Studies on signaling pathways induced by FLT3, an important oncogene in AML

Razumovskaya, Elena

2011

From the Department of Laboratory Medicine, Experimental Clinical Chemistry,

Lund University, Sweden

Studies on signaling pathways induced by FLT3, an important oncogene in AML

Elena Razumovskaya

LUNDS
UNIVERSITET

Faculty of Medicine

Academic dissertation

With the permission of the Faculty of Medicine, Lund University, to be defended at the lecture hall of the department of pathology, Entrance 78, Malmö University Hospital, on Wednesday the 21st of December 2011 at 9.15 a.m.

Faculty opponent:

Professor Bengt Hallberg, Department of Molecular Biology, Umeå University, Umeå, Sweden

Organization	Document name
LUND UNIVERSITY Department of Laboratory Medicine Division of Experimental Clinical Chemistry Malmö University Hospital Sweden	DOCTORAL DISSERTATION
	Date of issue 21 of December 2011
	Sponsoring organization
Author(s) Elena Razumovskaya	

Title and subtitle

Studies on signaling pathways induced by FLT3, an important oncogene in AML

Abstract

FLT3, a receptor tyrosine kinase, is expressed in hematopoietic progenitor cells. FLT3-ITD (internal tandem duplication) and D835 mutations are found in approximately 30% and 7% of Acute Myeloid Leukemia (AML) patients respectively, and correlate with a poor prognosis, thus making the mutated receptor a potential therapeutic target. FLT3 mutations cause constitutive activation of intrinsic tyrosine kinase of the receptor, leading to ligand-independent signal transduction.

The aims of my studies have been to analyze the kinetics and specificity of FLT3 autophosphorylation in wild-type FLT3 and mutants, to identify novel phosphorylation sites in FLT3 receptor, to find out PTPs which can affect the signaling activity of FLT3, to investigate a novel class of tyrosine kinase inhibitors, 3, 4-diarylmaleimides for their ability to inhibit FLT3 and to determine the role of MEK5/ERK5 signaling in FLT3-ITD mediated transformation.

Using phosphospecific antibodies, we have identified 3 novel phosphorylation sites in FLT3, Y726, Y793 and Y842, and studied their kinetics and specificity. The additional eight phosphorylated tyrosine residues in FLT3 were shown to have different phosphorylation characteristics in the wild-type FLT3 compared to the mutated receptors. We have found that the protein tyrosine phosphatase DEP-1 serves as a negative regulator of FLT3 activation and signaling. Furthermore, 3,4-diarylmaleimides inhibitors have been found to be able to inhibit FLT3-ITD in both transfected cells as well as primary ITD-positive AML blasts leading to induction of apoptosis in those cells. These inhibitors also reduced the phosphorylation of signaling molecules downstream of FLT3-ITD such as ERK and STAT5. The last paper in the thesis revealed the anti-apoptotic effect of MEK5/ERK5 on FLT3-ITD expressing leukemia cells.

Key words: FLT3, RTK, FLT3-ITD, FLT3-D835Y, acute myeloid leukemia, oncogenes, kinase inhibitors, ERK5, hematopoiesis

Classification system and/or index termes (if any):

Supplementary bibliographical information:	Language English
ISSN and key title: 1652-8220	ISBN 978-91-86871-59-8
Recipient's notes	Number of pages 138 / Price
	Security classification

Studies on signaling pathways induced by FLT3, an important oncogene in AML

Elena Razumovskaya

LUNDS
UNIVERSITET
Faculty of Medicine

ISBN 978-91-86871-59-8
ISSN 1652-8220

Lund University, Faculty of Medicine
Doctoral Dissertation
Series 2011:109
Printed by Tryckeriet i E-huset, Lund, Sweden 2011

To my Family and my people

Tur är det ställe där förberedelse och möjlighet möts.
J. Locke

Table of contents

Articles included in the thesis

I Oncogenic FLT3 receptors display different specificity and kinetics of autophosphorylation.
Razumovskaya E, Masson K, Khan R, Bengtsson S, Rönnstrand L.
Journal of Experimental Hematology 2009 Aug; 37(8):979-89.

II Protein tyrosine phosphatase DEP-1 controls receptor tyrosine kinase FLT3 signaling.
Arora D, Stopp S, Böhmer SA, Schons J, Godfrey R, Masson K, Razumovskaya E, Rönnstrand L, Tänzer S, Bauer R, Böhmer FD, Müller JP.
The journal of biological chemistry 2011 Apr 1; 286(13):10918-29

III 3, 4- Diarylmaleimides- a novel class of kinase inhibitors- effectively induce apoptosis in FLT3-ITD dependent cells.
F. H. Heidel, Tomas S. Mack, E. Razumovskaya, T.S. Mack, M-C.Blum, D. B. Lipka, A. Ballaschk, A-K. Borrmann, J-P. Kramb, S. Plutizki, L. Rönnstrand, G. Dannhardt, T. Fischer.
Annals of Hematology 2011 Sep 1; 10.1007/s00277-011-1311-3

IV Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD.
Razumovskaya E, Sun J, Rönnstrand L
Biochemical and Biophysical research communications 2011 Aug 26; 412(2):307-12.

Additional Article (not included in thesis)

I SRC is signaling mediator in FLT3-ITD but not in FLT3-TKD positive AML.

Leischner H, Grundler R, Albers C, Spiekermann K, Bohlander S, Razumovskaya E, Rönnstrand L, Götze K, Peschel C, Duyster J.

Submitted to Blood 2011

List of abbreviations

AA	Amino Acid
ALL	Acute LymphoblasticLeukemia
AML	Acute Myeloid Leukemia
AraC	Cytosine arabinoside
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
BM	Bone marrow
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CSF-1	Colony-stimulating factor-1
DAG	Diacylglycerol
DC	Dendritic cell
DEP-1	Density-enhanced phosphatase
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase

F	Phenylalanine
FGF	Fibroblast growth factor
FL	Flt3 ligand
FLK-2	Fetal liver kinase-2
FLT3	Fms-like tyrosine kinase 3
ITD	Internal tandem duplication
Fms	Feline McDonough sarcoma
FTI	Farnesyltransferase inhibitor
GMP	Granulocyte macrophage progenitor
HSC	Hematopoietic stem cell
Ig	Immunoglobulin
IL-3	Interleukin-3
JAK	Janus kinase
JM	Juxtamembrane
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated proteinkinase

MDS	Myelodysplastic syndrome
MEK	MAPK/ERK kinase
MEP	Megakaryocyte erythrocyte precursor
MPD	Myeloproliferative disorder
NES	Nuclear export signal
NK	Natural killer
NLS	Nuclear localization signal
p	Phosphorylated
PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphoinositide-dependent kinase 1
PH	Pleckstrin homology
PI3K	Phosphoinositide-3 kinase
PIP2	Phosphatidylinositol 4,5 bisphosphate
PIP3	Phosphatidylinositol 3,4,5 trisphosphate
PLC-γ	Phospholipase C-γ
PM	Point mutation
PP	Protein phosphatase
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RPTP	Receptor-like PTP
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SH2	Src-homology 2
SH3	Src-homology 3
SMI	Small molecular inhibitors
SRC	Sarcoma
STAT	Signal transducer and activator of transcription
TKD	Tyrosine kinase domain
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
WT	Wild-type
Y	Tyrosine

Introduction

The human body is composed of various cells that communicate with each other through signaling. The cells receive signals from the environment through proteins that are embedded in the cell membrane, called receptors. When receptor receives a signal it transmits the signal further into the cell which will result in a specific response.

