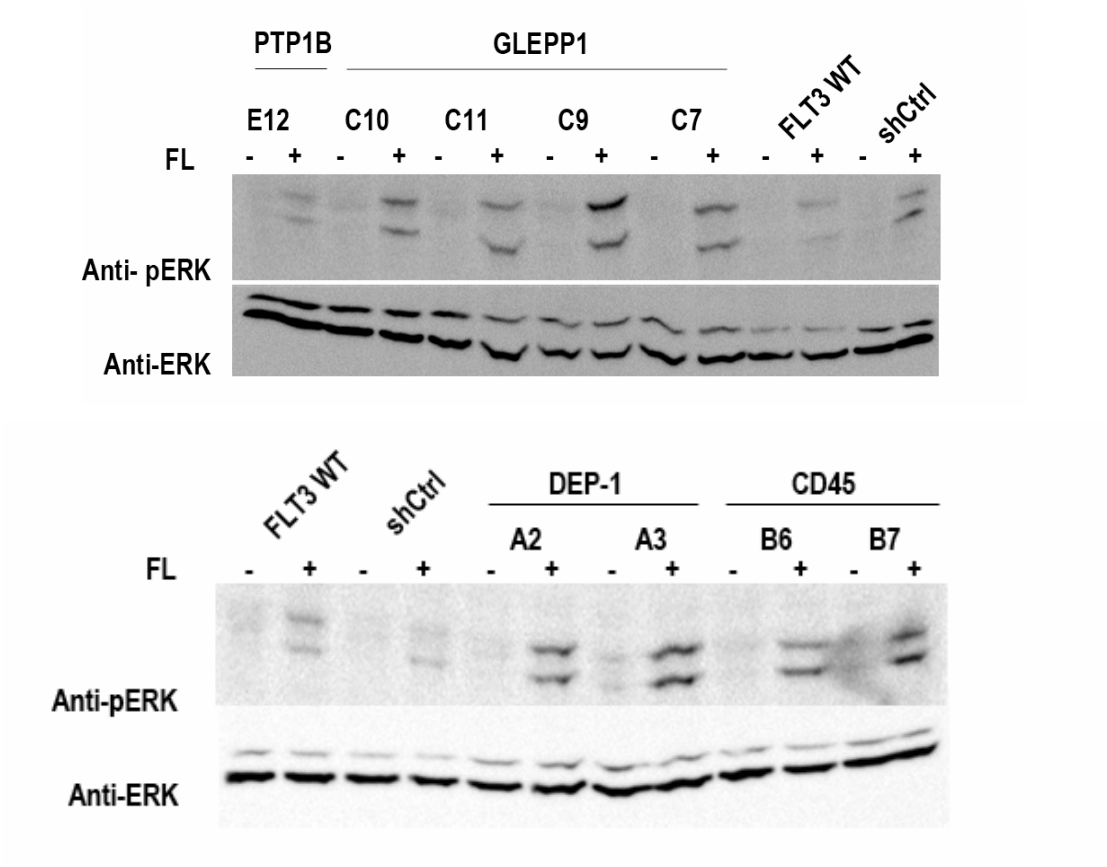


Figure 6-4. Increase in the levels of pERK in PTP depleted 32D muFLT3 WT expressing cells. 32D cells with stable PTP1B knockdown (E12), PTPRO/GLEPP1 (C10, C11, C9, C7), DEP-1 (A2, A3) and CD45 (B6, B7) were washed and starved in serum and IL3 deficient medium for 4 hr. The cells were stimulated with FL (50 ng/ml) for 10 minutes and lysed in RIPA buffer. The lysates were subjected to SDS-PAGE and western blotting and probed with antibodies recognizing pERK and ERK. The lines were compared with 32D cells expressing muFLT3 WT and 32D cells expressing muFLT3 WT and stably transfected with non targeting shRNA.

PTP	shRNA target	Score
OST-PTP (PTPRV)	A6	0
	A7	2
	A8	0
	A9	2
PTP alpha (PTPRA)	A11	2
	A12	1
	B1	-1
	B2	1
	B3	-1
DEP1 (PTPRJ)	A1	2
	A2	3
	A3	2
	A5	1
CD45R (PTPRC)	B4	-1
	B5	-1
	B6	2
	B7	3
	B8	-1
PTP Epsilon (PTPRE)	B9	-1
	B10	-1
	B11	-1
	B12	-1
	C1	1
PTPRL (PTPRU)	C2	-1
	C3	1
	C4	1
	C5	2
	C6	3
GLEPP1 (PTPRO)	C7	2
	C8	-1
	C9	2
	C10	3
	C11	2
PTP Sigma (PTPRS)	C12	-1
	D1	1
	D2	2
	D3	0
PTP beta (PTPRB)	D7	-1
	D8	-1
	D9	-1

TC-PTP (PTPN2)	E3	-1
	E4	0
	E5	-1
	E6	0
	E7	-1
PTPD2 (PTPN14)	E1	-1
	E2	-1
PTP-PEP (PTPN22)	A5	-1
	A6	1
	A7	-1
	A8	2
	A9	2
PTP-PEST (PTPN12)	F6	-1
	F7	-1
	F8	-1
	F9	-1
	F10	-1
FLP1 (PTPN18)	F11	-1
	F12	-1
	G1	-1
	G2	1
	G3	-1
MEG1 (PTPN4)	H2	3
	H3	-1
	H4	-1
	H5	2
	H6	2
MEG2 (PTPN9)	G10	
	G11	-1
	G12	-1
	H1	0
HePTP (PTPN7)	H7	0
	H8	0
	H9	-1
	H10	-1
	H11	0
PTP1B (PTPN1)	E12	-1
SHP2 (PTPN11)	F1	-1
	F5	1
SHP1 (PTPN6)	G6	3

Table 1 Results of the pERK based primary screen. List of the 20 Protein tyrosine phosphatases with the scores assigned based on western blotting result of ERK1/2 activation of FL stimulated and unstimulated lysates of muFLT3 WT expressing 32D cells with stable putative PTP knockdowns. For comparison, muFLT3 WT expressing 32D cells with non targeting shRNA were used. The scores refer to: -1 = Reduction of pERK compared to shCtrl, 0 = similar to shCtrl, 1 = slightly higher than shCtrl, 2 = higher than shCtrl, 3 = strongly enhanced than shCtrl. PTPs which were selected for confirmatory experiments are highlighted in bold.

6.1.3 Validation of PTP knockdown by RT-PCR

To establish whether the effects on FLT3 stimulation had occurred as a result of PTP depletion, a quantification of mRNA was performed for selected PTP candidates. The targets showing effects, primarily, increased phospho-ERK and therefore with high scores, were selected for analysis of mRNA levels. Other PTPs like PTP1B, SHP-2 were chosen additionally to possibly correlate earlier findings in earlier reports hinting at involvement of SHP-2 in FLT3 signaling (Müller, Schönherr et al. 2008).

Real time PCR was carried for eight PTPs, namely; CD45, DEP-1, SHP-1, SHP-2, PTP1B, PTP-α, OST-PTP, and GLEPP1. The knockdown efficiency varied in the different cases very much (see Figure 6-5). A knockdown of PTP1B (E12) and GLEPP1 (C9, C10) could not be confirmed by this method (not in the figure). Also, knockdown of SHP-1 (G6) and SHP-2 (F5) was not efficient. The knockdown of CD45 (B6, B7), DEP-1 (A2), OST-PTP (A9) and PTP-α (A11) was moderate. In all these cases, 50-60 % knockdown was determined in the cells obtained in the first round of preparation of stable knockdown lines.

Evidently, some of the effects on the ERK signaling in the cell lines where knockdown failed were “false-positive” results. Still, several cell lines with established knockdown were subjected to further investigation.

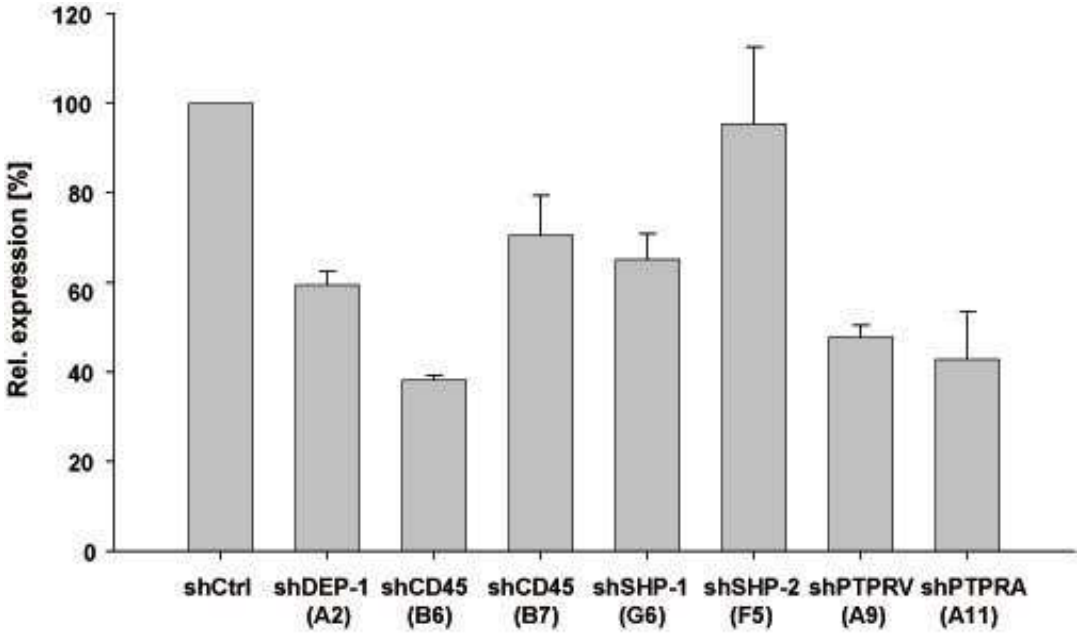
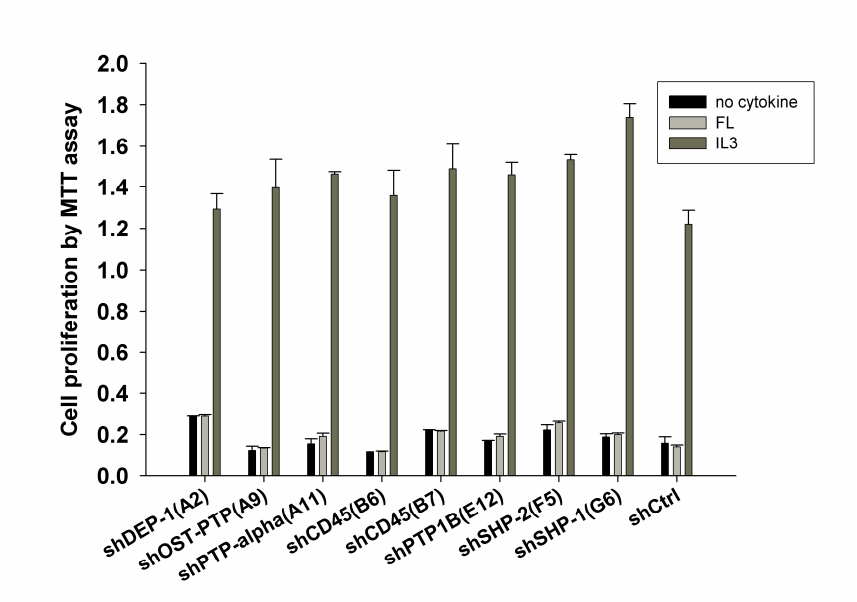


Figure 6-5. Verification of phosphatase knockdown by real-time PCR. Different pools of PTP targeting shRNA expressing muFLT3 WT 32D cells were harvested from which the RNA was extracted and quantified. 1 µg of RNA was used as input for cDNA preparation. The cDNA was used for detection of mRNA levels of knockdown PTP in a real time PCR. Respective primer assay was used for each knockdown cell line and β-actin was taken as a reference gene unaffected by lentiviral transduction. 32D shCtrl cells were used as a reference for calculation of relative expression in the knockdown lines. The relative expression was plotted as percentage (n = 2; mean±SD).

6.1.4 Improved growth effects upon depletion of some PTP

As a preliminary biological readout of phosphatase depletion, the growth characteristics were monitored by performing MTT assays and GFP-based proliferation assays (see Figure 6-6). The former detects the metabolic activity and therefore correlated with cell number and viability. The latter takes advantage of GFP expression in all cell lines, driven by a bicistronic Flt3-GFP expression construct. The proliferation experiment was conducted in parallel to verification of the PTP knockdown by real-time PCR, therefore all the PTP depleted cell pools namely, DEP-1 (A2), OST-PTP (A9), PTP-α (A11), CD45 (B6, B7), PTP1B (E12), SHP-2 (F5) and SHP-1 (G6) were used. The differences in the proliferation of 32D shControl cells and phosphatase depleted lines in response to FL were compared.

A)



B)

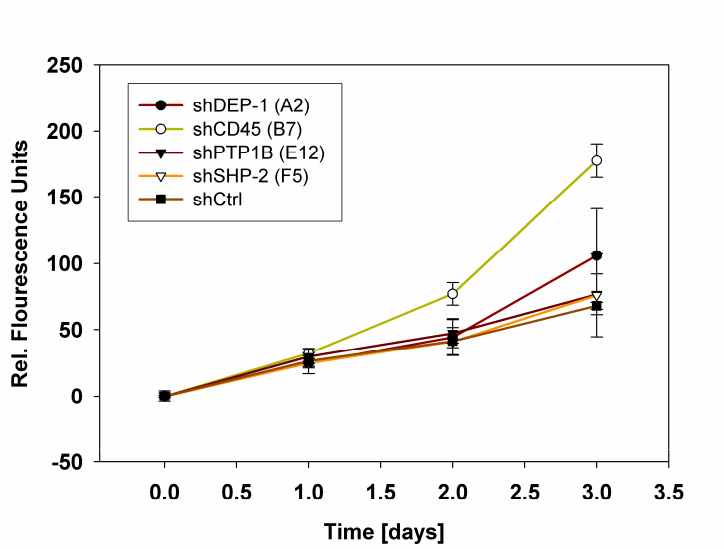


Figure 6-6. Proliferation of 32D cells with depletion of various PTPs. A) The cells were washed and seeded in 96 well-plates in the density of 20,000 cells /well in the presence of FL (20 ng/ml) or IL3 (2 ng/ml) or absence of growth factor and 0.5% heat inactivated serum and incubated for 48 hr after which MTT assay was performed. The absorbance was measured at 570 nm. The knockdown lines shDEP-1 (A2), shOST-PTP (A9), shPTP-α (A11), shCD45 (B6, B7), shPTP1B (E12), shSHP-2 (F5) and shSHP-1 (G6) were compared with the control transfected cell lines (shCtrl). B) GFP-based proliferation assay. Cells were washed twice and seeded in 96 well plates in the density of 20,000 cells /well in presence of FL (20 ng/ml) and 10% heat inactivated serum. The plate was incubated in a humid chamber for 3 days. GFP measurement was made on the day 0 (the day of seeding), day 1, day 2, and day 3. Cell lines shDEP-1 (A2), shCD45 (B7), shPTP1B (E12), and shSHP-2 (F5) were compared with the shCtrl.

The cell proliferation was terminated after 48 hr in MTT assay (see Figure 6-6 A). Overall, the tendency to proliferate in presence of IL3 was retained by all cell lines. The growth was compromised in cells in the absence of any growth factor, whereas, only limited proliferation could be observed in the presence of FL. The growth of DEP-1 and CD45 depleted cells was improved in presence of FL after 48 hr as compared to the shControl cell line. This indicates that, the increased activation of ERK seen in the primary screen correlates to the improved cell proliferation in PTP depleted lines particularly in DEP-1 (A2) and CD45 (B7).

Another growth assay was performed based on the green fluorescent protein for the following set of PTPs: DEP-1, CD45, PTP1B, and SHP-2 (see Figure 6-6 B). The data presented here shows the growth in the presence of FL over three days. It can be observed that the lines A2 and B7 show improved growth compared to the shRNA control cell line. The cells expressing PTP1B and SHP-2 targeting shRNA did not show any growth alteration which could be due to little or no PTP depletion in the respective cells. Based on the growth effects observed, only DEP-1 (A2) and CD45 (B7) were selected for further studies

6.1.5 DEP-1 and CD45: potential candidates for regulating FLT3 activity.

Enhanced downstream signaling in DEP-1 and CD45 depleted lines

DEP-1 and CD45 appeared to be putative regulators of FLT3 based on the screen results for ERK1/2 activation and growth patterns. Therefore, efforts were made to improve downregulation by performing new lentiviral transfections. Particularly, shRNAs A2 (DEP-1) and B7 (CD45) were effective previously and were therefore chosen for further experiments.

The new set of stable knockdown lines were produced which showed efficient down regulation of mRNA levels of DEP-1 and CD45. There was about 80% reduction in the expression in the knockdown (see Figure 6-7).

The signaling of 32D cells expressing FLT3 WT with DEP-1 and CD45 depletion was then analyzed by stimulating the starved cells with FL for 10 minutes. Activation of effectors of RAS-MAPK and PI3K-AKT was observed after stimulation in all cell lines (see Figure 6-8). The phosphorylation of ERK was enhanced in A2 and B7 lines. A weak elevation of phosphorylated AKT was also observed but this effect was not robustly reproducible.

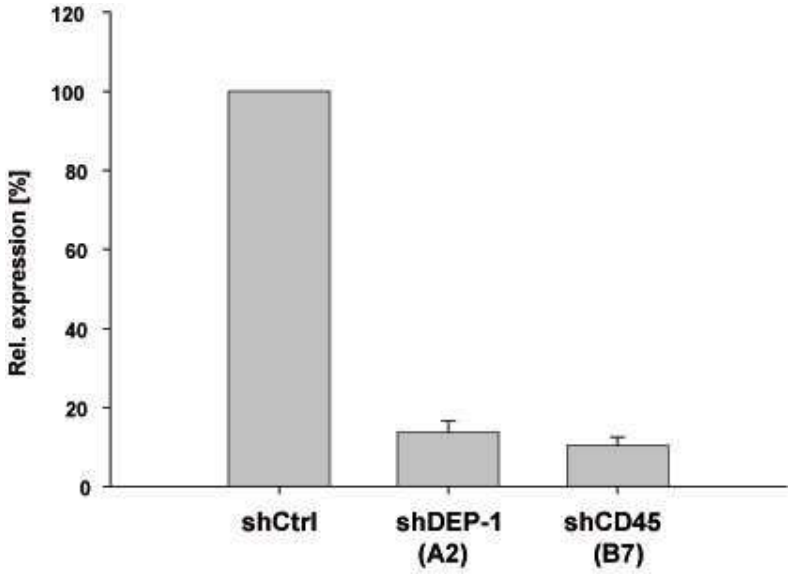


Figure 6-7. Expression levels of DEP-1 and CD45 in knockdown lines. 32D cells expressing muFLT3 WT were harvested from which the RNA was extracted and quantified. 1 µg of RNA was used as input for cDNA preparation. The cDNA was used for detection of mRNA levels of knockdown PTP in a real time PCR. β-actin was taken as a reference gene unaffected by lentiviral transfection. 32D shCtrl cells were used as reference for calculation of relative expression of DEP-1 and CD45 in the knockdown lines shDEP-1 (A2) and shCD45 (B7). The relative expression was plotted as percentage (n = 2; mean±SD).

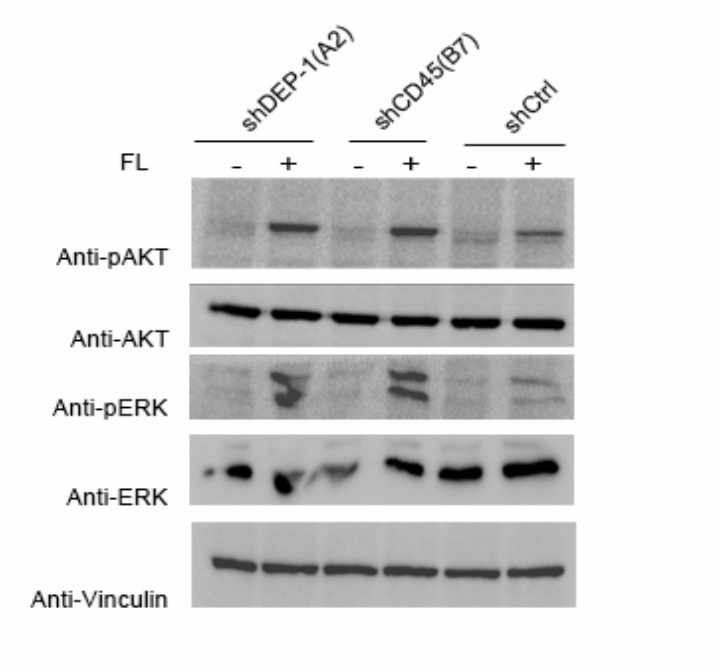


Figure 6-8. Activation of ERK in DEP-1 (A2) and CD45 (B7) depleted lines. 32D cells expressing muFLT3 WT were washed and equal amounts of cells were starved in serum and IL3 deficient medium for 4 hr prior to stimulation with FL (50 ng/ml) for 10 minutes. The cells were lysed in RIPA buffer and lysates were subjected to SDS-PAGE and immunoblotting. The lysates were probed with antibodies recognizing signaling proteins pAKT, and pERK, and total ERK and AKT protein levels. As an additional loading control, Anti-Vinculin was used.

Combinatorial knockdown of DEP-1 and CD45

After performing these experiments, DEP-1 and CD45 were considered as putative candidates controlling activity of FLT3. While this work was in progress, redundant functions f DEP-1 and CD45 were reported in the context of B-lymphocytes and myeloid cell functions (Zhu, Brdicka et al. 2008). Therefore, an approach to knockdown both DEP-1 and CD45 in muFLT3 WT expressing 32D cells was followed to evaluate if this would have stronger effects on FLT3 signaling. To achieve double-transduction, the puromycin resistance cassette of the shRNA expression constructs was replaced with a neomycin resistant cassette for one of the shRNA constructs CD45 (B7). A second round of infection of puromycin selected DEP-1 depleted cells was done. The cells were then selected with G418 to obtain double knockdown cells. The knockdown was verified by qRT-PCR. The percentage expression of DEP-1 in A2B7 was 15.3% while the expression

of CD45 was 52.9% (see Figure 6-9). The down regulation of CD45 upon secondary infection was less efficient than that of DEP-1.

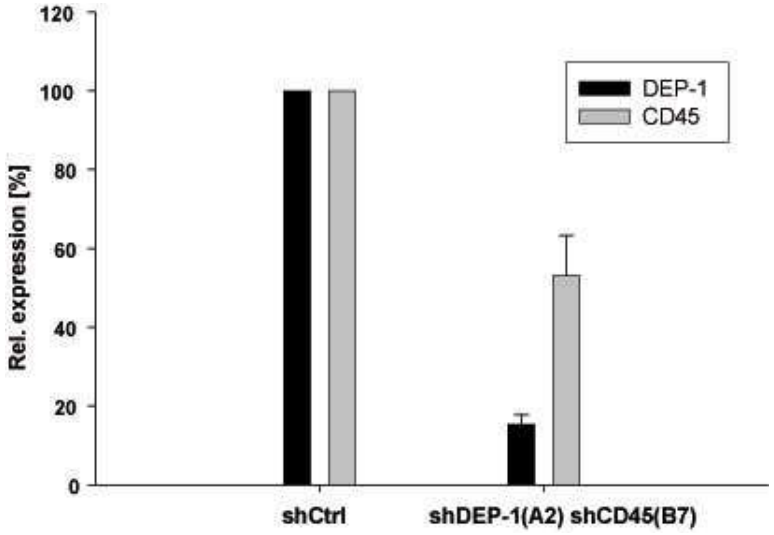


Figure 6-9. DEP-1 and CD45 mRNA expression levels in a double knockdown shDEP-1(A2) shCD45(B7) 32D cell line. 32D cells expressing muFLT3 WT were washed and RNA was prepared. 1 µg RNA was used for preparation of cDNA for real time PCR of DEP-1 and CD45. β-actin was used as a reference gene not affected by viral transduction. 32D shCtrl cells were used as reference for calculation of relative expression which is expressed as percentage (n = 2; mean±SD).

The DEP-1 and CD45 double knockdown (A2B7) cells were subjected to proliferation assays. GFP-based proliferation revealed that DEP-1 and CD45 double knockdown cell lines appeared to grow better in presence of FL from any of the single knockdown cells (see Figure 6-10).

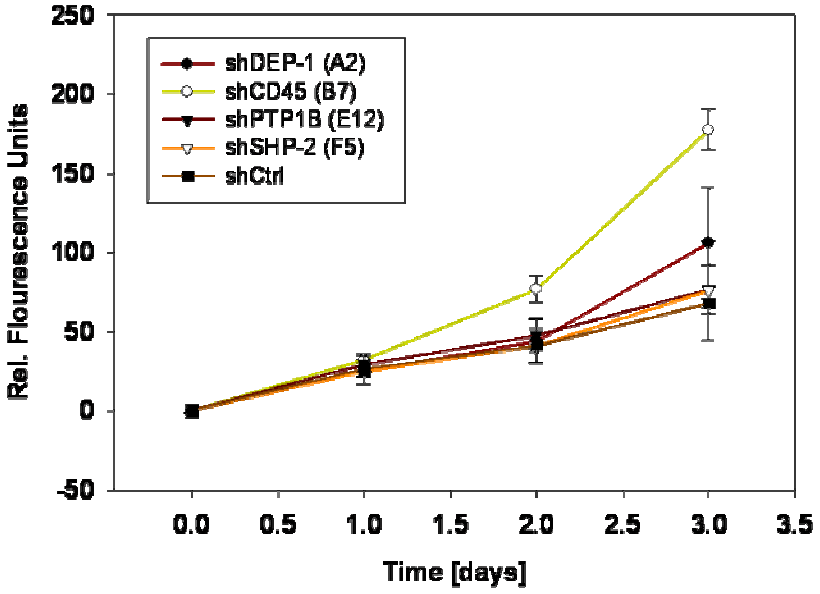


Figure 6-10. Growth of 32D cells with knockdown of DEP-1 (A2), CD45 (B7) and double knockdown of DEP-1 and CD45 (A2B7). 32D cells expressing muFLT3 WT were washed twice and seeded in 96 well-plates at the density of 20,000 cells/well in the presence of FL (20 ng/ml) and 10% heat inactivated serum for 6 days and relative (rel.) fluorescence units were measured each day at absorbance 485-520 nm. The experiment was done in triplicates (n = 3 mean±SD).

However, it was later observed by immunoblotting (data not shown) that, the combined DEP-1 and CD45 knockdown cells attained higher FLT3 levels over time. The growth results obtained therefore, in the double knockdowns could have been caused by heightened signaling activity of more receptor on the surface. When these lines were subjected to FACS analysis, there were two cell populations in the double knockdown cells, out of which one population correlated with the FLT3 levels in the shControl cell line and another population which expressed clearly more FLT3 (data not shown). The cells were therefore sorted using a FACS sorter to obtain a population with comparable levels of FLT3 as in the control cell line. The sorted double knockdown cells were subjected to an initial analysis of FLT3 signaling and cell proliferation, however did not show enhanced FLT3 signaling as compared to cells with DEP-1 knockdown only (not shown). Therefore, further analysis was focused on the effects of DEP-1 depletion. Only cell pools were further on compared which expressed similar FLT3 levels.

6.1.6 Optimization of DEP-1 depletion

The data above indicated that DEP-1 may play an important role in FLT3 signaling since; some of the effects were observed in lines with maximal DEP-1 knockdown. Attempts were made to improve downregulation of DEP-1 by optimizing the lentiviral mediated transduction of shRNA targeting DEP-1. For this, the lentiviruses expressing the shRNA constructs were concentrated by special columns (as described in 5.4) as this would ensure high viral titres for infection of 32D cells and therefore increased transduction efficiency. Two shRNA constructs for targeting DEP-1 were used; shRNA DEP-1(A2) and shRNA DEP-1(A3) as these targets showed higher phospho-ERK in the screen.

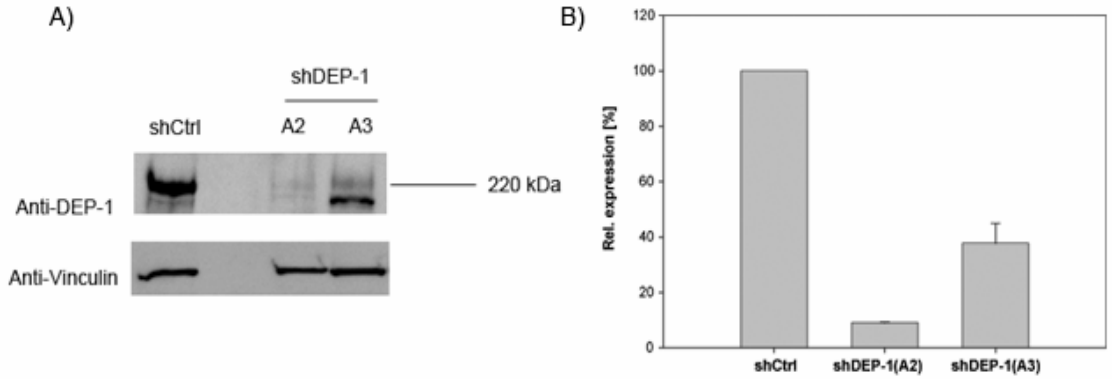


Figure 6-11. Knockdown of DEP-1. 32D cells expressing muFLT3 WT transduced with shRNA targeting DEP-1 (A2 and A3) were washed and lysed in RIPA buffer. The lysates were subject to SDS-PAGE and western blotting. The lysates were probed with antibodies recognizing muDEP-1 and vinculin. A) Anti-DEP-1 blot showing reduction in protein levels in DEP-1 knockdown lines A2, and 3. As loading control, the blot was probed with anti-vinculin. B) Relative expression in percentage of DEP-1 mRNA levels in DEP-1 knockdown line A2 and A3. The shControl (shCtrl) line was taken as reference for DEP-1 expression to calculate reduction in DEP-1 in A2 cell line and β-actin was used as the reference gene not affected by viral transduction (n = 2; mean±SD).

As seen from Figure 6-11, the DEP-1 proteins levels were reduced in A2 and A3. The maximal reduction at protein levels was however achieved in cell line A2. The mRNA levels of shDEP-1 A2 were reduced to 10% of the DEP-1 present in shCtrl cell line. For further experiments similar FLT3 expressing cell pools were used (see Figure 6-12).

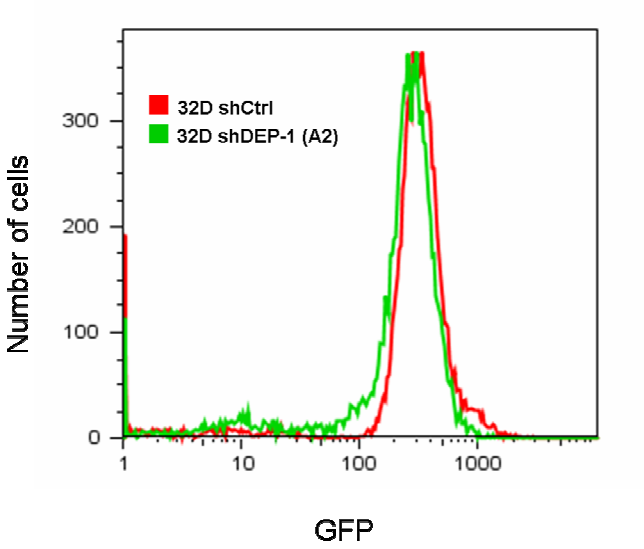


Figure 6-12. Expression of GFP in 32D cells expressing muFLT3 WT shCtrl and shDEP-1 (A2). The cells were washed and resuspended in 1X PBS and were then analyzed using flow cytometry. The graph shown here is an overlay of 32D cells expressing muFLT3 WT transduced with non targeting shRNA (red) or DEP-1 targeting shRNA (A2) (green).

6.1.7 DEP-1 depletion results in moderately enhanced cell proliferation

Cell growth in cell pools with efficient DEP-1 knockdown was monitored by MTT assay. Cells were washed off IL3 and serum, and plated with or without growth factors. It was observed that upon DEP-1 depletion, the FL-dependent growth was somewhat higher than in the control cell line (see Figure 6-13). The cells did not grow well in the absence of cytokine. However, cytokine independent growth was possibly was also somewhat higher in the DEP-1 depleted cells. The growth was similar and maximal in the presence of IL3 (data not shown). FLT3 signals via the RAS-MAPK pathway upon binding of the specific ligand FL. Activation of this signaling pathway should result in activation of genes involved in cell proliferation. Elevated proliferation of DEP-1 depleted cells was therefore expected from the elevated ERK activation seen in the screen. However, the effect on cell growth was overall rather mild.