Receptor tyrosine kinases are membrane-bound proteins having essential roles in cell growth, division, maturation, metabolism, adhesion, survival and motility. Receptor tyrosine kinases are usually activated by specific ligands, e.g. growth factors. An important process in propagation of a signal cascade is the post-translational modification, called protein phosphorylation. When the ligand binds to the receptor it often causes dimerization of the receptor which leads to activation of the intrinsic tyrosine kinase activity of the receptor. Constitutive, uncontrolled signaling through the receptor causes cell transformation and uncontrolled cell growth, which is found in many kinds of human cancers.

This thesis is focused on FMS-like tyrosine kinase-3 (FLT3) studies. FLT3 is a receptor tyrosine kinase, which is expressed on hematopoietic progenitor cells. Mutations in FLT3 have been implicated as a major cause of transformation in acute myeloid leukemia (AML). There are two major *FLT3* gene mutations that have been identified in AML: an in-frame duplication of the gene segment encoding the juxtamembrane region, the so-called- Internal Tandem Duplication (ITD) and a point-mutation of D835Y (Asp835Tyr) in the activation loop of the second part of the kinase domain. These mutations cause constitutive activation of FLT3 receptor and therefore lead to ligand-independent signal transduction. These alterations occur in approximately 30% of AML patients and are associated with a poor prognosis. High occurrence of these mutations in AML patients makes FLT3 receptor one of the most interesting therapeutic targets.

In paper **I**, we have identified three novel phosphorylation sites of FLT3 receptor in living cells and further compared the stoichiometry and kinetics of phosphorylation of additional sites in wild-type FLT3, FLT3-ITD and FLT3-D835Y mutations by use of our in-house produced phosphospecific antibodies directed against specific tyrosine residues in FLT3. Phosphorylated tyrosines 589, 591, 726, 768, 793 and 842 of the FLT3 receptor were investigated and shown to be differentially activated in wild-type versus the mutated receptor. Our data help to further understand the mechanisms of signaling pathways of the FLT3 receptor that can be involved in many biological responses and also aid to

identify the mechanism by which FLT3-ITD and D835Y mutants function in pathological conditions.

In paper **II**, we have studied the proteintyrosine phosphatase (PTP) DEP-1 (density-enhanced phosphatase-1) and its role in FLT3 signaling. PTPs are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues of the protein. We have identified DEP-1 to be involved as a negative regulation of FLT3 phosphorylation and signaling. Acute depletion of DEP-1 in a human AML cell line leads to increased FLT3 and ERK phosphorylation as well as increased cell growth and survival of these cells. Our data show that DEP-1 is negatively regulating FLT3 signaling activity and its absence can contribute to leukemic cell transformation.

In paper **III,** we have studied 3, 4-Diarylmaleimides, a type of kinase inhibitors which are able to inhibit FLT3 and thus downregulate the phosphorylation of downstream signaling molecules, such as STAT5, AKT and ERK. This novel group of inhibitors induces apoptosis in FLT3-ITD cells even at low concentrations. Since these inhibitors are more efficient against FLT3-ITD than wild-type FLT3, they could be used as a part of the therapy for AML patients. The uniqueness of this study is that it shows not only how the overall phosphorylation of FLT3 is affected by inhibitors, but how every phosphorylation site in FLT3 is influenced by the inhibitor.

In paper **IV**, we have investigated how ERK5 is involved in FLT3-mediated signaling and transformation by using a specific inhibitor of kinase upstream of ERK5, MEK5, called BIX02188. It turns out that cell survival and growth is inhibited when cells are treated with this inhibitor, especially cells expressing oncogenic FLT3-ITD receptor. We have also examined the signaling pathways which could be involved in these processes. We have found that BIX02188 negatively regulates the phosphorylation of AKT and ERK1/2. This study shows that ERK5 is involved in FLT3-mediated signaling and especially in FLT3-ITD induced hematopoietic transformation. This finding may contribute to a novel specific potential future therapy for patients with FLT3-ITD positive leukemia.

In conclusion, these studies show that FLT3 signaling is altered depending on the mutation it carries. Furthermore, we have investigated the role of novel inhibitors in FLT3 signaling which could in future contribute for more specific therapies. In general, our studies show that still there is a lot to evaluate to be able to select the specific therapy for every AML patient.

Background

In multicellular organisms, cells communicate with each other with the help of specific extracellular molecules, e.g. growth factors. These signals are further transmitted to the cell's interior through the plasma membrane proteins, called the signal-transducing receptors. Every signal elicits a specific biological response of the cell. These responses are usually cascades of reactions which cause different effects within the cell, for example change in gene expression, cell growth, proliferation and differentiation. Both specificity and selectivity of the signaling molecules contribute to a healthy physiological state. A genetic aberration in these receptors causes an altered signal cascade leading to pathological conditions such as cancer.

1. Receptor Tyrosine kinases (RTKs)

Receptor tyrosine kinases (RTKs) comprise a large family of enzyme-linked transmembrane proteins with the presence of a tyrosine kinase domain in their intracellular region as a common feature. To date 58 mammalian RTKs are known, which are distributed into 20 structurally different subfamilies (**Fig.1)** [1]. Receptor tyrosine kinases contain a glycosylated, extracellular ligand binding domain, a single transmembrane region and an intracellular domain containing the catalytic protein tyrosine kinase (PTK) domain [2, 3]. In the absence of ligand, RTKs reside as monomers in inactive conformation in the cell membrane, but upon ligand binding they dimerize leading to activation of the intrinsic kinase activity of the receptors, which then phosphorylates intracellular signal transduction molecules and relay the signal into the cell. RTKs play crucial role in many cellular processes such as cell survival, differentiation, proliferation, migration and cell cycle control [4].

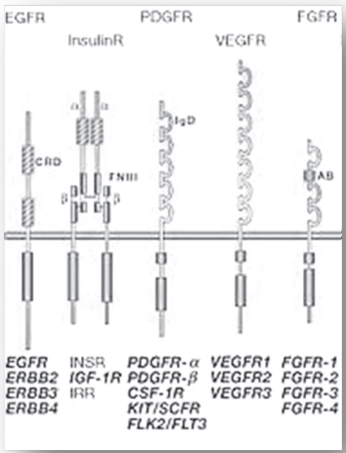

Figure 1. Receptor tyrosine kinases. Schematic view of some representatives of RTK families (modified from [1]). EGFR, epidermal growth factor receptor; Insulin R, insulin receptor; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor. Other abbreviations: CRD, cysteine-rich domain; FNIII, fibronectin type III-like domain; IgD, immunoglobulin-like domain; AB, acidic box.