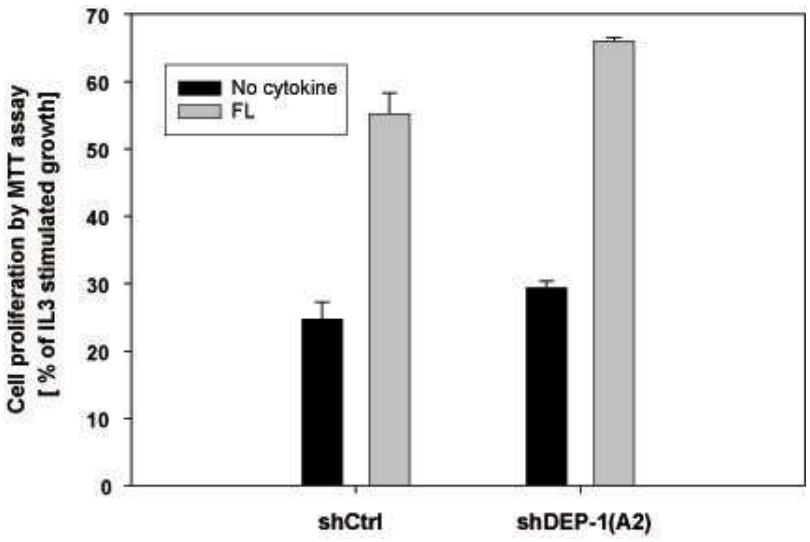
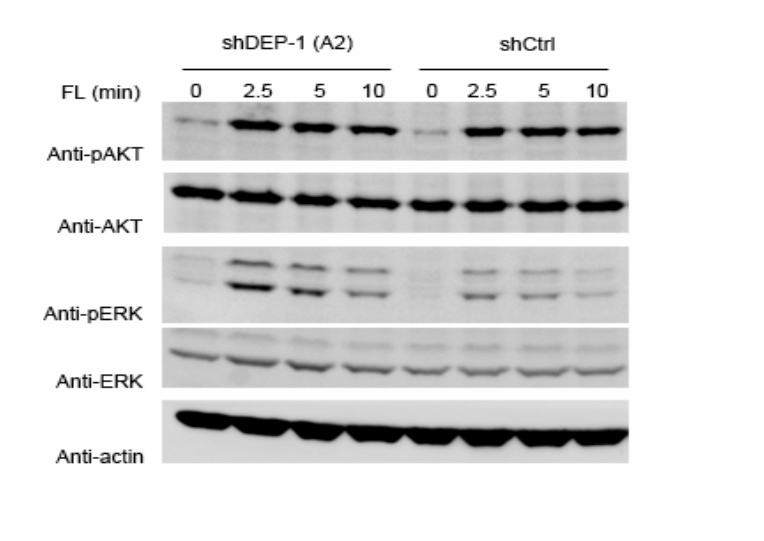


Figure 6-13. Enhanced proliferation in DEP-1-depleted cells shDEP-1 (A2). 32D cells expressing muFLT3 WT were washed and seeded in the density of 20,000 cells/well in a 96 well-plate. The cells were incubated in 0.5% hi FCS in presence of absence of growth factors; FL (20 ng/ml) and IL3 (2 ng/ml) for 48 hr after which the growth was terminated by addition of MTT. The experiment was performed in triplicates. The absorbance was read at 485-520 nm. The proliferation was normalized to growth in presence of IL3.

6.1.8 DEP-1 depletion results in activation of downstream FLT3 signaling

To further elucidate effects of DEP-1 depletion on FLT3 mediated downstream signaling, the cells were starved and subjected to FL stimulation for varying time points, and activation of different signaling proteins as detected using western blotting. The DEP-1 depleted line showed a clearly detectable activation of phospho-ERK and possibly a weak activation of phospho-AKT as compared to the control cell line (see Figure 6-14). The activation was observed at 2.5, 5 and 10 minutes. This indicates that depletion of DEP-1 results in some activation of the RAS-MAPK pathway but little or no activation of AKT. Activation of STAT5 was also analyzed by detecting pSTAT5. This was of particular interest since STAT5 activation correlates with cell transformation and is normally not seen in FLT3 WT expressing cells (see 3.2.2). It was seen that STAT5 was also weakly activated in DEP-1 depleted lines. There was little or no STAT5 activation observed for control cells, as expected.

A)



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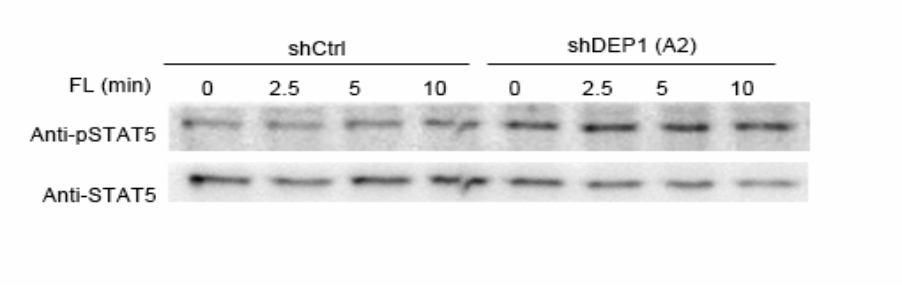

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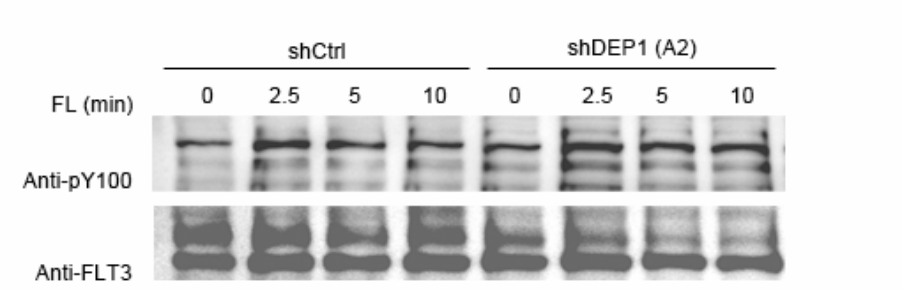


Figure 6-14. Elevated signaling in DEP-1 depleted muFLT3 WT expressing 32D cells. Cells were washed and equal amounts of cells were starved in serum and IL3 deficient medium for 4 hr prior to stimulation with FL (50 ng/ml) for the indicated time points. The cells were lysed in RIPA buffer and lysates were subjected to SDS-PAGE and immunoblotting. A) The lysates were probed with antibodies recognizing signaling proteins pAKT, and pERK, and total ERK and AKT protein levels. As an additional loading control, anti-actin was used. B) and C) The cells were treated in a similar manner and the lysates were prepared in RIPA buffer. Following SDS-PAGE and western blotting, the lysates were probed with antibodies recognizing pSTAT5, STAT5, phospho-FLT3 (pY100) and pan-FLT3.

In conclusion, the principal signaling phenotype in DEP-1 depleted cells which were detected in the screen i.e. elevated ERK activation could be confirmed. Interestingly, in addition a weak activation of STAT5 occurred in the DEP-1 depleted cells, which is the characteristic of only transformed 32D cells.

6.1.9 FLT3 is hyperphosphorylated upon depletion of DEP-1

An obvious explanation of enhanced FLT3 signaling in DEP-1 depleted cells would be that the DEP-1 phosphatase can directly dephosphorylate the FLT3 protein. To obtain definitive data on this issue in the muFLT3 WT expressing cell lines proved out to be technically difficult, in part because immunoprecipitation of muFLT3 was inefficient (data not shown). Therefore 32D cell lines expressing huFLT3 were used for these experiments. Immunoprecipitation of huFLT3 was efficiently possible. Moreover, these experiments offered the possibility to analyze individual phosphorylation sites in FLT3 due to the availability of a unique set of phospho-site selective antibodies. These have been previously used to analyze differences in activation of phosphotyrosine sites in FLT3 WT and FLT3 ITD. (Razumovskaya, Masson et al. 2009). Phosphorylation of different sites in growth factor receptors mediate binding and activation of signaling proteins involved in the signaling cascade after receptor activation. For example, activation of STAT5, as we observed in DEP-1 depleted muFLT3 WT expressing 32D cells is believed to require phosphorylation of the juxtamembrane phosphorylation sites pY589 and pY591 in FLT3 (Rocnik, Okabe et al. 2006). To know if any phosphorylation site of FLT3 is hyperactivated in DEP-1 knockdown cells, the FLT3 receptor was immunoprecipitated from stimulated 32D cell containing huFLT3 WT and control shRNA, or 32D cells huFLT3 WT with stable DEP-1 knockdown. For these experiments, a double DEP-1 knockdown was performed by employing a similar strategy used for producing DEP-1 and CD45 combinatorial knockdown cells. The double DEP-1 knockdown was made from the shRNA targets A2 and A3 and a good knockdown of DEP-1 in these lines was achieved (data not shown). A number of site-specific FLT3 phosphotyrosine antibodies were used for elucidating the difference in phosphorylation patterns between both the lines.

Important sites were shown to be differentially phosphorylated in DEP-1 knockdown cell lines. Notably, phosphotyrosines at positions 589 and 591 were shown to be hyperphosphorylated in the DEP-1 depleted cells (see Figure 6-15). Another important site pY591 involved in STAT5 activation was shown to be hyperphosphorylated after DEP-1 depletion. Clearly, DEP-1 knockdown caused hyperphosphorylation of FLT3 in these cells.

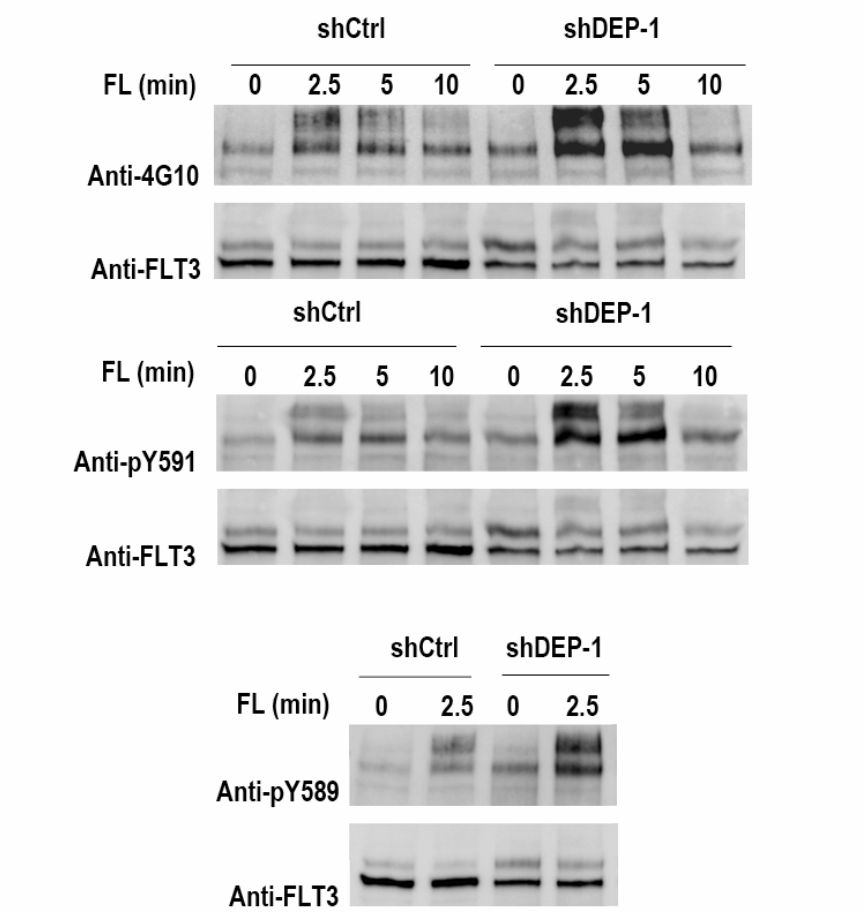


Figure 6-15. Hyperphosphorylation at specific sites in huFLT3 WT upon DEP-1 depletion. 32D cells expressing huFLT3 WT and DEP-1-specific shRNAs (A2A3), or a control shRNA were starved and stimulated with FL (100 ng/ml) for indicated time points. The cells were lysed in lysis buffer and FLT3 was immunoprecipitated and analyzed by immunoblotting with Anti-FLT3 or phospho-FLT3 (pY591, pY589 and pY4G10) antibodies.

These experiments revealed that FLT3 is presumably a direct substrate of DEP-1 since it is hyperphosphorylated upon loss of DEP-1.

6.1.10 Elevated FLT3 signaling activity in transient knockdown of DEP-1 in THP-1 cells

It appeared of interest to confirm the effect of DEP-1 knockdown on FLT3 signaling in a different cell background, preferentially a human cell line with endogenous FLT3. Therefore transient knockdown by using siRNA of DEP-1 was performed in the human AML cell line THP-1 which expresses FLT3 WT (see 5.5.2). The knockdown was efficiently achieved after 48 hr. analysis of the activation of signaling proteins in control and knockdown cells, revealed that phospho-ERK was weakly activated in the DEP-1 transient knockdown cells (see Figure 6-16). There was weak or no activation observed for AKT. These findings confirmed the results obtained upon stable DEP-1 depletion in 32D cells. FLT3 phosphorylation was also analyzed. The phosphorylation of sites pY589, and pY599 was reproducibly increased upon DEP-1 transient knockdown.

6.1.11 Radiation induced apoptosis in DEP-1 depleted cell lines

According to previous studies, cells expressing the constitutive active form of FLT3 are resistant to radiation-induced apoptosis (Mizuki, Fenski et al. 2000). In contrast, cells expressing FLT3 WT is susceptible to apoptosis after exposure to γ-radiation. To analyze whether, DEP-1 depletion would result in conferring apoptosis resistance, 32D cells with expressing muFLT3 WT or without DEP-1 knockdown were subjected to gamma-radiation. Cells were cultured with or without growth factors for 96 hr to assess apoptosis. The cells without growth any growth factor underwent rapid apoptosis after gamma irradiation. The cells evaded apoptosis in the presence of IL3 (data not shown). However, presence of FL (see Figure 6-17) did not rescue the cells from undergoing apoptosis.

The cell line with DEP-1 knockdown, showed slight or no evasion to radiation induced apoptosis after 48 hr of irradiation than the control cell line. The percentage of surviving cells appears to be come up after 48 hr and is very small fraction of the parental cell population.

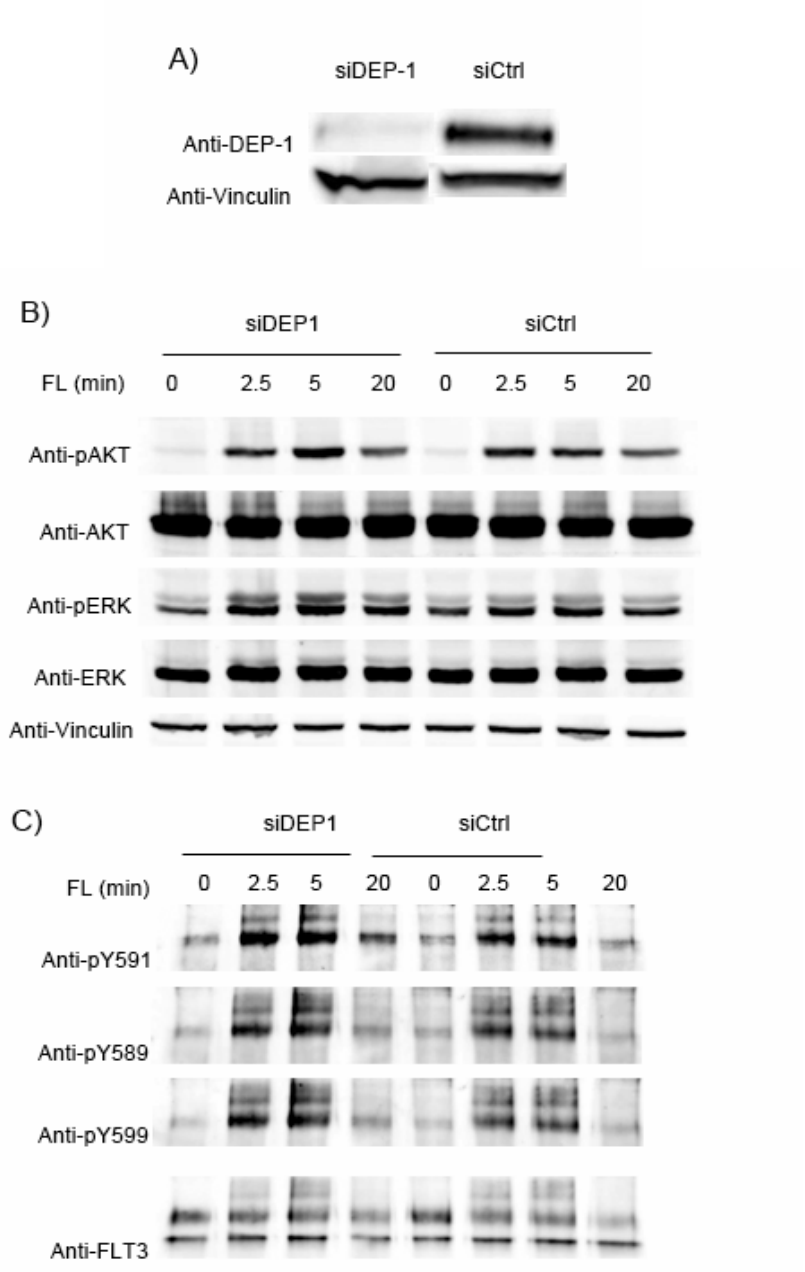


Figure 6-16. Effect of depletion of DEP-1 in THP-1 cells. THP-1 cells were transfected with siRNA targeting DEP-1 (siDEP-1) or a nontargeting control siRNA (siCtrl). After 48 hr, cells were starved for 4 hr and stimulated with FL (100 ng/ml) for the indicated time points. The cells were lysed and subjected to SDS-PAGE and western blotting. A) The lysates were probed with anti-DEP-1 and anti-vinculin antibodies. B) The lysates were probed with antibodies recognizing phosphorylated signaling proteins AKT or ERK and reprobed for total signaling proteins ERK and AKT in addition to vinculin as loading control, C) Subsequent to stimulation and cell lysis, FLT3 was immunoprecipitated and analyzed by immunoblotting with site-specific phosphotyrosine FLT3 antibodies as indicated, and reprobed for total immunoprecipitated FLT3.

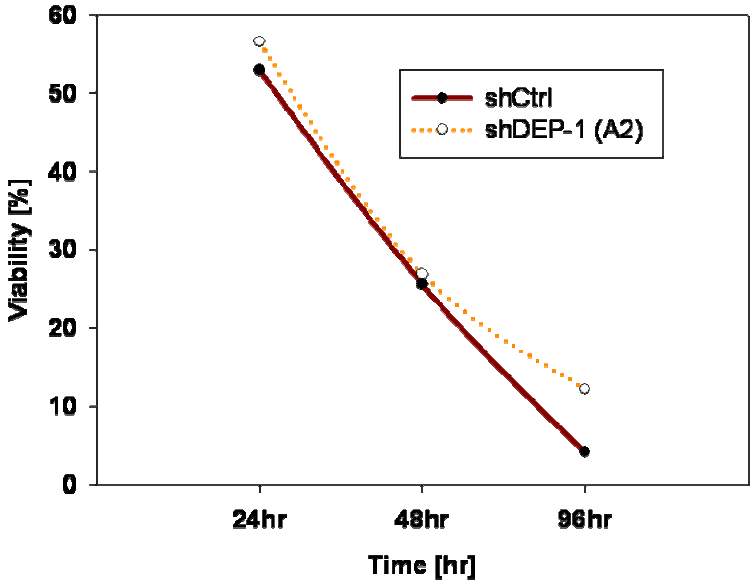


Figure 6-17. Ligand dependent evasion of apoptosis in response to DEP-1 depletion in shDEP-1(A2) cells. Cells were washed and equal numbers of cells were starved in 6 or 12 well-plate for 4 hr. prior to exposure to 5 Gy γ-radiation. The cells were incubated with 10% hi FCS in presence of FL (100 ng/ml) for indicated time points. The cells were stained with Annexin-V and 7-AAD. The non-stained or double-negative cells were considered as viable cells. The percentage viable cells from the total population were plotted. The graph is representative of two independent experiments performed.

6.1.12 FL-dependent colony formation in DEP-1 depleted 32D cells

Depletion of DEP-1 had positive effects in FLT3 mediated proliferation, and caused stimulation of phosphorylated ERK. Also, a weak activation of phospho-STAT5 was observed in DEP-1 depleted cell lines. STAT5 is an important mediator of cellular transformation. Therefore DEP-1 depleted cells were analyzed for the ability to form colonies in methylcellulose containing medium. Colony formation under these conditions is considered as indirectly cell transformation.

Cells were seeded with or without growth factors in the assay mixture containing methylcellulose. The cell lines with DEP-1 depletion showed growth in methylcellulose in the presence of FL, while the control cell lines did not show colonies in presence of FL

indicating that the transformation is FLT3 specific (see Figure 6-18). The lines did not show any differences in IL3 dependent growth and there was no transformation in absence of growth factor in the DEP-1 knockdown line.

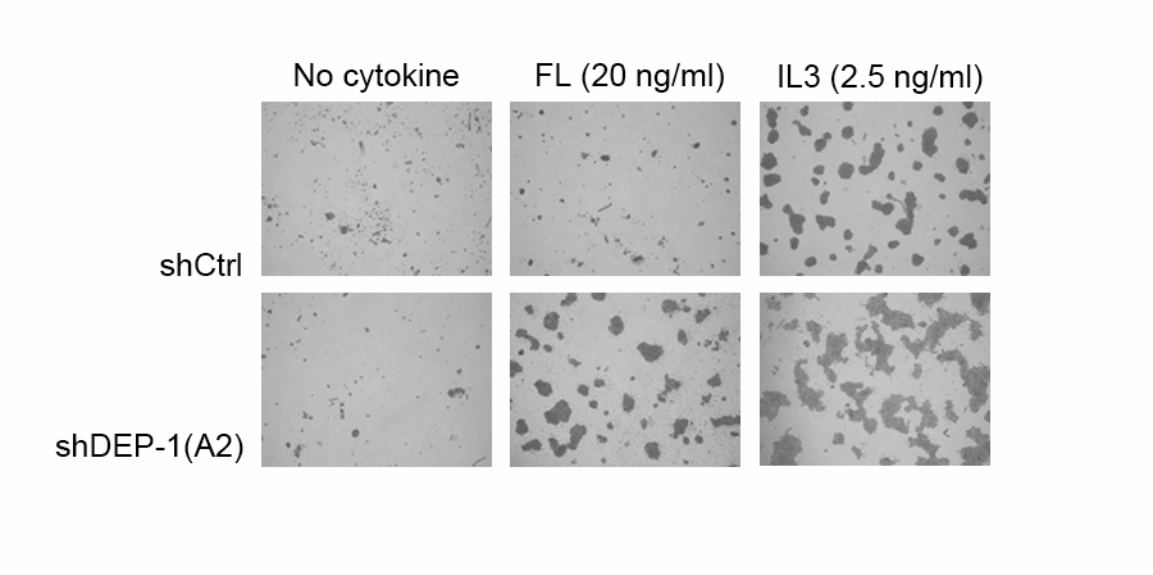


Figure 6-18. FL-dependent clonal growth of muFLT3 WT expressing 32D cells upon DEP-1 depletion in methylcellulose. Cells were washed to remove IL3, suspended at a density of 3000 cells/ml in methylcellulose containing IMDM medium and seeded per well in a 12 well-plate. The medium contained growth factor FL (20 ng/ml) or IL3 (2.5 ng/ml) and 20% hi FCS. The assay was plated in duplicates. Pictures were taken after 6 days (IL3-incubated samples) and 12 days (FL-incubated samples).

Notably, colony formation in DEP-1 depleted cells took longer than in IL3-stimulated cells. Still, these findings indicate that DEP-1 loss can cause a transformed phenotype by enhancing FLT3 signaling.

DEP-1 depletion does not lead to transformation in mice

Since the stable DEP-1 knockdown in 32D cells with FLT3 WT resulted in colony formation in semi-solid medium in presence of FL, it was considered that the DEP-1 down regulation could also induce formation of leukemia like disease in syngenic mice. For such experiments C3H/HeJ mice can be used, which are syngenic with 32D cells. It has been shown previously that 32D cells with FLT3 ITD resulted in leukemia like disease in mice within 2-3 weeks.(Müller, Schönherr et al. 2008). 32D cells with stable

DEP-1 depletion were injected in the mice. The mice however did not show any signs of disease or tumor formation within 3 months observation time. This finding indicates that DEP-1 is not sufficient to induce *in vivo* transformation. Still, DEP-1 loss may co-operate with other factors to induce leukemogenesis in AML.

6.2 Expression of protein tyrosine phosphatases in AML cells and its regulation by FLT3 ITD

Survey of PTP mRNA in AML cells

The expression of protein tyrosine phosphatases in AML has not yet been systematically analyzed. Therefore, the screening analysis in the first part of the thesis was based on rather limited information. PTPs may play multiple roles in the transformation for example, while the phosphatases may pose a mechanism in controlling activity of FLT3 WT, the expression of certain phosphatases could be altered by FLT3 ITD which is one of the most frequently occurring mutations AML. Mutated FLT3 (FLT3 ITD) could perhaps down regulate phosphatases to exert an unrestricted growth advantage in cells. During the course of this thesis, a unique possibility for analyzing PTP expression became available through interaction with Drs. Rob Hooft van Huijsduijnen and Monique Van Den Eijnden (Merck Serono, Geneva) in the context of the Marie-Curie PTPNET. They developed a platform to comprehensively measure mRNA expression of 92 human PTP genes by semi automatic qRT-PCR.

6.2.1 PTP screen in Human AML blasts and AML cell lines

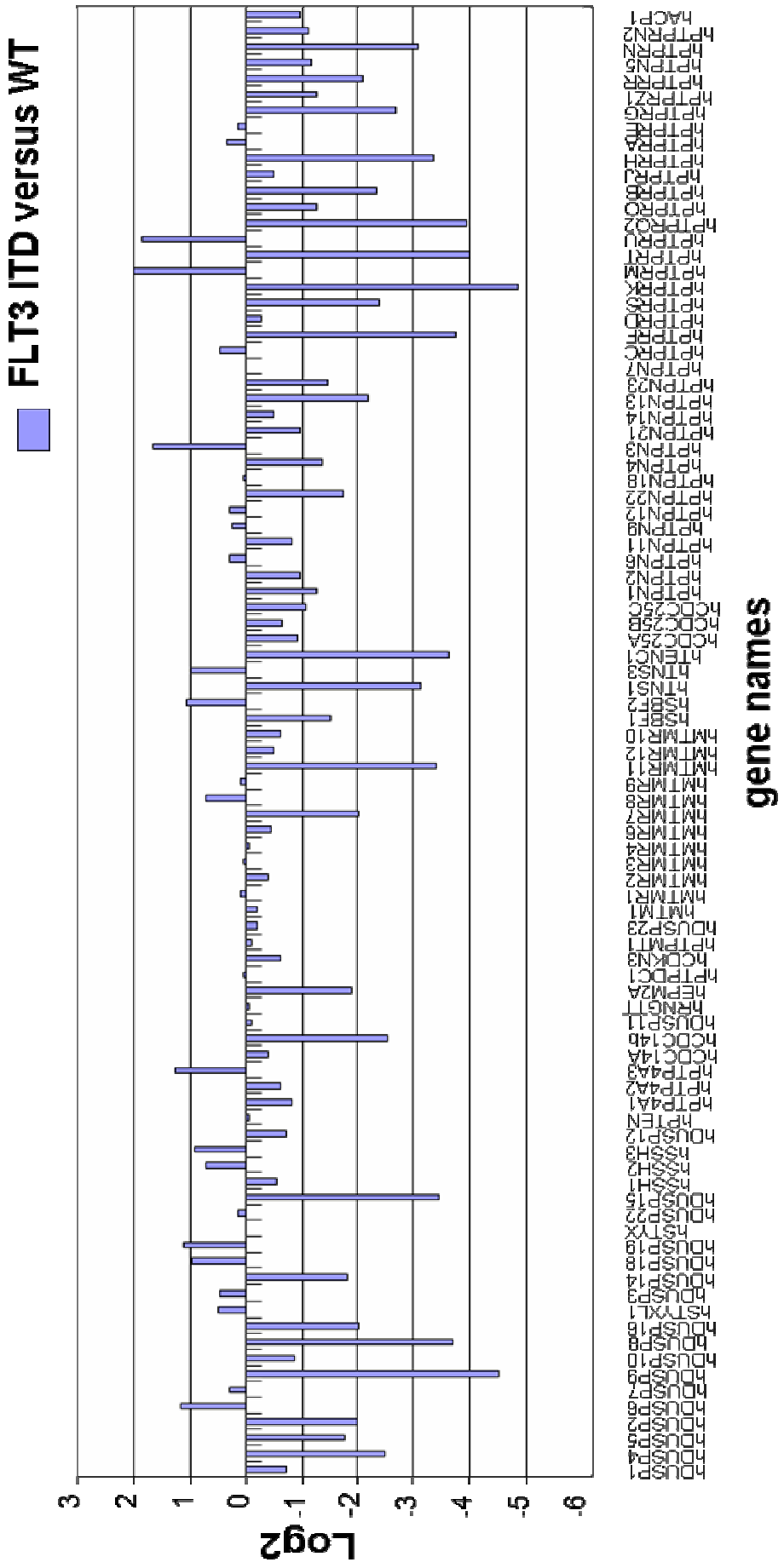
To identify PTPs which are expressed in AML cells and which may be regulated by FLT3 ITD, a number of human AML blast samples which expressing the ITD form of FLT3 or FLT3 WT were studied. Additionally, the AML derived cell lines EOL-1, THP-1, RS4-11 expressing FLT3 WT and MV4-11 expressing FLT3 ITD were studied. RNA and cDNA of these samples were analyzed and subjected to qRT-PCR.

By employing this platform, several observations were made inspecting this data set. An overview of PTP expression for the physical analysis in a small number of patient samples is shown in Figure 6-19. Many PTP, especially the receptor type PTP were expressed in very low levels in both WT and ITD expressing blasts e.g., PTPRD, PTPRK whose expression was as low as 0.1% and 0.03%, therefore, these PTPs were not studied further. Several DUSPs (Dual Specificity Phosphatases) were expressed in abundance, with some appeared down regulated in FLT3 ITD blasts and some appeared up regulated when compared with the FLT3 WT blasts (see Table 2).

Expression in the AML cells lines was variable (see Table 3). Still, some expression differences seen in the initial patient sample set were re-capitulated upon comparison of RS4-11 and MV4-11 cells. These two cell lines were considered as better comparable since they differ in FLT3 status but harbor the same translocation (4-11).

Expression differences seen at mRNA levels could in part be confirmed at protein level, including the DUSP6 (MV4-11>> RS4-11), ACP-1/LMW-PTP (RS4-11> MV4-11), and PTPN2/ TC-PTP (RS4-11> MV4-11) (B. Tölle, A. Böhmer, unpublished data).

Figure 6-19. Expression profile of 92 huPTPs in AML blast samples. cDNA was prepared form human AML blasts expressing FLT3 WT (n = 4) or FLT3 ITD (n = 5). The samples were then plated in a 384 well format pre-filled with 92 human PTPs and β-actin, PSMB3 and CANX as HKGs. Real time PCR was performed and delta Ct values were calculated as described in Materials and Methods. The difference between the delta Ct values of FLT3 WT and FLT3 ITD blasts was calculated (delta-delta Ct). The fold change in gene expression was calculated using 2^ (-delta-delta Ct) and log2 values were computed and plotted.


FLT3 ITD versus WT
Log2
3
2
1
0
-1
-2
-3
-4
-5
-6
gene names

	FLT3 WT _AML		FLT3 ITD _AML	
PTP	**blasts**	**Std Dev.**	**blasts**	**Std Dev**
DUSP1	114.64	76.85	62.36	28.39
DUSP2	27.83	31.87	5.02	2.50
DUSP4	11.32	7.07	2.10	1.24
DUSP6	30.20	16.37	74.93	52.54
DUSP8	13.33	9.73	2.36	2.45
PTP4A1	25.45	11.76	13.83	3.58
PTPN2	22.34	6.84	11.34	3.07
PTPRJ	3.40	3.05	2.80	2.08
ACP1	33.81	17.06	16.28	4.70
SBF1	13.61	9.60	4.27	1.80

Table 2. Expression profile of few PTP in human AML samples. FLT3 WT (n = 4) and FLT3 ITD (n = 5) expressing human blasts were compared for PTP expression profile. 92 human PTPs were analyzed, out of which 10 PTPs are presented in this table. Three HKGs were used for normalization of expression. The values represent expression in percentage.

Analysis of PTP mRNA expressing in an expanded set of patient samples

A number of interesting PTP seemingly regulated by FLT3 ITD could be identified from the data obtained. Selected PTPs which appeared differentially expressed in both AML types, we analyzed the mRNA expression in additional patient samples, and also included PTPRJ (DEP-1) which has been studied before functionally in FLT3 WT expressing 32D cells. While the expression of DEP-1 mRNA remains unaffected by either type of FLT3 receptor. Several other PTP mRNA was differentially expressed in FLT3 WT and FLT3 ITD harboring patient blasts also when more patients were included (see Table 4). These comprised for sample DUSP2, DUSP6, DUSP8 and PTPN2. It should be noted that patient to patient variations were large, and the data was therefore not subjected to statistical analysis. The number of human AML blasts analyzed for the expression analysis was limited; the data obtained may not be a true representation of the PTP expression in FLT3 WT and FLT3 ITD blasts.