2. Concepts of Phosphorylation and Dephosphorylation

2.1 Phosphorylation

Protein phosphorylation plays a major role in the regulation of many biological processes. There are around 520 protein kinases and 130 protein phosphatases which control the processes of protein phosphorylation and dephosphorylation. When balanced, the protein switches between phosphorylated and unphosphorylated form. Phosphorylation is one of the post-translational modifications in which a covalently bound phosphate group is added to a protein. Phosphorylation can occur on serine, threonine or tyrosine residues. Recent studies have shown that lysine, arginine and histidine residues can also be phosphorylated. Ligand binding to the receptor causes its dimerization (the process when two receptors get attached to each other via non-covalent bonds) which results in autophosphorylation of specific tyrosine residues on the receptor's cytoplasmic region. The autophosphorylation causes the activation of the receptor and it creates docking sites

for intracellular proteins containing Src homology 2 (SH2, phosphotyrosine binding) - and protein tyrosine binding (PTB)-domains. Not only a phosphotyrosine residue but also a specific recognition amino acid sequence is required for high affinity binding of phosphotyrosine site of the receptor to the SH2 domain- containing signaling protein [1, 2, 5]. These proteins then serve as docking sites for adaptor proteins which further regulate downstream signaling cascade or have an enzymatic activity of their own.

2.2 Protein tyrosine phosphatases (PTPs)

The control of the receptor activity is maintained by protein tyrosine phosphatases, enzymes that remove a phosphate group from its substrate resulting in formation of a free hydroxyl group, the process called dephosphorylation. The PTPs can be divided in two groups, non-transmembrane (cytoplasmic) and receptor-like PTPs (RPTP, transmembrane). RPTPs consist of 7 families which differ in extracellular domains. They all have a single transmembrane segment and usually contain two tyrosine phosphatase domains. The main role of RPTPs is to control signaling of proteins via the process of ligand-modulated dephosphorylation of tyrosine residues [3, 6-8]. Several studies have demonstrated that disruption of different PTPs can lead to hyperphosphorylation of proteins. Moreover, if the cells are treated with PTP inhibitors, the phosphorylation of RTKs stays activated [4]. In general, PTPs can modify signaling of RTKs in a positive and negative manner. An example of a positive regulator of RTK signaling is the RPTP CD45, which can activate Src family kinases. It is expressed on the surface of all white blood cells. CD45 has an essential role in the lymphocyte activation [9]. Another positive mediator is PTP SHP-2, which is required for the activation of the Ras–ERK pathway in response to growth factors [10, 11].

One of the well-known negative modulators is the PTP-1B. It plays role in negative regulation of insulin receptor (IR) and epidermal growth factor (EGF) signaling. Another PTP is SHP-1 that is found to be involved in signaling of colony-stimulating factor-1 (CSF-1), stem cell factor (SCF) and EGF receptors. Both PTP-1B and SHP-1 can be activated by tyrosine-specific phosphorylation [12-14].

One of the PTPs is a density-enhanced phosphatase-1 (DEP-1). It has been earlier described as a PTP which can dephosphorylate various RTKs, such as EGF receptor [15, 16], PDGFβ receptor [17], VEGF receptor [18, 19] and c-Met [20], (**Fig.2**). In this study, we have shown that RPTP DEP-1 is expressed in hematopoietic cells and negatively regulates the phosphorylation and signaling of FLT3 receptor tyrosine kinase [21].

In summary, it appears that PTPs are not very selective, i.e. many PTPs can interact with various RTKs and vice versa. Together, the main role of PTPs is to ensure that phosphorylation of tyrosines is short and that the level of phosphorylation in unstimulated, resting cells is low.

Because dysregulated RTK signaling is involved in cell transformation, PTPs, regulators of this process, become interesting targets for novel strategies for control of oncogenic RTK signaling.

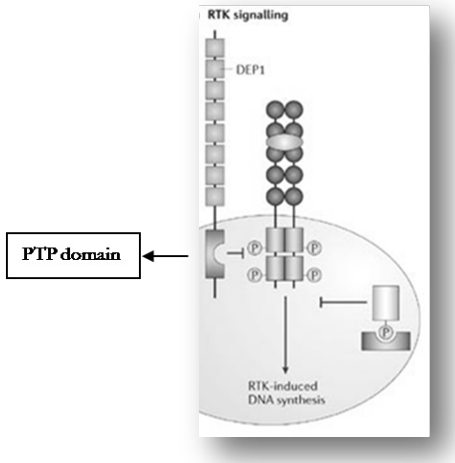

Figure 2. DEP-1-mediated dephosphorylation of RTK. Schematic view of the RTK which gets dephosphorylated by DEP-1 binding, adapted from [7]. Through dephosphorylation and inactivation of inhibitory components, some PTPs can also promote signaling of RTK.

3. Receptor Tyrosine Kinase signaling and adaptor proteins

Cell signaling is tightly regulated by the balance between phosphorylation and dephosphorylation. Signaling through tyrosine kinases plays important role in many biological activities such as cell growth, cell shape, cell cycle control, transcription, proliferation, survival, apoptosis, embryogenesis and development. Tyrosine kinase receptors signal through their cytosolic regions phosphorylating different target proteins upon ligand binding to their extracellular domains. RTKs are activating mostly the same set of signaling pathways. Among them the most common are phosphoinositide 3-kinase (PI3K)/AKT, MAPK (ERK), STAT and other pathways.

For proper signaling many signal transduction pathways need additional binding partners, so-called adaptor proteins that serve as bridges for specific protein interactions. Adaptor proteins are proteins that lack intrinsic enzymatic activity themselves and are unable to directly activate effector proteins. Instead they contain several different domain combinations (e.g. SH2 and SH3 domains) which function as docking sites for other

proteins. In case of multiple binding of signaling proteins together in a functional signaling complex the adaptor proteins are also called scaffold proteins. It is suggested that these signaling complexes enhance the speed, efficiency, and specificity of cell responses. Adaptor proteins (e.g., GRB2, Nck, Crk, SHC), mediate specific protein-protein interactions which are very important for the amplification and regulation of downstream signaling. Shortly, adaptor proteins link one signaling protein to another, without themselves conveying the signal [4].

The RTK family consists of several classes of proteins which have distinct structural features. Members of subclass III RTKs consist of the extracellular domain and the intracellular part containing the kinase domain which is divided in two parts by "interkinase" insert. The kinase insert is unique for each member of the subclass III and is known to play role in specific substrate bindings [2, 22]. Members of this group have major functions in proliferation, differentiation and survival of normal hematopoietic cells [23].