PTP	THP-1	Std dev	EOL-1	Std dev	RS4-11	Std dev	MV4-11	Std dev
DUSP1	1.07	0.90	1.90	2.03	8.05	2.00	0.59	0.76
DUSP2	3.56	1.20	4.18	2.05	1.78	0.29	2.75	0.72
DUSP4	0.11	0.08	0.30	0.09	0.27	0.05	0.15	0.05
DUSP6	5.23	1.67	5.46	0.40	1.99	0.61	10.79	3.48
DUSP8	0.04	0.03	0.01	0.01	0.10	0.07	0.02	0.01
PTP4A1	17.18	1.26	19.40	4.84	39.82	6.54	8.92	1.39
PTPN2	4.26	0.81	7.68	1.41	10.55	0.68	6.12	0.17
PTPRJ	4.09	0.73	1.93	0.29	1.17	0.34	1.69	0.67
ACP1	11.95	1.06	21.47	1.80	52.68	2.59	18.05	0.95
SBF1	1.90	0.56	2.58	0.26	6.01	1.56	2.63	0.40

Table 3. Comparison of mRNA expression of selected PTP in human AML cell lines. Human AML cell lines were compared for the mRNA expression profile of PTPs. Three independent cultures of each cell lines were used. Out of 92 human PTPs analyzed, 10 PTPs are tabulated which appeared in part to be differentially expressed in FLT3 WT and FLT3 ITD expressing cell lines. THP-1, EOL-1, RS4-11 express FLT3 WT while MV4-11 express FLT3 ITD. Three house keeping genes (HKG) were used for normalization of expression. The values represent expression in percentage (n = 3; mean±SD).

	FLT3 WT _AML		FLT3 ITD _AML	
PTP	blast	Std Dev	blast	Std Dev
DUSP1	79.91	50.45	83.49	76.85
DUSP2	22.30	27.58	13.48	18.68
DUSP4	11.98	9.28	18.89	33.27
DUSP6	41.40	26.31	75.12	44.87
DUSP8	7.47	8.29	4.41	5.71
PTP4A1	28.05	17.54	20.26	13.89
PTPN2	22.65	32.93	18.08	17.07
PTPRJ	4.90	3.10	4.69	4.29
ACP1	25.30	16.98	38.51	61.19
SBF1	9.83	8.82	19.99	34.38

Table 4. Expression profile of selected PTP in human AML samples. FLT3 WT (n = 17) and FLT3 ITD (n = 11) expressing human blasts were compared for PTP expression profile. Out of 92 human PTPs analyzed, 10 PTPs were selected based on considerable difference between the two blast types. Three HKG were used for normalization of expression. The values represent expression in percentage.

The results of these expression analysis lead to initiation of functional testing of some of the PTPs for relevance in FLT3 signaling, which is however, not the subject of this thesis.

6.2.2 Phosphatase expression regulation by FLT3 ITD in murine cell lines

Given that several PTP mRNA appeared differentially expressed in cells harboring FLT3 ITD, further analysis was performed to clarify if FLT3 ITD is indeed causing changes in expression. This was done initially in 32D cells. Parental 32D cells or 32D cells stably transfected with muFLT3 WT or muFLT3 ITD allows discriminating the contribution of FLT3 variants to regulate expression of PTPs in comparable cell backgrounds. 10 PTP were selected for this further analysis in 32D cell lines. After performing qRT PCR from cDNA obtained from starved cells, it was seen that the expression of PTP4A1 and PTPN2 which appeared downregulated in FLT3 ITD AML blasts and human AML cell lines, also show decreased expression in muFLT3 ITD expressing 32D cells (see Table 5). DUSP4 and DUSP8 expression was generally rather low or even not detectable in 32D cells. Therefore, no conclusion of regulation of these PTPs can be drawn. DUSP6 expression showed a 2-fold increase in 32D FLT3 ITD cells when compared to 32D FLT3 WT cells. These results indicate that expression of some PTP mRNA seems indeed regulated by FLT3 ITD. Most clearly, expression of the dual specificity phosphatase DUSP6 seemed to be induced by FLT3 ITD. Increased levels were also seen in FLT3 WT expressing 32D cells as compared to parental cells possibly indicating regulation by basal FLT3 activity.

PTP	32D Parental	Std Dev	32D muFLT3 WT	Std Dev	32D muFLT3 ITD	Std Dev
DUSP1	1.48	0.66	2.14	0.48	0.71	0.15
DUSP2	3.13	0.01	1.85	0.55	1.05	0.10
DUSP4	0.58	0.07	0.54	0.11	1.53	1.02
DUSP6	1.54	0.14	5.55	2.27	10.14	1.75
DUSP8	0	0.00	0	0.00	0	0.00
PTP4A1	7.39	1.79	16.56	3.75	11.25	3.01
PTPN2	9.47	5.29	17.75	2.36	8.21	2.01
PTPRJ	1.03	0.25	1.33	0.11	0.47	0.38
ACP1	17.66	6.02	23.28	5.50	17.75	2.80
SBF1	9.56	1.98	18.49	6.49	10.08	2.63

Table 5. Comparison of expression of selected PTPs in 32D cells. Cells were starved overnight and then were used for preparation of RNA and cDNA and profiling for expression of PTPs in cells with or without FLT3 WT or FLT3 ITD expression, as indicated. Two independent cultures of each cell lines were used for the expression profiling. Three HKG were used for normalization of expression. The values represent expression in percentage (n = 2; mean±SD).

To reproduce the effect of different FLT3 variants in yet another cell background, Ba/F3 cells were chosen. Ba/F3 cells are derived from mouse bone marrow and are also factor-dependent, similar as in 32D cells. Three cell lines with Ba/F3 background i.e.; Ba/F3 parental, Ba/F3 –FLT3 WT, and Ba/F3-FLT3 ITD were used to study the expression pattern of DUSP1, DUSP2, and DUSP6. qRT-PCR data obtained in Ba/F3 cells confirmed the increase in the expression of DUSP6 in presence of FLT3 ITD (see Table 6).

PTP	Ba/F3 Parental	Std Dev	Ba/F3 huFLT3 WT	Std Dev	Ba/F3 huFLT3 ITD	Std Dev
DUSP1	1.03	0.54	1.25	0.260	1.37	0.125
DUSP2	0.99	0.02	0.89	0.115	0.99	0.027
DUSP6	4.46	0.38	4.90	0.946	7.22	1.360

Table 6. Comparison of expression of selected PTPs in Ba/F3 cells. Cells were starved overnight and then were used for preparation of RNA and cDNA and profiling for expression of PTPs in cells with or without FLT3 WT or FLT3 ITD expression, as indicated. Two independent cultures of each cell lines were used for the expression profiling. Three HKGs were used for normalization of expression. The values represent expression in percentage (n = 2; mean±SD).

6.2.3 DUSP6 expression regulation by FLT3 ITD

DUSP6 mRNA is abundantly higher expressed in cells with FLT3 ITD compared to FLT3 WT expressing cells. It was therefore interesting to analyze if elevated mRNA levels would also result in elevated DUSP6 protein levels. DUSP6 protein levels were therefore analyzed in 32D cells with or without FLT3 receptor. DUSP6 is expressed as a 44 kDa band in 32D cells expressing FLT3 WT and FLT3 ITD but was nearly absent in 32D cells parental cells (see Figure 6-20). A more intense band of DUSP6 was seen in FLT3 ITD cells and comparatively a weaker band observed in lysates of FLT3 WT expressing 32D cells. This finding indicates that increased mRNA seen in FLT3 ITD expressing cells indeed translates to higher DUSP6 protein levels.

Interfering with FLT3 ITD signaling activity leads to lowered DUSP6 mRNA expression

Elevation of DUSP6 levels may play a role in FLT3 ITD mediated cell transformation. In any case, the signaling pathways leading to DUSP6 induction in the AML cells appeared to be of interest. Therefore, pathways for DUSP6 induction were analyzed using pharmacological inhibitors. Since the expression of DUSP6 is induced by FLT3 ITD, blocking the receptor tyrosine kinase activity should inhibit DUSP6 expression. To test

this, two independent sets of experiments each were performed where the FLT3 kinase inhibitors AG1295 and 1020 were used.

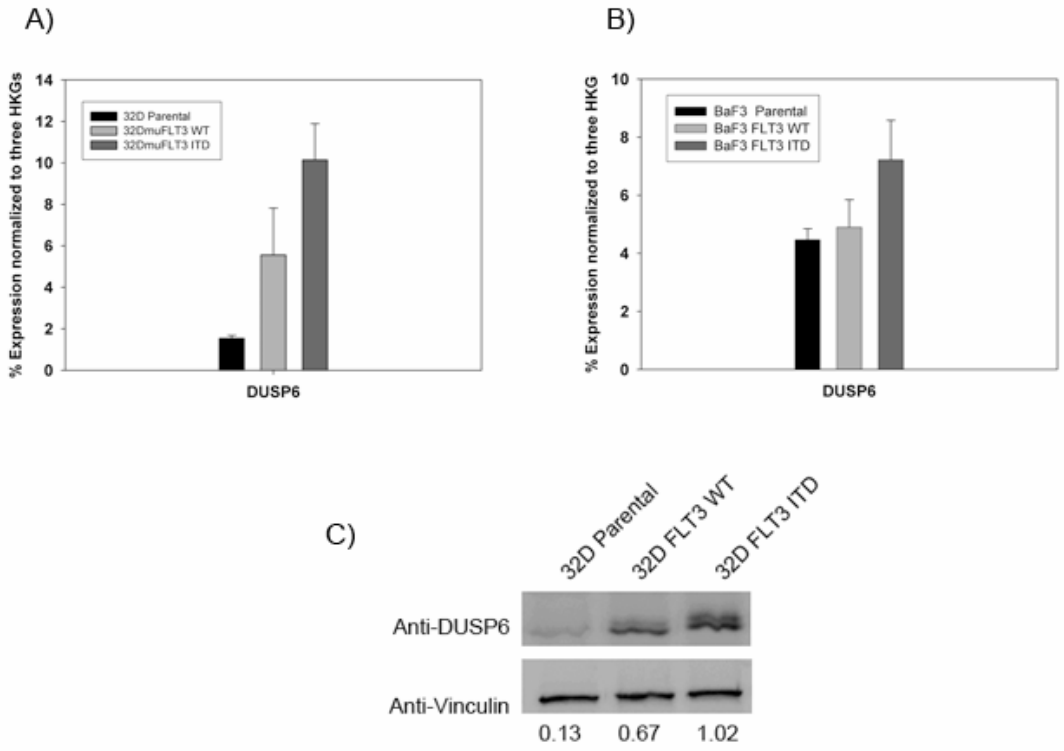


Figure 6-20. FLT3 ITD in expression of DUSP6 in 32D cells and Ba/F3 cells. The cells were starved overnight and RNA was prepared followed by cDNA preparation. Equal amounts were adjusted by using 1 µg RNA as input for cDNA synthesis. Expression of DUSP6 was calculated relative to three house keeping genes; β-actin, CANX, PSMB3. Two independently growing cultures of A) 32D cells and B) Ba/F3 cells were used. Values are means of triplicates (n = 3; mean±SD). C) 32D cells were starved for 4 hr and harvested and cell lysates prepared. 40 µg protein lysate was loaded for per lane and subjected to SDS-PAGE and western blotting. The lysates were probed with anti-DUSP6 and reprobed with anti-vinculin as loading control. The numbers represent DUSP6 and vinculin ratio as quantified using the MultiGuage software.

Both inhibitors are relatively specific for FLT3 inhibition. Both, 32D cells expressing FLT3 ITD and MV4-11 cells were treated with 20 µM AG1295 or 1 µM 1020 for time points 1 hr, 3 hr and 6 hr. these inhibitor concentrations are known to cause complete FLT3 inhibition. As postulated, the DUSP6 showed a distinctive decrease in mRNA

expression following inhibition. Maximum inhibition with AG1295 was observed after 6hr. Almost 60% reduction in DUSP6 mRNA levels was seen in the 32D FLT3 ITD cells (see Figure 6-21). Nearly, 80% inhibition of DUSP6 expression was observed after treatment with inhibitor 1020 after 3 hr. At 6 hr, the expression was again increased, possibly indicating loss of inhibitor activity.

Inhibitor treatment in MV4-11 cells to inhibit endogenous FLT3 ITD appeared even more effective than in the 32D cells. Treatment with AG1295 resulted in only negligible residual DUSP6 mRNA expression while, treatment with inhibitor 1020 also resulted in strongly diminished DUSP6 expression (see Figure 6-21). Interestingly, a strongly increased expression of DUSP6 mRNA after 1 hr was seen form the graph which may relate to some feedback effects known to play a role in DUSP6 regulation (see Discussion). Also, the DUSP6 protein levels were reduced upon kinase inhibitor AG1295 and 1020 treatments. These data confirm a role of FLT3 ITD activity for DUSP6 expression level.

Inhibition of PI3K, MAPK and STAT5 reduces variably the DUSP6 mRNA expression

FLT3 activates the RAS-MAPK and PI3-kinase pathways upon activation. The mutated form FLT3 ITD is constitutively active and activates these pathways and additionally STAT5 independently of the ligand. Inhibitors of these signal transduction pathways were used to assess the role the individual pathways downstream of FLT3 signaling for DUSP6 mRNA expression.

Wortmannin (WM) is a potent and irreversible inhibitor of phosphatidylinositol 3-kinase (PI3-kinase) Treatment of 32D cells expressing FLT3 ITD or of MV4-11 cells resulted in lowering of DUSP6 mRNA levels. Maximum inhibition was observed after 3 hr of treatment in both cell types, where the expression of DUSP6 was only around 20% (see Figure 6-22). Interestingly, the expression was increased again after 6 hr in both cell types.

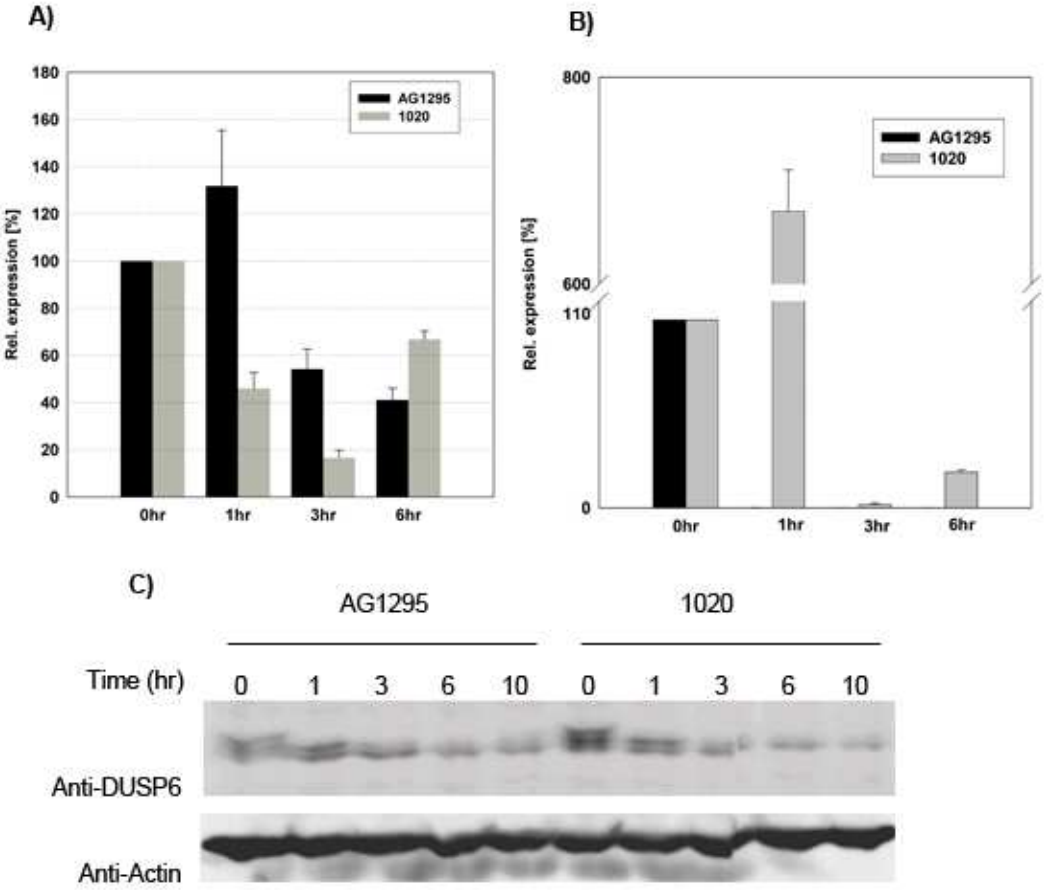


Figure 6-21. FLT3 inhibitor treatment on DUSP6 expression in 32D FLT3 ITD and MV4-11 cells. A) 32D FLT3 ITD cells were washed and starved in 10% hi FCS overnight before treatment with AG1295 (20 µM) and 1020 (1 µM) for the indicated time points; B) MV4-11 cells were washed and starved in serum deficient medium overnight before treatment with AG1295 (20 µM) and 1020 (1 µM) for the indicated time points. The cells were harvested and RNA was prepared followed by cDNA preparation. Equal amounts were adjusted by using 1 µg RNA as input for cDNA synthesis. Expression of DUSP6 was calculated relative to DMSO-treated cells and β-actin was used as housekeeping gene. The experiment was performed in triplicates and the expression of DUSP6 is represented as percentage (n = 3; mean±SD). C) Inhibitor treated MV4-11 cells were harvested and lysed in RIPA buffer. 60 µg protein form each sample was run on 8% acrylamide gel. The lysates were subjected to western blotting and probed with anti-DUSP6. Actin was used as loading control.