4. The Receptor Tyrosine Kinase FLT3

Feline McDonough Sarcoma (Fms)-like tyrosine kinase 3 (FLT3) belongs to the class III RTK family together with the stem cell (Steel) factor receptor (KIT), macrophage colony-stimulating factor (M-CSF), receptor-FMS and the platelet-derived growth factor receptors (PDGFRα and β). FLT3 shares around 30% of homology with other family members. The *FLT3* gene was isolated using placenta cells by O. Rosnet et al. in 1991 [23, 24] and cloning of human *FLT3* gene followed a few years later [25]. At the same time another group identified FLT3 from murine enriched stem cell population of fetal liver cells and gave the name Flk-2 (fetal liver kinase-2) [26]. The sequence of human FLT3 is homologous to the murine FLT3. The *FLT3* gene is located on chromosome 13 at band q12 [23]. FLT3 receptor is normally expressed on the cell surface of hematopoietic progenitor cells but expression is lost upon cell maturation. The expression of FLT3 has been also detected in brain, bone marrow, placenta and gonads where its function is unknown [24, 26, 27]. FLT3 expression can be found in different human and mouse cell lines of both myeloid and lymphoid lineages, but the distribution is quite different [28-30]. Many studies indicate that FLT3 has a crucial role in development, survival and proliferation of normal stem/progenitor cells [31].

FLT3 exists in two forms: a 160 kDa glycosylated membrane-bound protein and 130-140 kDa partially glycosylated protein [32]. FLT3 consists of five immunoglobulin-like (5 Ig-like) extracellular domain, a transmembrane region, a juxtamembrane domain (JM) and an intracellular kinase domain with the kinase insert (**Fig.3**) [33].

FLT3 receptor is activated by FLT3 ligand, FL. The ligand binding causes dimerization (formation of homodimer) of the receptor and its autophosphorylation on multiple

tyrosine residues. When receptor gets activated it undergoes conformational changes leading to activation of its intrinsic kinase activity, which in turn leads to phosphorylation of downstream signaling molecules eventually leading to different cell responses like cell survival, differentiation, proliferation, and apoptosis depending on the tissue type [31, 34, 35]. The phosphorylation of FLT3 receptor usually occurs within first 5-15min after ligand binding. After phosphorylation the complex of FLT3 and FL is rapidly internalized and degraded, similar to the other member of subfamily III, the KIT receptor [29].

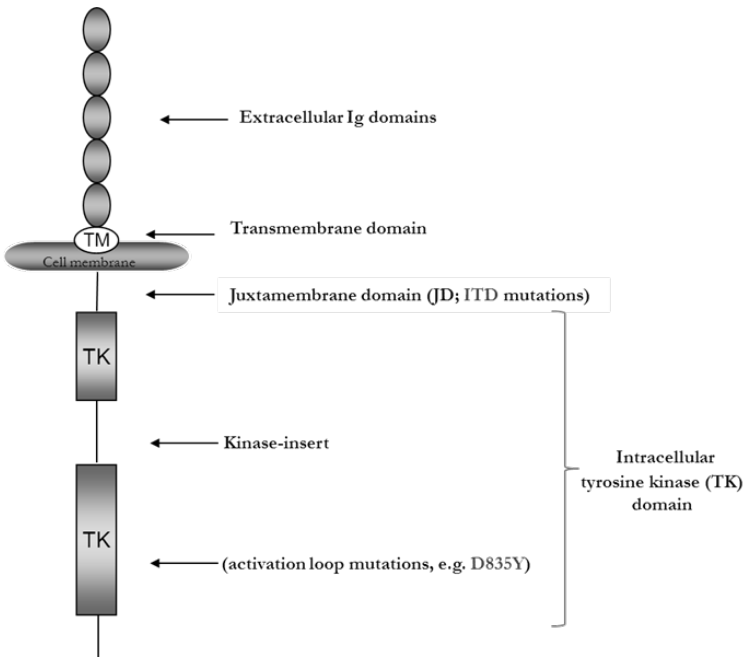

Figure 3. The structure of FLT3 receptor. The FLT3 receptor consists of 5 immunoglobulin-like domains, transmembrane domain, juxtamembrane domain (Internal Tandem Duplication mutations can occur in this region) and kinase domain which is divided into two parts by kinase-insert (D835 mutation can occur in activation loop region).

5. FLT3 ligand (FL)

The FLT3 ligand regulates early hematopoiesis through stimulating the FLT3 receptor and activation of downstream signaling pathways.

The murine FLT3 ligand (FL) was cloned in 1993 by Lyman, et al. FLT3 ligand is a type I transmembrane protein which is similar in both size and structure to the ligands of other members of the same subfamily, the colony-stimulating factor-1 (CSF-1) and the Steel factor (stem cell factor). FL consists of signaling peptide, extracellular domain, transmembrane domain and a small cytoplasmic tail [36]. The ligand can exist in both membrane-bound and soluble (as a result of a proteolytic cleavage) forms [37]. The human FLT3 ligand was cloned a year later using fragment of the murine FL as a probe. Interestingly, both forms of human or murine ligands are biologically active and are capable to stimulate the proliferation and colony formation of either the mouse or human hematopoietic progenitor cells at similar concentrations, demonstrating the lack of species specificity [38, 39]. The murine and human FLs show 72% amino acid identity. Both mouse and human FL occur in multiple isoforms, although the biological significance of those is still unknown. FL can also function in synergy with other growth factors, like IL-3, IL-6 and SCF [38-40]. Furthermore, additional synergestic partners have been identified, such as IL-11, IL-12, GM-CSF, G-CSF and CSF-1 with the different synergetic strength ability. FL alone is a weak proliferative stimulus but when acting in synergy with other growth factors, the stimulation of cell growth/proliferation is enhanced [41]. FL is expressed by hematopoietic bone marrow stroma cells and T-lymphocytes, as well as its mRNA is widely expressed in a variety of tissues including hematopoietic organs, prostate, ovary, lung, kidneys, testis, heart, placenta, etc. The fact that FL is expressed by most tissues and FLT3 receptor is expressed only by hematopoietic progenitor cells indicates that expression of FLT3 is more crucial for its activation and function in the cells. Study on FL-deficient mice shows reduction in lymphoid progenitor cells, supporting the previously suggested role for FL in promoting early lymphoid development from hematopoietic stem cell [42].

Another important role of FL is in immune response. FL has been found to stimulate the increased production of natural killer (NK) cells and all types of dendritic cells (DC) *in vivo* [43, 44].

6. Downstream signaling of FLT3

Upon ligand binding FLT3 undergoes dimerization and conformational changes opening binding sites for specific proteins as well as activates phosphorylation process to be able to send the signal further into the cell. Using chimeric receptor kinases, phospholipase C-γ (PLC-γ), SH2-containing transforming protein C1 (SHC1), growth factor receptor-bound protein 2 (GRB2) and SRC family kinases were found to directly associate with the receptor. The activated FLT3 receptor results in tyrosine phosphorylation of CBL, SHC, SHP-2, VAV, FYN, GAB1, GAB2, GRB2, PI3K, SHIP, STAT5A and MEK5/ERK5

effector proteins. FLT3 is involved in several main signaling pathways like RAS/MAP-kinase and AKT/PI-3 kinase and STAT signaling pathways [31, 45].