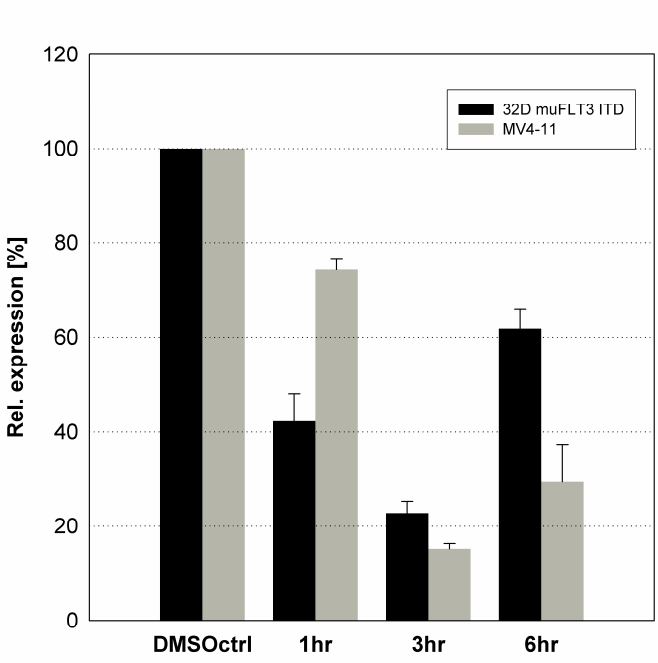


Figure 6-22. Effect of a PI3 Kinase inhibitor on DUSP6 mRNA expression in 32D FLT3 ITD and MV4-11 cells treatment. 32D FLT3 ITD cells or MV4-11 cells were washed and suspended in 10% hi FCS or serum deficient medium, respectively and incubated overnight. The cells were then treated with Wortmannin (100 nM) for the indicated time points, subsequently harvested and RNA was prepared followed by cDNA preparation. Equal amounts were adjusted by using 1 µg RNA as input for cDNA synthesis. Expression of DUSP6 was calculated relative to DMSO treated cells and β-actin was measured as housekeeping gene. The experiment was performed in triplicates and the expression of DUSP6 is represented as percentage (n = 3; mean±SD).

U0126 is a selective and potent inhibitor of RAS-MAPK pathway since it inhibits immediate upstream activators of ERK; MEK1 and MEK2. MEK inhibition by U0126 resulted in very strongly diminished mRNA levels of DUSP6 in the 32D cells expressing FLT3 ITD as well as in MV4-11 cells (Figure 6-23). A very pronounced down regulation was achieved in MV4-11 cells after 3 hr of U0126 treatment after which there was only a slight increase in DUSP6 mRNA expression. In 32D cells, the expression was also lowered to as little as 10% after 3 hr, persisting even after 6 hr of inhibitor treatment.

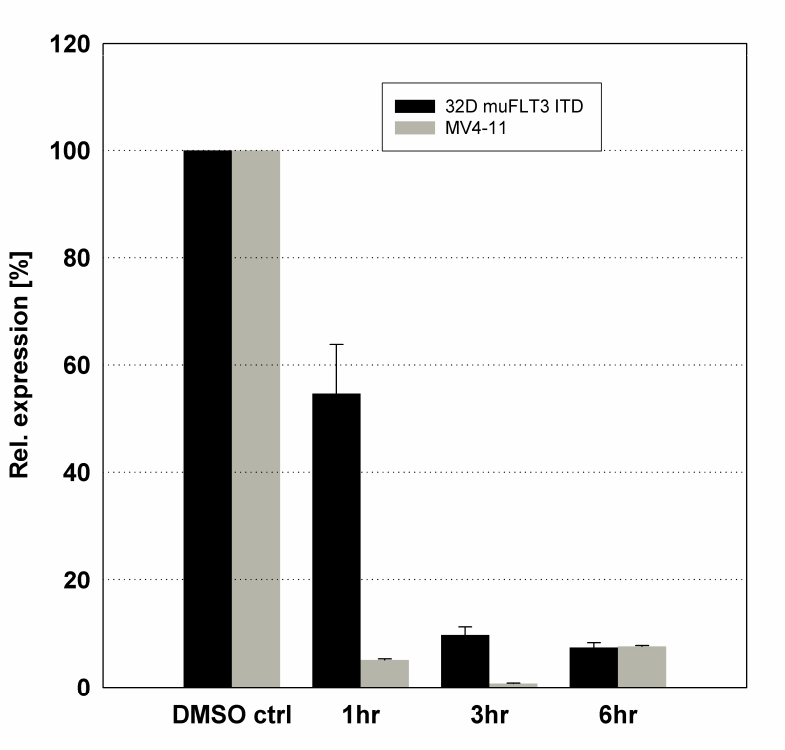


Figure 6-23. Pronounced inhibition of mRNA expression of DUSP6 in 32D FLT3 ITD and MV4-11 cells by MEK inhibitor treatment. 32D FLT3 ITD cells and MV4-11 cells were washed and suspended in 10% hi FCS and serum deficient medium respectively and incubated overnight. Thereafter, the cells were treated with U0126 (10 µM) for the indicated time points. Cells were harvested and RNA was prepared followed by cDNA preparation. Equal amounts were adjusted by using 1 µg RNA as input for cDNA synthesis. Expression of DUSP6 was calculated relative to DMSO treated cells and β-actin was used as housekeeping gene. The experiment was performed in triplicates and the expression of DUSP6 is represented as percentage (n = 3; mean±SD).

A hallmark of FLT3 ITD signaling is activation of the STAT5 pathway, which is essential for cellular transformation. The FLT3 WT does not activate STAT5 and is therefore not transforming. To inhibit this transforming pathway of FLT3 ITD, a pharmacological inhibitor of STAT5 (SI) was used which selectively targets the SH2 domain of STAT. STAT5 is the preferred target of this compound compared to STAT1 and STAT3 (Müller, Sperl et al. 2008). Treatment of FLT3 ITD expressing 32D cells with this compound completely abrogated colony formation (Rinesh Godfrey,

unpublished data). The DUSP6 mRNA expression in 32D cells expressing FLT3 ITD was lowered by treatment with the compound but not drastically (see Figure 6-24).

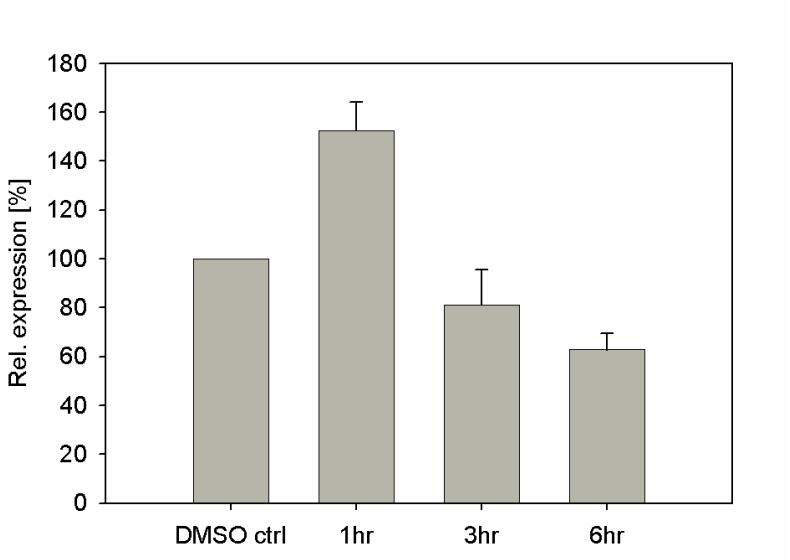


Figure 6-24. Expression of DUSP6 mRNA in 32D FLT3 ITD after STAT5 inhibitor treatment. 32D FLT3 ITD cells were starved in 10% hi FCS and incubated overnight and then treated with STAT5 inhibitor SI (200 µM) for the indicated time points. The cells were harvested, and RNA was prepared followed by cDNA preparation. Equal amounts were adjusted by using 1 µg RNA as input for cDNA synthesis. Expression of DUSP6 was calculated relative to DMSO treated cells and β-actin was used as housekeeping gene. The experiment was performed in triplicates and the expression of DUSP6 is represented as percentage (n = 3; mean±SD)

The expression was as high as 70% even after treatment for 6 hr. This finding indicates that the STAT5 pathway which is quite independent of both RAS-MAPK and PI3K-AKT pathway, when blocked does not affect the downstream signaling of FLT3 ITD via the other signaling cascades and plays only a minor role in DUSP6 expression regulation.

Acknowledgments: The experiments showing site specific hyperphosphorylation of FLT3 in DEP-1 depleted cell pools were performed by Sabine Stopp and have been described in detail in the "Diplomarbiet" presented to FSU, Jena in 2009. The Figure 6-15 is used with her consent. Transient depletion of DEP-1 in THP-1 cells and signaling effects were performed by Annette Böhmer and the data in Figure 6-16 was kindly provided by her. The cell sorting experiments were performed with the help of Simone Tänzer, Fritz Lipmann Institute, Jena. The experiments on mice were conducted by Prof. Reinhardt Bauer, CMB, Jena. Detection of GLEPP1 by SDS-PAGE using specific antibodies (data not shown) was done with the help of PTPNET collaborators Drs. Ari Elson and Vasudeva Akepati Reddy at The Weizmann Institute of Science, Rehovot, Israel.

7 Discussion

7.1 Identification of PTPs involved in negative regulation of FLT3

There have been indications from the previous studies of some PTPs like PTP-PEST, SHP-1, PTP1B dephosphorylating FLT3 when overexpressed (Schmidt-Arras, Bohmer et al. 2005) and SHP-2 being implicated as a positive mediator of FLT3 dependent proliferation in 32D cells (Müller, Schönherr et al. 2008). To identify PTPs involved in FLT3 regulation by a more rigorous approach, a panel of PTPs was targeted by shRNA expressing lentiviral particles in 32D cells, which were also expressing the muFLT3 WT. PTP depleted cells were screened to assess the possible effects on FLT3 signaling. When the screen was initiated the shRNAs available were not validated. Therefore, all available shRNAs targeting a given PTP were used for screening. FLT3 signaling activates downstream the RAS-MAPK pathway resulting in proliferation of cells. PTPs regulating the activity of FLT3 will affect the downstream FLT3 pathways like RAS-MAPK and PI3K signaling. The effect on the activation of ERK was assayed after depletion of the PTPs. Another screening method was tested which was based on the transcriptional activation of PU.1, a transcription factor regulated by FLT3 (Mizuki, Schwable et al. 2003). However, this approach could not be successfully used as a robust screening strategy, and was thus replaced with the phosphorylated ERK screen. From the phosphorylated ERK screen, cell pools expressing shRNAs targeting some PTPs showed elevated levels of phosphorylated ERK after stimulation with FL. The data obtained from all the cell pools were analyzed qualitatively and scores were assigned based on the extent of increase in phosphorylated ERK in comparison to cells harboring the non targeting shRNA.

From the PTP screen it became clear that out of 4-5 available shRNA sequences targeting a PTP, if any, usually only 1-2 sequences had an observable effect on the FL-induced ERK activation. For example, only two shRNA sequences; A2 and A3 which target the

expression of PTPRJ/DEP-1 resulted in increased phosphorylation of ERK, while other sequences did not show detectable differences compared to the non targeting control.

At the time of the screen, the level of depletion of mRNA of the PTPs achieved was not analyzed for all 20 PTPs. Therefore only PTPs were selected which showed high scores in relation to phospho-ERK (see Table 1) to analyze mRNA levels of the PTPs. The selected cell pools with the shRNA sequence targeting these PTPs were used to analyze downregulation of the mRNA level. Knockdown efficiency was determined for OST-PTP, PTP-α, DEP-1, CD45, GLEPP1, SHP-1, SHP-2, and PTP1B using quantitative real time PCR. The knockdown efficiency varied among the different shRNA sequences. A knockdown of PTP1B could not be confirmed. Strikingly, GLEPP1 targeting shRNAs C7, C9, C10, and C11 all of which resulted in maximal ERK activation after stimulation of FLT3, could not be detected at mRNA levels in muFLT3 WT expressing 32D cells. Additionally, efforts were made to detect GLEPP1 at protein level using a panel of antibodies (This was done with the help of PTP-NET collaborators Drs. Ari Elson and Vasudeva Akepati Reddy at The Weizmann Institute of Science, Israel). It was confirmed that indeed GLEPP1 is not expressed in 32D cells. The fact that it was identified as a positive “hit” from the phosphorylated ERK screen, but not expressed in the 32D cells, classified GLEPP1 as a “false positive” result of the screen. One possible explanation for the increase in phosphorylated ERK levels in the false-positives could be off-target effects of the shRNAs. Alternatively, these cell pools might have had elevated FLT3 levels and thus, enhanced signaling altogether. Alterations in FLT3 levels were observed after selection of the cell pools but were recognized only after the screen had been finished. As mentioned earlier, only 1-2 which were analyzed out of 4-5- sequences were effective with respect to PTP knockdown. In some cases knockdown failed and therefore, the possibility of “false negative” results cannot be ruled out. Some relevant PTPs might have been overlooked because of the lack of an efficient knockdown. Thus, in a blind screen, the occurrence of such false positives and negatives has to be considered.

The above mentioned PTPs depleted 32D cell pools were also analyzed for growth properties in presence or absence of growth factors. The improvement in cell growth was

observed for some selected candidates, especially for CD45 and DEP-1 depleted cell pools. This could be a consequence of elevated activation of ERK in these cell pools as observed in the screening FLT3 WT receptor upon stimulation with FL is activated and initiates phosphorylation of downstream signaling proteins, the mechanisms have been outlined (Schmidt-Arras, Schwable et al. 2004). Knockdown of DEP-1 or CD45 in 32D muFLT3 WT expressing cells appeared to result in increased activity of the FL-stimulated FLT3 kinase, which strongly activates signaling through the RAS-MAPK pathway. These initial experiments along with the reduction of mRNA levels of DEP-1 and CD45 indicated a negative regulatory effect on the FLT3 signaling. Efforts were made to therefore, more efficiently and repeatedly downregulate DEP-1 and CD45, and a second set of cell lines was created independently in which the possible negative role of these PTPs in FLT3 was confirmed.

Notably, activation of ERK as reproduced a number of times in independently derived cell pools depleted of DEP-1 or CD45. This correlated with a somewhat improved cell growth with specific stimulation of FLT3. Moreover, transient depletion of DEP-1 in human AML blast cells line THP-1 cells expressing FLT3 WT resulted in elevated phosphorylation of tyrosine sites 589, and 599. Although, there was marginal activation of RAS-MAPK pathway in transiently DEP-1 depleted THP-1 cells. Thus in this study, using a loss-of-function approach, DEP-1 was identified as a negative regulator of FLT3 signaling.

The position at which DEP-1 interferes with the signaling of FLT3 was another question to be addressed. It was observed that DEP-1 depleted 32D cells exhibited hyperphosphorylation based on detection of total phosphotyrosine on the receptor as well as of specific tyrosine phosphorylation sites. Notably, pTyr sites 589 and 591 appeared to be hyperphosphorylated upon DEP-1 depletion. These sites in the juxtamembrane region of FLT3 mediate and activate STAT5 in FLT3 ITD expressing 32D cells (Rocnik, Okabe et al. 2006). The activation of STAT5 is one of the most striking features exclusive to FLT3 ITD and is required for the transforming potential of the mutant receptor. The sites pY589 and pY591 are also hyperphosphorylated in FLT3 ITD as compared with the

wildtype receptor (Razumovskaya, Masson et al. 2009). Hyperphosphorylation of these sites in DEP-1 depleted cell pools indicate the possibility of activation of STAT5 as a consequence of DEP-1 depletion in 32D cells expressing FLT3 WT.

This was indeed observed, however the activation was very moderate. In human breast cancer cell lines, DEP-1 has been shown to dephosphorylate of the c-Met/HGF receptor (Palka, Park et al. 2003). c-Met dephosphorylation occurred preferentially at C-terminal phosphorylation sites. In another study performed in PDGFR-β expressing porcine aortic endothelial cells, induction of DEP-1 overexpression resulted in dephosphorylation of PDGFR-β receptor (Kovalenko, Denner et al. 2000). This study and additional *in vitro* experiments revealed that DEP-1 prefers a C-terminal site of the PDGFR-β receptor for dephosphorylation (Persson, Engström et al. 2002). Also, DEP-1 knockout fibroblasts exhibited an increased PDGFR-dependent signaling and transient knockdown in vascular smooth muscle cells resulted likewise hyperactivation of PDGFR induced signaling (Kappert, Paulsson et al. 2007). In yet another model *C. elegans,* a homolog of DEP-1 is identified as a negative regulator of EGFR which dephosphorylates EGFR thereby affecting the EGFR/RAS-MAPK signaling pathway (Berset, Hoier et al. 2005). These data might indicate that DEP-1 may function as a general phosphatase for RTKs rather than being specific. Moreover, site selectivity of RTK dephosphorylation appears to be another common observation.

It has been shown that 32D cells expressing FTL3 ITD but not FLT3 WT, are capable of clonal growth *in vitro* (Mizuki, Fenski et al. 2000). WT FLT3 does not mediate colony formation in presence of FL, whereas, FLT3 ITD is transforming even in absence of any growth factor. When we subjected DEP-1 depleted 32D cells expressing FLT3 WT to *in vitro* transformation assay in a semi-solid media, colony formation was observed in cells incubated with FL. No colony growth was observed of cells without growth factor. This indicated that all the transformation resulting in colony growth was mediated by specific stimulation of FLT3. The colony formation in DEP-1 depleted 32D cells expressing FLT3 may be attributed to the activation of STAT5. In addition, the transforming potential of these cells however, did not result in myeloproliferative disease in syngenic

C3/HeJ mice. It can be inferred that though loss of DEP-1 may contribute to leukemogenesis, it is not sufficient to promote cell transformation to the extent FLT3 ITD is capable of.

Whether DEP-1 indeed dephosphorylates FLT3 directly, is a matter of further investigation. In general, direct substrates of DEP-1 have not been conclusively elucidated, but some candidates have been proposed. For example, DEP-1 has been shown to associate directly with ERK1/2 in intact cells resulting in dephosphorylation (Sacco, Tinti et al. 2009). In the same study, using a phosphopeptide screen other putative substrates of DEP-1 have been identified e.g. EGFR, Grb2, and Raf1 among others. In a recent study to identify PTPs interacting with EGFR a siRNA library consisting of all human PTPs was used. DEP-1 and PTPRK were shown as PTPs targeting EGFR, whereas, direct interaction was demonstrated for DEP-1 and EGFR (Tarcic, Boguslavsky et al. 2009). RTK for which a direct interaction with PTP was demonstrated is VEGFR and VE-PTP (Mellberg, Dimberg et al. 2009). It was observed that VEGFR and VE-PTP are in close association in resting cells, which is disrupted after activation of VEGFR. The binding resumes as the activity of receptor is decreased. VE-PTP is structurally similar to DEP-1 in having extracellular fibronectin type III domains and a single intracellular domain. Based on these observations, it could be postulated that there is also an association between FLT3 and DEP-1 which may be required for suppressing basal receptor activity in resting cells. In some experiments (S. Stopp, J. Müller, unpublished data) indeed weakly elevated basal activity of FLT3 upon DEP-1 depletion was seen. Alternatively, DEP-1 could be recruited in a complex only after FL-stimulation. Depletion of DEP-1 mainly promotes increased FLT3 activity upon FL-stimulation, which could be based on ligand-dependent FLT3-DEP-1 association. The effects of DEP-1 depletion were quite robust at the level of FLT3 phosphorylation suggesting direct interaction. Signaling effects (such as on ERK1/2) were comparatively weak. It is well possible that there are yet unidentified signaling molecules hyperactivated in DEP-1 depleted cells, involved in yet unidentified signaling pathways. Their identification may unravel novel pathways of regulation. For example, an unidentified phosphoprotein has been found associated with FLT3 in THP-1 cells only if they are transiently depleted of

DEP-1 (Annette Böhmer, unpublished data). Further work on this aspect is being undertaken.

In addition to DEP-1, another PTP which appeared as a negative regulator of FLT3 was CD45. Depletion of CD45 resulted in activation of the downstream RAS-MAPK pathway and cell proliferation but did not activate STAT5 or induce transformation *in vitro*. Both DEP-1 and CD45 are structurally distinct RPTPs but overlapping regulatory functions of both have been indicated in macrophages of B-cells (Zhu, Brdicka et al. 2008). In these cells, both of them are required for the optimal dephosphorylation of src-family kinases (SFKs). The DEP-1 and CD45 double knockout mice had severe B-cell and macrophage signaling defects, whereas the effects of a single DEP-1 or CD45 knockout were very mild. One could assume that a similar redundancy of DEP-1 and CD45 also occurs in FLT3 signaling. It was therefore attempted to stably deplete both of these PTPs in 32D cells expressing FLT3 WT. Strikingly, the "double knockdown cell pools" and in part also the CD45 depleted cell pools exhibited an elevated surface expression of FLT3 at protein level, which resulted in enhanced growth effects. This seemed to be a persistent outcome of stable targeting of CD45 in 32D cells, but the undergoing mechanisms remained unclear. It could be speculated that SFKs may play a role in this phenomenon.

Src and SFKs which include Yes, Lyn, Lck, Src, Fyn, Fgr, Yrk, Hck and Blk, have diverse and key functions in the cell proliferation, differentiation and survival. Src has been associated with various sub-cellular locations like plasma membrane, cytoplasmic and perinuclear spaces as well as the nucleus, where also functions have been proposed (Bjorge, Jakymiw et al. 2000). One of the mechanisms of Src activation involves dephosphorylation of the inhibitory C-terminal phosphotyrosine, in src the pTyr527 site. PTPs shown to aid in this process are PTP1B, SHP-1, SHP-2 among the cytosolic PTPs and CD45, and PTP-α among the transmembrane PTPs. On the other hand, dephosphorylation of the activating site in Src pTyr416 by PTP-BAS decreases Src activity (Roskoski Jr 2005). Internalization and trafficking regulate the activity of RTK-mediated signaling events. Receptor internalization is often mediated by clathrin-coated pits. Non-clathrin coated pits have also been implicated in the process but the mechanism

is poorly understood (Orth, Krueger et al. 2006). RTK internalizations related to the presence of some special structures called circular dorsal ruffles (CDR) or "waves". These are dynamic and transient actin-based structures on the cell surface which presumably serve an endocytotic function in that they can specifically sequester and subsequently internalize up to 50% of RTK, such as the EGF receptor (EGFR) and PDGFR. The formation of these structures is dependent on the GTPase dynamin and on cortactin, in addition to proteins like Rac, Ras, and c-Abl. Also, SFKs play a role in this process. For example, SFKs are reported to be involved in PDGF-induced actin dorsal ruffle formation (Veracini, Franco et al. 2006). The mechanism by which Src signals for dorsal ruffle formation still remains unclear. The receptor internalization is regulated by Src activity, and this is in turn partly affected by CD45 and depletion of CD45 may affect surface FLT3 expression through related mechanisms. Reduction of CD45 in 32D cells expressing FLT3 could result in increase in the cellular pool of phosphorylated (pTyr527) and therefore inactive Src and thereby reducing the Src mediated receptor internalization, the overall effect being elevated FLT3 levels on the cell surface. However, this hypothesis needs to be investigated in detail.

While CD45 may regulate FLT3 signaling indirectly via alterations of trafficking, DEP-1 was found to be a presumably direct negative regulator of FLT3 signaling. Loss of DEP-1 elevated signaling and conferred a growth advantage to FLT3 expressing 32D cells. Loss of DEP-1 resulted in *in vitro* transformation, but was insufficient to cause myeloproliferative disease in syngenic C3H/HeJ mice. The concept of the "two-hit" model to explain leukemogenesis has been discussed earlier; where one class of mutations is required for a proliferation advantage and a second class of mutation is required to block differentiation of the blast cells (see 3.1.2). For example, it is known that activating mutations in FLT3 induce myeloproliferative disease but are not sufficient to cause AML. It was reported that PML/RARα can cooperate with FLT3 ITD to induce an APL-like disease in the mouse (Kelly, Kutok et al. 2002). Approximately 37% of t(15;17) APL patients have mutations that constitutively activate the FLT3 tyrosine kinases (Kottaridis, Gale et al. 2001). Mutant FLT3 may play a role as a second event in pathogenesis of APL.

According to the findings in this thesis, DEP-1 represents as a possible target for mutations contributing to AML progression by causing deregulation of RTK signaling. However, this could clearly not be the sole factor for AML progression and other contributing mutations should be essential for disease formation. For example, it was recently shown that PTPN2/TCPTP inactivation increased proliferation and cytokine sensitivity of T-ALL cells (Kleppe, Lahortiga et al.). TCPTP was reported to be a negative regulator of NUP214-ABL1 kinase activity, loss of TCPTP was apparently supplemented by aberrant expression of the transcription factor oncogene TLX1 to result in leukemic transformation. In A549 lung adenocarcinoma cells, a phosphatome screen identified DEP-1, TCPTP and PTEN among the potential negative regulators of the Ras pathway (Omerovic, Clague et al.). Loss of function of these PTPs accelerate the activity of Ras, however this depended on the presence of activating Ras mutations signaling. Likewise, in addition to loss of DEP-1, other factors appear to be required to result in myeloproliferative disorder in mice or perhaps contribute to human AML.

Having described the possible importance of DEP-1 in FLT3 WT signaling, the probable role of DEP-1 in mutated FLT3 remains to be addressed. If DEP-1 is a negative regulator of FLT3, which may relate to a tumor suppressive function in AML, its loss could also enhance mutant FLT3 signaling. Alternatively, FLT3 mutations could perhaps affect DEP-1 expression or function to dampen its inhibitory role. When the AML blasts expressing FLT3 WT or FLT3 ITD were used for expression analysis, it was found that expression of DEP-1 remained nearly similar in both blast types. It has however, been shown that FLT3 ITD produces excessive intracellular reactive oxygen species (ROS), which causes DNA damage and contributes to aggressive AML (Sallmyr, Fan et al. 2008). This could potentially affect DEP-1 function. The mechanisms explaining regulation of PTPs have been outlined (in the Introduction), where importance of oxidation has been mentioned. ROS can oxidize the catalytic cysteine of the active domain of PTPs reversibly, and this oxidation makes the enzyme inactive. Further work investigating the oxidation and inactivation of DEP-1 by FLT3 ITD induced ROS production is being performed (Rinesh Godfrey, unpublished data).

A schematic representation summarizing the negative regulation of FLT3 by DEP-1 is being shown in Figure 7-1. The transmembrane protein DEP-1, possibly in proximity with FLT3, dephosphorylates the tyrosine kinase residues in the receptor. DEP-1 mediated dephosphorylation of FLT3 may exert a basal level of control over FLT3 signaling. Reduction in DEP-1 activity due to altered protein levels, or altered function may disrupt the basal control, thereby activating the receptor.

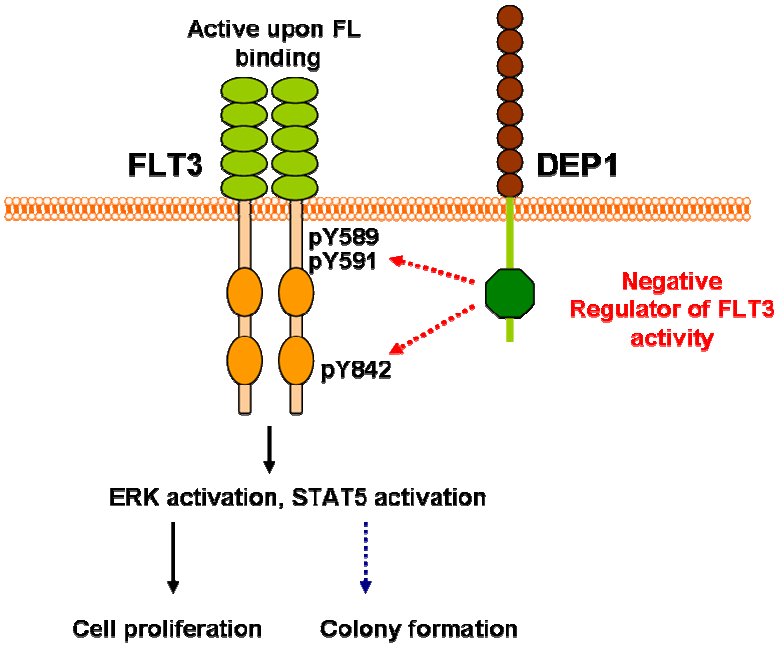


Figure 7-1: Hypothetical scheme of regulation of FLT3 by DEP-1. Ligand binding induces dimerization of FLT3 on the cell surface, and phosphorylation of tyrosine kinase residues of the receptor. The activated receptor mediates signaling through RAS-MAPK and PI3K-AKT pathways resulting in cell proliferation, survival and differentiation. DEP-1 which is a transmembrane PTP, dephosphorylates tyrosine residues pY589, pY591, and pY842 on FLT3 thereby, lowering FLT3 mediated RAS-MAPK signaling. The black solid arrows indicate positively regulated well described pathway and red dashed arrows indicate steps which are negatively regulated by DEP-1. STAT5 activation causing colony formation appears to be a feature of DEP-1 depleted cells (blue).

7.2 Expression regulation of PTPs by FLT3 ITD

Dysregulation of PTPs is increasingly recognized as feature of different cancer types. Putative tumor suppressor PTPs are inactivated or downregulated, while oncogenic PTPs are in part activated or upregulated (Andersen, Jansen et al. 2004) (Alonso, Sasin et al. 2004). To identify PTPs which are expressed in AML cells and thereby putatively relevant for leukemia biology, as well as such which might be regulated by FLT3 ITD, the expression pattern of 92 human PTP was analyzed. Both human AML blasts and cell lines expressing either FLT3 WT or FLT3 ITD were compared. To start with, human AML blasts of a few patients were used to get the PTP profile pattern. It was observed that the mRNA expression of many receptor type PTPs for example PTPRK, PTPRO, and PTPRD was very low or undetectable in AML blasts of both types. These PTPs are unlikely to play a role in leukemia biology. A reservation which needs to be mentioned here is that no comparison with a relevant hematopoietic normal cell population like CD34 positive stem cells was made. Potentially some RPTPs may not have been detected because of loss in the AML cells. In contrast, many dual specificity phosphatases were expressed abundantly for example DUSP1/MKP1, DUSP2/PAC1, DUSP6/MKP3, and ACP1/LMWPTP.

In the following, some potentially interesting examples of expression alterations and their relevance in AML shall be discussed. Comparisons of PTP mRNA expression made between human AML blast cell lines and AML blasts expressing either FLT3 or FLT3 ITD revealed that PTP4A1 and PTPN2 mRNAs appeared downregulated upon FLT3 ITD expression. Conversely, DUSP6 was consistently upregulated in FLT3 ITD expressing cells. Other PTPs like DUSP1, DUSP2 and DUSP4 appeared to be regulated by FLT3. Recently, PTP expression data from a set of over 200 AML patients was made available, obtained by a group in Rotterdam (Wouters, Jorda et al. 2007). The samples used in the study were characterized and stratified in various groups and subgroups of mutation types occurring in the samples. While analyzing this larger dataset, it was observed that expression of DUSP1 and TNS1 was downregulated in AML blasts expressing FLT3 ITD (see Figure 7-2). In our experiments with all the patient AML blast samples we analyzed, for example, there was a trend of downregulation of DUSP1 expression in AML blasts

and cell lines expressing FLT3 ITD, however, there was large variation between the patient samples we analyzed. FLT3 ITD AML blasts exhibited reduced TNS1 expression in a small set of AML blast samples which were analyzed (data not shown). These samples were only segregated on the basis of FLT3 status; other features have not yet been considered to classify the patients, notably given the low patient numbers. This is one of the possible explanations of higher variations observed in the PTP expression profile. Therefore, proper characterization of the patient AML blasts for further mutations and cytogenetic features is customary. Confirmation of the results obtained with the limited number of human AML blasts should be done by comparison with a larger dataset of samples which are characterized explicitly in terms of these parameters.