6.1 The MAP-kinase Signaling Pathway

The mitogen-activated protein kinases (MAPKs) or Extracellular signal-regulated protein kinases (ERKs) pathway is one of the best studied signaling cascades. It transduces signals by phosphorylation and plays an essential role in cell proliferation, differentiation, migration, survival, gene expression, cell cycle arrest and apoptosis. Several MAP kinase subfamilies have been identified, i.e. ERK1/2 (extracellular signal-regulated kinases), ERK5, p38 and JNK1/2/3 (c-Jun amino-terminal kinases) [46]. MAPK kinases are activated upon tyrosine/threonine phosphorylation in Thr-Glu-Tyr (TEY) activation motif of kinase domain. The classical activation occurs in three layer cascade: MAPKKK (MEKK/RAF) activates MAPKK (MEK1/2) which in turn activates MAPK (ERK1/2) [47, 48]. Activation of ERK pathway results in distinct biological outcomes depending on many factors, like cell line specificity, cell-surface receptor density, cell environment, etc. [49].

Ras (acts as a small G protein- protein that bind and hydrolyze GTP) is the first member of MAPK pathway to be activated by receptor tyrosine kinase via complex of several other adaptor proteins, like Sos/GRB2 and SHC. Ras members (H-Ras, N-Ras and K-Ras) were discovered as oncogenes of murine sarcoma viruses and activating mutations of Ras family have been found in almost one third of all human cancers [50].

Activated Ras associates with Sos/GRB2 complex, and binds further to Raf-1 kinase with the high affinity. The main function of Ras is to promote the membrane association and activation of Raf-1 [51]. More recent studies have shown that activated Ras can be also detected in Golgi's, endoplasmic reticulum's (ER) and endosomal membranes where kinetics of Ras activation will differ depending on compartmentalization [52, 53]. SHP-2 tyrosine phosphatase has been found to positively regulate Ras activation. It can bind to either RTK or an adaptor protein and it contributes to Ras signaling from both Golgi and plasma membrane [53].

Raf, is a key effector of Ras. There are 3 members of mammalian Raf protein family: Raf-1 (C-Raf), A-Raf and B-Raf (several alternatively spliced forms) [54, 55]. It has been shown that Ras mediates the activation of Raf through very complex and multiple-step activation process, reviewed in [56, 57]. *In vivo* studies have demonstrated that interaction of Ras and Raf-1 is not enough to activate the last one, which indicates that other factors than just Ras are required to activate Raf-1 [58, 59]. Several years later, two Ras binding sites in Raf-1 have been discovered. One is required for Raf-1 translocation and another one is required for full activation of Raf-1 [60]. Recent studies have revealed that protein phosphatases (PPs) are also involved in Raf activation. PP1 and PP2 have been shown to

regulate Raf activity in positive manner through dephosphorylation of Raf inhibitory binding site and interact with Ras resulting in its recruitment to the membrane [61, 62]. In addition to three Raf kinases, Ras can activate a number of other proteins which are discussed in review by Campbell et al. [54]. Once activated, Rafs can activate MEK1/2 and thereafter ERK1/2 by phosphorylation of their kinase domains. Activated ERK1/2 in turn phosphorylates many substrates in cytoplasm and nucleus (e.g. transcription factors like Elk1 and c-Myc, protein kinases such RSK) which result in diverse cellular responses [63, 64].

Extracellular signal-regulated protein kinase 5 (ERK5) is a relatively newly identified MAP kinase, was cloned in 1995 by Dixon and co-workers [65]. At the same time another group identified an identical protein and named it a big MAPK1 (BMK1) because of its size which is more than twice the size of the other MAPKs, around 98 kDa [66]. The N-terminal kinase domain shares 50% homology with ERK1/2 [67, 68]. The kinase domain is important for cytoplasmic targeting, interaction with the upstream MAPK kinase MEK5 and oligomerization. MEK5 can be activated by MEKK2/3 (but not MEKK1), members of MAPKKK family [69-71]. It has been shown that ERK5 interacts directly with MEK5 but not with MEK1/2 [65]. The C-terminal domain is unique for ERK5. It encodes two proline-rich regions (PR1 and PR2), nuclear localization and nuclear export signals (NLS and NES), which are responsible for nuclear shuttling of ERK5 [72]. The C-terminal domain of ERK5 undergoes autophosphorylation at several sites upon its activation which lead to a conformational change that exposes the docking site and the NLS [72]. The phosphorylation of C-terminus plays role in the regulation of gene expression [73-75]. ERK5 can be activated by oxidative stress, hyperosmolarity, serum [76], and several growth factors like nerve growth factor (NGF), epidermal growth factor (EGF) [77-80] and platelet-derived growth factor (PDGF) via dual phosphorylation of TEY motif by MEK5 [81-84]. ERK5 is associated with cell survival, proliferation and migration [68, 85, 86]. Important role of ERK5 has been shown in phosphorylation of BAD and AKT (PKB), suppression of caspase-3 cleavage and inhibition of nuclear apoptotic alterations, which shows anti-apoptotic function of ERK5 [83, 86, 87]. ERK5 plays also a key role in neural differentiation and cardiovascular development. Furthermore, it has been shown previously that PDGF receptor can activate ERK5 and thereby generate positive signals for cell proliferation and decrease motility [82]. As FLT3 belongs to the same family as PDGF receptor we have investigated if FLT3 also can activate ERK5 and if it plays any role in FLT3 signaling. Our study shows for the first time that ERK5 can be activated by FLT3 receptor stimulation. Moreover, inhibition of ERK5 causes a decrease in AKT activation in murine pro-B cells (Ba/F3) expressing wild-type FLT3 or oncogenic FLT3-ITD receptor. With existing knowledge and understanding of the MEK5/ERK5 signaling pathway, there are still many questions remain unanswered [88]. FLT3 activates the MAPK (ERK1/2) pathway through the interaction of GRB2/Sos with tyrosines 768, 955 and 969 on FLT3 receptor. Association

of GRB2 to GAB2 recruits SHP-2 which leads to activation of ERK phosphorylation [89].

The discovery of MAPK family has provided a significant insight into major signaling pathways, which play role in many aspects of normal physiological development.

6.2 PI-3 kinase signaling pathway

PI3K is a lipid kinase that phosphorylates phosphoinositides on the inositol ring. PI3K consists of a catalytic p110 and p85 regulatory subunits. Five isoforms have been identified for each subunit [90, 91]. The p85 subunit consists of two SH2 domains (involved in protein-protein interactions), an SH-3 domain, and two proline-rich domains [92, 93]. The p85 also contains a GTPase-responsive domain (GRD) and inhibitory domain which play role in lipid kinase activity and activation of PI3K signaling pathway [94, 95]. The p110 contains p85-interacting domain, Ras-binding domain and PI3K catalytic domain [92]. The members of PI3K are divided into three classes. Members of class I are activated by receptor tyrosine kinases and convert plasma membrane lipid PIP_2 (phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$]) to PIP_3 (phosphatidylinositol-3,4,5-trisphosphate[$PI(3,4,5)P_3$]). Proteins containing pleckstrin homology (PH) domains such as AKT (PKB) and phosphoinositide-dependent kinase 1 (PDK1) get phosphorylated by binding directly to PIP_3. The activated PDK1 phosphorylates AKT, which in turn activates other proteins involved in growth, cycle, survival, proliferation, motility, transcription of cells, along with protein synthesis and metabolism [96-100]. Class II and III are found to be involved in membrane trafficking and metabolism.

Protein kinase B (AKT) is a serine/threonine kinase, the most studied downstream target of PI3 kinase pathway. In mammals, three isoforms of AKT protein have been identified. AKT consists of N-terminal lipid-binding PH regulatory domain, a hinge region and a kinase domain. The substrates can bind to different parts of AKT [101, 102]. Activated PI3K causes translocation of AKT to the plasma membrane through its PH domain and its phosphorylation on T308 site by PDK1. The phosphorylation of another site, S473 is regulated by the mammalian target of rapamycin complex 2 (mTORC2) [103]. For activation of AKT both residues are needed to be phosphorylated. The dephosphorylation of PI3K is controlled by a lipid phosphatase, a tumor suppressor gene *PTEN* [104, 105], phosphoinositide phosphatases: SHIP1 [106] and SHIP2 [107, 108].

In most cases phosphorylation of AKT causes inhibition of target proteins. For example, apoptosis-inducing protein BAD is prevented from binding to Bcl-2 upon AKT activation resulting in cell survival [109]. Another example is a Forkhead family of transcription factors called FOXO proteins. When FOXO proteins are not activated, they contribute to apoptosis. Phosphorylation of FOXO proteins by AKT inhibits their ability to activate transcriptional target genes leading to stimulation of cell growth. The

study on involvement of FOXO proteins in signaling of oncogenic FLT3-ITD receptor showed that FLT3-ITD signaling suppresses phosphorylation of FOXO3A proteins thereby preventing cells from default programmed cell death (apoptosis). FLT3-ITD signaling induced constitutive activation of AKT and, in turn, inactivated its substrate FOXO3A which led to increased survival, proliferation and leukemic transformation of myeloid cells [110, 111].

Only murine form of FLT3 has a binding motif for the p85 subunit of PI3K and therefore can directly associate with PI3K, whereas human FLT3 can only activate PI3K indirectly, via GAB2. GRB2/GAB2 binds to tyrosines 768, 955 and 969 of FLT3 receptor and thereby mediates signaling via PI3K (AKT) [89, 112]. In a closely related receptor c-KIT, p85 can bind directly to pY721 or indirectly to pY703 and pY936 via GRB2/GAB2 which in turn activates AKT and PI3K pathway leading to increased cell survival [113-115].

The PI3K pathway is implicated in major human diseases like type 2 diabetes (caused by defects in the pathway) and cancer (caused by hyperactivation of the pathway). Because of high frequency of alterations in PI3K pathway in cancer patients it is of big interest to develop PI3K inhibitors/drugs for treatment of patients. Currently, there are two inhibitors, LY294002 and Wortmannin (inhibit p110 and AKT phosphorylation) which are widely used in the laboratory studies [116].

6.3 Src Family Kinases (SFK)

Src was the first tyrosine-specific protein kinase to be identified back in the late 70s [117-119]. It belongs to a family of non-receptor (intracellular) tyrosine kinases which consists of 8 members: Src, Yes, Fyn, Lyn, Lck, Hck, Fgr and Blk which are expressed by different human cell types [120-122]. Expression of Src, Fyn and Yes is found in most tissues whereas expression of other members of the family is more restricted to hematopoietic cells [122, 123]. Src members are 52-62 kDa proteins composed of distinct functional regions: the unique region, the SH3 domain, the SH2 domain, the catalytic domain, and a short negative regulatory tail [124]. The functions of structural domains of Src kinases are described in details in review by Thomas, S.M. and Brugge, J.S. [122]. The unique domain plays role in mediating specific for each family member interactions with receptors/proteins. The SH2 and SH3 domains play crucial role in regulating catalytic activity of Src [125, 126]. Src PTKs can function in many distinct cells and in distinct subcellular locations (e.g. caveolae, endosomes, and focal adhesions) [122]. The *c-src* is a proto-oncogene which can transform if mutated and/or overexpressed [118]. Src is able to interact with a various RTKs (e.g. EGF receptor family), cell-cell adhesion molecules, G-protein-coupled receptors, STATs, integrins, adaptor proteins (e.g. Cbl, GAB2 and SHC). SFKs are overexpressed and activated in numerous human malignances and are

shown to lead to the development of cancer and progression of metastases [127]. For example, SFKs have been found to negatively regulate human c-KIT through its effect on ligand-induced internalization, ubiquitination and degradation [128, 129]. Additionally, Src activation of PDGF receptor and EGF receptor cause their down regulation [130, 131]. Interestingly, the Src activation acts as a downstream target of FLT3 receptor in FLT3 signaling. Mitina, O. et al. have found that Hck, Lyn and less Fyn can associate with FLT3 through tyrosine residues located in the JM domain of the receptor [132]. Activated FLT3 recruits Src to Y589 and Y591 binding sites in the JM region and cause its phosphorylation [133]. Several Src inhibitors, including Dasatinib, are currently under investigation for usage in treatment of different tumors [134].

6.4 JAK/STATs signaling pathway

Janus kinase (JAK) has got its name from the Roman God who has two faces as JAK contains a pseudokinase and a kinase domain in tandem. JAKs are cytoplasmic tyrosine kinases playing important role in cytokine signaling. In mammals there are four members of JAK family: JAK1, JAK2, JAK3 and TYK2 [135, 136]. Upon activation JAKs phosphorylate the signal transducers and activators of transcription (STATs), which are transcription factors that regulate expression of specific genes. JAK can also activate SFKs, adaptor proteins and phosphatases. JAK members consist of a set of unique JAK homology domains (JH1-JH7), but only JH1 catalytic domain contains kinase activation loop and considered as a functional whereas JH2 is only a pseudokinase domain [137].

There are 7 members of STATs that have been identified in mammalian cells: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 [136].

The tyrosine phosphorylation of STATs is mediated by cytokine activation of the upstream receptors via JAK. Then STATs get dimerized and translocated into the nucleus where the expression of target genes is modulated. STATs consist of SH2 domain which differs for every STAT member thereby providing specific binding to various receptors, N-domain (involved in oligomerization of STAT dimers) [138, 139] and the C-terminal domain containing serine residue (transcription activation) [140].

FLT3 receptor contains two docking sites for STAT5 activation, Y589 and Y591. STAT5 has been found to be strongly activated by mutated FLT3 receptor (FLT3-ITD) but not by wild-type FLT3. These results have been verified in primary AML blasts containing ITD mutation on FLT3 receptor [89, 141, 142]. STAT5 gets activated from the ER by FLT3-ITD but not by wild-type membrane-bound FLT3, leading to the cell survival and growth [143, 144]. Mice transplanted with bone marrow containing FLT3-ITD and the Y589/Y591 mutations did not develop myeloproliferative disease [145].