10 PTPs namely, DUSP1, DUSP2, DUSP4, DUSP6, DUSP8, DEP-1, PTPN2, PTP4A1, SBF1, and LMWPTP were selected for further investigation in a larger set of patient samples from the total of 92 human PTPs. The criterion to choose these genes were; trends of alteration in the qRT-PCR analysis of the array dataset related to FLT3 status, very high expression levels, and interest from functional point of view. In addition, these PTPs were then tested for mRNA expression in murine myeloid-derived 32D cells expressing no FLT3, FLT3 WT or FLT3 ITD. As observed in human AML blasts and human AML cell lines, DUSP6 mRNA was upregulated in FLT3 ITD expressing 32D cells. Interestingly, the 32D cells without FLT3 expression displayed low levels of DUSP6, and expression of FLT3 alone resulted in increase of basal DUSP6 expression. This was also observed at the DUSP6 protein level. In yet another murine cell line, the bone marrow-derived Ba/F3 cells, DUSP6 appeared to be upregulated with expression of FLT3 compared to Ba/F3 cells not expressing FLT3, and even more with FLT3 ITD expression. The findings in human AML blasts and cell lines along with data obtained in two different murine cell models, confirmed that DUSP6 mRNA expression is driven by FLT3 and is even elevated by expression of FLT3 ITD. The expression of DUSP6 was observed to be causally linked with FLT3 ITD signaling, as blocking the FLT3 activity by using kinase inhibitors AG1295 and 1020, resulted in lowering of DUSP6 mRNA in murine 32D cells and human AML cell line MV4-11 cells, both expressing FLT3 ITD.

The results obtained with DUSP6 mRNA were consistent with those observed at DUSP6 protein levels.

The three central signaling pathways active in FLT3 ITD are RAS-MAPK, PI3K-AKT, and STAT5. Pharmacologic inhibition of all the three pathways was performed in murine and human cell lines expressing FLT3 ITD to assess their relevance for DUSP6 induction. Inhibition of the RAS MAPK pathway was done with MEK inhibitor U0126, and treatment with this compound resulted in acutely reduced mRNA levels of DUSP6, indicating it to be the main pathway controlling DUSP6 expression. Interaction between RAS-MAPK and PI3K pathways has been shown in COS-7 cells By using PI3K inhibitors, positive cross-talk between both pathways was observed upstream and downstream of Ras (Wennstrom and Downward 1999). When 32D cells and the human AML cell line MV4-11 expressing FLT3 ITD were treated with the PI3K inhibitor Wortmannin, the DUSP6 mRNA level was reduced. However, the effect of the PI3K inhibitor was milder in comparison to direct inhibition by MEK inhibitor U0126. It can however be speculated, that this effect may occur indirectly via affecting RAS-MAPK signaling due to PI3K inhibition.

Genes previously reported to be upregulated by constitutively activated FLT3 include Pim-1 and DUSP6 among others (Small 2006). The transforming potential of FLT3 ITD is mediated by activation of STAT5, which induces the expression of serine threonine kinase Pim-1 (Kim, Baird et al. 2004). Thus, induction of Pim-1 is important for FLT3 mediated leukemogenesis. To test whether the STAT5 pathway influences expression of DUSP6, a STAT5 inhibitor was used, but STAT5 inhibition did not reduce DUSP6 mRNA expression to a great extent, indicating that both pathways are nearly independent of each other.

According to one study in cultured cells like PC12 and MM14 muscle cells, stimulation of ERK pathway by growth factors results in induction of DUSP6 transcripts (Mourey, Vega et al. 1996) (Muda, Boschert et al. 1996). The induction of DUSP6 by nerve growth factor (NGF) and fibroblast growth factor (FGF) suggests a possible role in proliferation

and differentiation. As recently reported DUSP6 is an important regulator of PDGF-induced phosphorylation of ERK and is involved in both positive and negative feedback loops in the signaling cascade (Jurek, Amagasaki et al. 2009). They reported that in early phase, stimulation with PDGF-BB causes MEK dependent degradation of DUSP6 which is required for activation and phosphorylation of ERK. Upon prolonged stimulation, the DUSP6 gene is induced and DUSP6 is synthesized which then dephosphorylates and inactivates ERK. Also, specific downregulation of DUSP6 resulted in hyper activation of ERK. Since FLT3 and PDGFR are close relatives, several of these features may apply also to FL-stimulated cells. This is currently being explored (Benjamin Tölle, unpublished data).

Some reports have previously addressed the possible role of DUSP6 in cancer. For example, in non-small cell lung cancer (NSCLC) DUSP6 expression was shown to be regulated by ERK signaling and that DUSP6 exerts a negative feedback regulation (Zhang, Kobayashi et al.). In pancreatic cancers, downregulation of DUSP6 has been correlated with hypermethylation of its promoter (Xu, Furukawa et al. 2005). Upregulation of DUSP6 has been associated with other cancer types. For example, prolonged activation of ERK1/2 leads to an increased expression of DUSP6 in human breast cancer cell lines and this activation of DUSP6 is linked to transcription of Ets2 transcription factor (Nunes-Xavier, Tárrega et al.) . Also, strong upregulation of DUSP6 has been noted in separate breast cancer datasets with HER-2 positive tumors (Lucci, Orlandi et al.). Microarray analysis in melanoma cell lines harboring mutations of Ras (N-Ras) and B-RAF, revealed DUSP6 as one of the strongly upregulated genes (Bloethner, Chen et al. 2005). Numerous studies have indicated that DUSP6 expression correlates with ERK1/2-activation and upregulation of DUSP6 may contribute to hyperactivation of ERK, however, the mechanism in contribution towards transformation needs to be understood.

As indicated by the high expression levels of both mRNA and protein, there might be possible involvement of DUSP6 in FLT3 ITD mediated transformation. To further test this hypothesis, the functional significance of DUSP6 was analyzed in human and murine

cell lines expressing FLT3 ITD. Targeted disruption of DUSP6 resulted in enhanced phosphorylation of ERK in the human AML cell lines MV4-11 and RS4-11 expressing FLT3 ITD and FLT3 WT respectively. However, in murine 32D cells expressing FLT3 ITD, stable depletion of DUSP6 did not affect the transforming potential greatly (Benjamin Tölle, unpublished data). These findings may indicate that high DUSP6 levels do not solely contribute to FLT3 ITD-mediated transformation. However, downregulation of DUSP6 in the stable cell pools was only partially possible, limiting conclusions.

In most AML samples, the mutations in FLT3 and Ras are almost always known to be mutually exclusive (Small 2006). This seems to be in conformation with the "two-hit" model of leukemogenesis, mutations in either FLT3 or Ras are sufficient to drive cell proliferation and to induce transformation. FLT3 ITD which activates RAS-MAPK pathway constitutively in the absence of FL, results in elevated levels of activated ERK.

As discussed above, affymetrix data of expression profile of all PTPs in over 200 hundred AML blasts were available (Wouters, Jorda et al. 2007). The AML blasts used for this study are classified into various commonly occurring sequence mutations and chromosomal abnormalities. On analyzing the data, it was found that the expression regulation of DUSP1 and DUSP6 by FLT3 was not significant (see Figure 7-2) in this dataset. Interestingly, dual specificity PTP TNS1 was significantly downregulated in presence of FLT3 ITD. Data obtained in our experiments revealed similar trends in the samples we tested, but there was a large variation (this issue has been discussed already). The expression of DUSP1 and TNS1 was therefore also analyzed in murine Ba/F3 and 32D cells with or without FLT3 expression to test for FLT3 driven differences in PTP expression. Both TNS1 and DUSP1 were downregulated at mRNA level in the murine cells (see Figure 7-3).

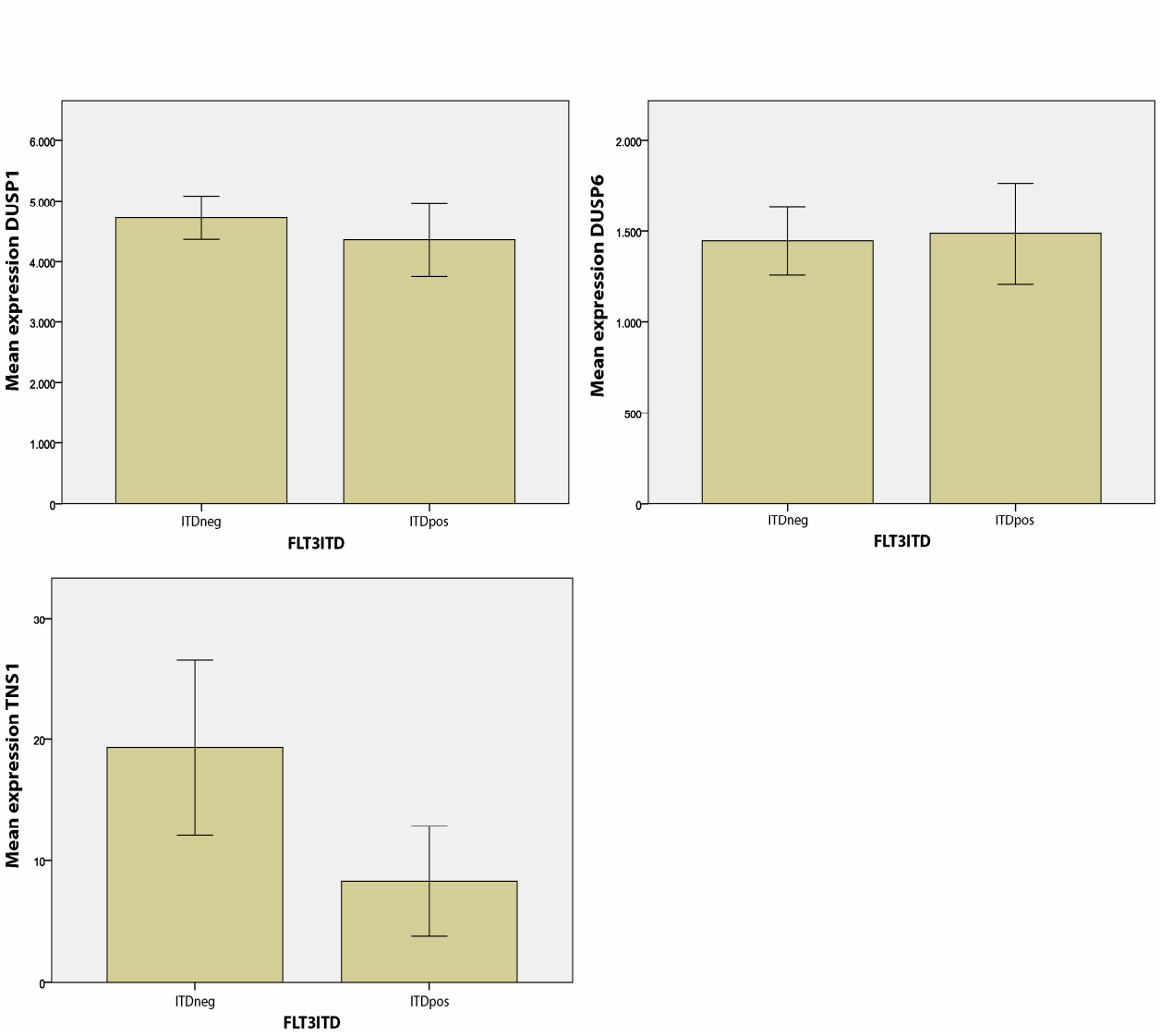


Figure 7-2. Expression of DUSP1, DUSP6 and TNS1 in AML patient blasts analyzed using Affymetrix HGU133A Genechip. The mean expression is depicted; 95% confidence limits (Data analysis: Susanne Köthe).

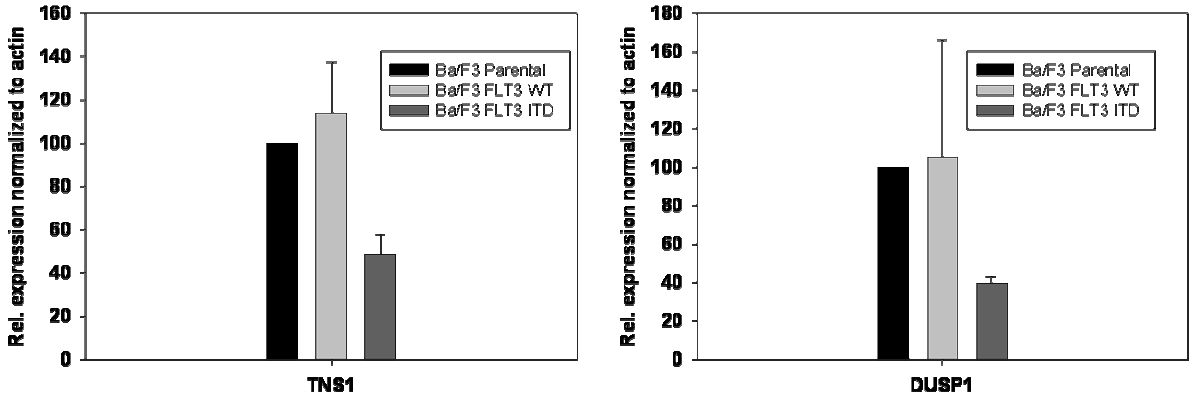


Figure 7-3 mRNA expression of DUSP1 and TNS1 in Ba/F3 cells. The cells were starved overnight and RNA was prepared followed by cDNA preparation. Equal amounts were adjusted by using 1 µg RNA as input for cDNA synthesis. Expression of DUSP1 and TNS1 was calculated relative to the house keeping gene- actin. Two independently growing cultures of Ba/F3 cells were used. Values are means of triplicates (n = 3; mean±SD).

Taken together with the data available for the AML blasts and data indicated in murine 32D cells, it can be thus stated that expression of PTPs, especially, DUSP6 and TNS1 is regulated by FLT3 ITD. However, DUSP1 also appears to be a PTP which is downregulated by FLT3 ITD in AML. DUSP1/MKP1 is primarily a nuclear phosphatase which is specific for p38 and JNK. It has been shown to be overexpressed in NSCLC, gastric carcinomas and oral cancers, and upregulated in some hepatocellular carcinomas and prostate cancers (Pulido and van Huijsduijnen 2008). TNS1, 2 and 3 belongs to the Tensin family of intracellular proteins which mediate signaling for maintaining cell shape and motility. They have been proposed as suppressors of metastasis in kidney, and their loss is correlated with greater tumor cell motility and consequent metastasis (Martuszewska, Ljungberg et al. 2009). The role of these interesting PTPs for transformation of AML cells need to be analyzed in detail.

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9 Appendix

9.1 Abbreviations

7-AAD: 7-Aminoactinomycin D

ALL: Acute lymphoblastic leukemia

AML: Acute myeloid luekemia

AML/ETO: Acute myeloid luekemia/ Eight twenty one

APL: Acute promyelocytic leukemia

ATP: Adenosine triphosphate

DEP-1: Density Enhanced Phosphatase

DUSP: Dual specificity phosphatase

EGFR: Endothelial growth factor receptor

FL: FLT3 ligand

FLT3: *Fms* like tyrosine kinase

GEP: Gene expression profile

GFP: Green fluorescent protein

IL3: Interleukin-3

ITD: Internal tandem mutation

JM: Juxtamembrane

MAPK: Mitogen activated protein kinase

MDS: Myelodysplastic syndrome

MTT: (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

p14ARF: Alternate reading frame product of cyclin dependent kinase N2A

PDGFR: Platelet derived growth factor

PI3-K: Phosphatidylinositol 3-kinase

PML/RAR α: Promyelocytic leukemia/ Retinoic acid receptor α

PTP: Protein tyrosine phosphatase

qRT-PCR: Qualitative real time polymerase chain reaction

ROS: Reactive oxygen species

Rpm: Revolutions per minute

RTK: Receptor tyrosine kinase

SFK: Src-family kinase

SI: STAT5 inhibitor

STAT: Signal transducers and activators of transcription

TKD: Tyrosine kinase domain

VEGFR: Vascular endothelial growth factor receptor

WT: wildtype

WM: Wortmannin

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9.3 Curriculum Vitae

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9.4 Declaration

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of the Friedrich Schiller University, Jena that the submitted thesis was written only with the assistance and literature cited in the text.

The thesis has not been previously submitted either to the Friedrich Schiller University, Jena or to any other University.

Jena, July 23rd 2010 ----------

Deepika Arora