6.5 Phospholipase C-γ

Phospholipase C-γ (PLC-γ) catalyzes the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) by hydrolyses of phosphatidylinositol 4,5-bisphosphate (PIP2). IP3 binds to specific receptors on the endoplasmic reticulum which leads to release of $Ca^{2}+$ while DAG remains bound to the plasma membrane and binds and activates various isoforms of protein kinase C [146-148]. There are two isoforms of PLC-γ: PLC-γ1 and PLC-γ2. While PLC-γ1 is ubiquitously expressed, PLC-γ2 is restricted to hematopoietic cells. They both consist of two SH2 domains, an SH3 domain, a PH domain and a catalytic domain. During signaling cascade PLC-γ is recruited to the activated receptor and get activated by tyrosine phosphorylation and through plasma membrane translocation via its PH domain [149].

PLC-γ has been found to interact with autophosphorylation sites in the C-terminal tail of the EGF receptor [150], the fibroblast growth factor (FGF) receptor [151] and the PDGFβ receptor [152]. The ability of c-KIT to activate PLC-γ is still not clear. There are few studies showing that PLC-γ can be activated by ligand-induced phosphorylation of chimeric PLC-γ and c-KIT overexpressing them in HEK293 cells [153, 154]. Another study suggests that only membrane-bound SCF can promote PLC-γ activation upon c-KIT receptor stimulation [155].

The first data on PLC-γ association with FLT3 dates back to 1993 but since all experiments in that study were performed on chimeric receptor: extracellular domain of FMS and intracellular domain of FLT3 [34]. It has not been clear whether the normal, wild-type FLT3 does also activate PLC-γ. This year we could demonstrate the activation of PLC-γ upon FLT3 stimulation in DEP-1 depleted 32D cells [21].

6.6 Cross-talk

Cross-talk between different receptors and their signaling pathways increase the complexity of FLT3 signaling studies and contribute to a diversity of signaling networks. For example, cross-talk occurs between the different members of MAPK family leading to a signal complexity and higher variety of signal response. There is also a cross-talk between distinct pathways, for example Ras can activate MAPK but also affect the phosphoinositol 3-kinase (PI3K) pathway. It can occur through direct phosphorylation of Raf-1 and B-Raf by AKT [156-158]. Receptors lacking direct p85 binding site (EGF, FGF and FLT3) can activate PI3K through Gab1, Gab2 or Ras [159].

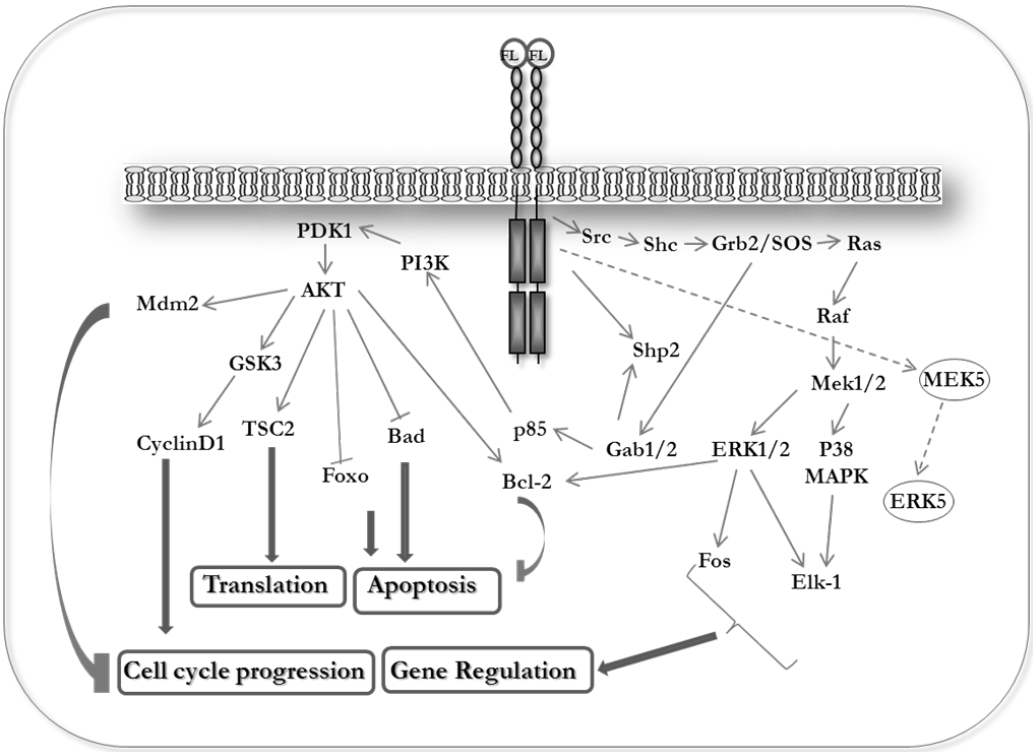

Figure 4. The main signal transduction pathways of the wild-type FLT3 receptor. Binding of FLT3 ligand (FL) to FLT3 leads to receptor dimerization and activation via phosphorylation of multiple tyrosine kinases. The activated receptor recruits a number of proteins, e.g. Src, SHC and GRB2 to form a complex of protein-protein interactions, which lead to activation of downstream pathways including, PI3K, AKT, ERK1/2 and ERK5, resulting in various cellular responses such as cell growth, proliferation, differentiation, and apoptosis.

7. Hematopoiesis

Hematopoiesis is a process of blood cell formation. It is a very well organized system with a strict hierarchy and steady-state production of around 10^{12} cells per day/ per person [160]. All hematopoietic lineages (all blood cells) originate from the common pluripotent hematopoietic stem cell (HSC), identified by Till and McCulloch in 1961 [161]. Matured HSCs are located in the bone marrow. HSCs are defined as clonogenic cells with the unique feature possessing the self-renewal ability and differentiation

through the whole life of a living organism. The HSCs give rise to multiple hematopoietic lineages with the distinct functions: the erythroid lineage (red blood cells, mediate oxygen transport), the lymphoid lineage (T-, B- and natural killer (NK) cells- adaptive immune system) and the myeloid lineage (granulocytes, megakaryocytes and monocytes/macrophages- innate and adaptive immunity), as well as dendritic cells [162, 163].

To date, HSCs are considered to be the most studied adult stem cells that have been isolated. Mature blood cells have a relatively short life span ranging between several hours (granulocytes) and several weeks (erythrocytes) except some lymphoid cell types which have a longer lifespan. The balance between different cell types is provided by well-organized HSCs functions. In case of infections, bleeding or low oxygen levels, the system is able to rapidly response to maintain the homeostasis in the blood system [164]. During the classical hematopoiesis HSCs give rise to either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs). CLPs differentiate further into pro-B, pro-T, pro-NK and dendritic cells. CMPs give rise to either granulocyte-macrophage progenitors (GMP) or megakaryocyte-erythrocyte progenitors (MEP). GMPs further differentiate into dendritic cells, granulocytes, or macrophages whereas MEPs into platelets and red blood cells (**Fig. 5)** [165].

7.1 The hematopoietic hierarchy

There have been found different cell surface markers for isolating various hematopoietic stem cells at different stages of their development. For example, using markers for FLT3 and Vascular cell adhesion molecule-1 (VCAM-1) a population of hematopoietic cells (FLT3+, VCAM-) defined as lymphoid primed multipotent progenitor cells (LMPPs) can be isolated. These cells give rise to granulocytes and macrophages [166, 167].

7.2 The bone marrow HSC niche

The HSC niche is located in the bone marrow (BM) microenvironment, which is comprised of stromal cells, fibroblasts, smooth muscle cells, endothelial cells and others. The HSC represent only around 0.01% of all BM cells. All these cells have several crucial functions like involvement in hematopoietic cell development, production of transmembrane ligands, extracellular matrix components and soluble proteins. Here, in the bone marrow, the fate of the cell is decided [168, 169]. A loss of control over those decisions can lead to cellular transformation and cancer.

7.3 Regulation of HSC and hematopoiesis

HSC and hematopoiesis are regulated by either intrinsic factors like transcription factors or extrinsic factors like growth factors, called cytokines [170-172]. Cytokines are found to be involved in HSC development and expansion. Cytokines exist in both soluble and membrane- bound forms, which bind to cell-membrane receptors thereby promoting cell survival and proliferation [160, 173]. A number of studies have shown that cytokines, like IL-3, IL-6, IL-11, FLT3 ligand and stem cell factor (SCF) promote proliferation, self-renewal to some extent and differentiation either working alone or in synergy with each other [160, 173-175].

A role of FLT3 in HSCs regulation has been suggested based on the findings where reconstitution ability of FLT3 receptor deficient BM cells is reduced [176]. On the contrary, studies on $FL^{-/-}$ mice have shown normal HSC levels, which suggest a redundant role of FL in HSCs regulation [42]. Thus, it is still not clear whether FLT3 signaling is involved in HSC regulation.

In some cases deregulation of HSC functions can cause severe disease - a blood cancer (leukemia).

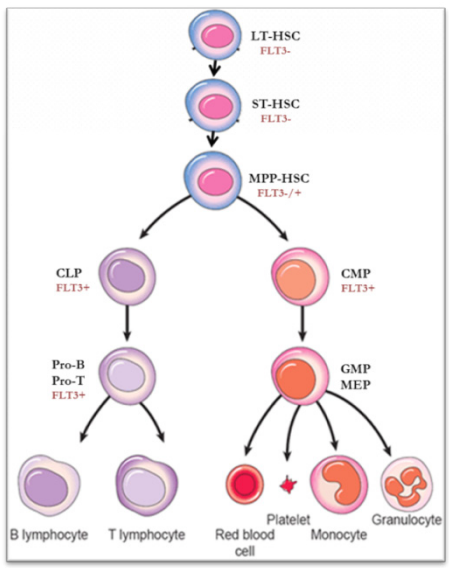

Figure 5. Schematic view of the human hematopoietic system and FLT3 expression during blood cells maturation. Hematopoietic stem cells (HSCs) can be long-term (LT) and short-term (ST) self-renewing, which generate multipotent progenitor stem cells (MPPs). These further generate common lymphoid (CLP) or myeloid (CMP) progenitors. CLPs give rise to B- and T-cells (as well as natural killer cells and dendritic

cells (DCs), not shown on the picture). All blood cells except DCs lose the expression of FLT3 upon maturation. CMPs differentiate into either GMPs (granulocyte-macrophage precursors) or MEPs (megakaryocyte-erythrocyte precursors) which further give rise to various mature blood cells.

8. Cancer

Cell survival, proliferation and death are essential processes of the living organisms which are tightly regulated. When the control that regulates these processes breaks down, an uncontrolled cell growth and differentiation occur which result in formation of mass of cells, called a tumor. Some tumors are not serious health hazards, but those consisting of cells that spread throughout the body usually cause the disease, called cancer. There are a few key features for cancer determination. Cancer is either caused by inherited mutations or by newly arising somatic mutations. Second, the cancer usually is a result of several mutations (3-20 mutations), depending on the type of cancer and other factors. The process of transformation of a normal cell into a cancer cell is called tumorogenesis. There are three types of genes responsible for this process: oncogenes, tumor-suppressor genes and stability genes. Oncogenes are the genes that are constitutively active or active when wild-type genes are usually not. These can be induced by chromosomal translocations, gene amplifications or mutations affecting crucial residues of the product of the gene. Tumor-suppressor genes can contain mutations which lead to reduction of the protein activity. These mutations can be caused by missense mutations of residues regulating the activity of the gene product and mutations resulting in truncated protein (from deletions/insertions of different sizes). The stability genes, also called caretakers, include the genes responsible for repairing mistakes during DNA replication or genes controlling mitotic recombination and chromosomal segregation, and others. These genes prevent genetic alterations and their inactivation cause a higher incidence of mutations in other genes. On the other hand, oncogenes and tumor-suppressor gene mutations operate through stimulation of cell birth or the inhibition of cell death or cell-cycle arrest leading to an increased number of tumor cells. Cancer can be subdivided into several groups depending on the tissue specificity: leukemias (blood or bone marrow), lymphomas (lymphatic cells of the immune system), sarcomas (tissues that develop from embryonic mesoderm) and carcinomas (epithelial cells). Based on structural differences cancers can be divided into solid (immobilized tumor cells) and non-solid tumors (mobile cancer cells) [177-179].

There are two main hypotheses that explain tumor progression: the stochastic and, more common, hierarchical. The first one suggests that any cell within a tumor can form and maintain tumor mass whereas another hypothesis assumes that there are only a few cells within a tumor mass which possess stem phenotype, called cancer stem cells (CSCs) and

only these cells can initiate and maintain survival and growth of the tumors. These cells belong to a particular niche, usually have a higher resistance to cancer treatments and often responsible for the disease relapse or spreading it to other tissues/organs forming metastases [180].

9. Leukemia

Leukemia (from the Greek *leukos* - white, and *haima* – blood) is a cancer of the blood system, caused by deregulation of HSC functions and rapid proliferation of abnormal blood cells. The first case to be described in medical literature dates to 1827 when a French doctor Velpeau described a patient who developed the symptoms such as fever, weakness, urinary stones, enlargement of the liver and spleen. He noticed that the blood of this patient had a consistency like "gruel". Rudilf Virchow was the first to describe the abnormal amount of white blood cells in patients and called the disease "leukemia" in 1856. In 1889, Wilhelm Ebstein presented the name "acute leukemia" to the leukemias that are rapidly progressive and fatal. Finally, in 1900 Naegeli divided leukemias into myeloid and lymphocytic based on cell specificity. Nowadays leukemias are divided into several major groups: acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL).

10. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is the most common type of leukemia. AML accounts for approximately 30% of all adult leukemias, affecting mostly elder people and its incidence increases with the age. AML is a relatively rare disease occurring in approximately 10,500 cases per year in the United States and around 300 cases per year in Sweden [181, 182].

To date, there are approximately 200 different aberrations that have been described in AML [183]. Therapy-related AML or AML arising after a myelodysplastic or myeloproliferative syndrome are usually more resistant to treatment compared to *de novo* AML [184]. A number of risk factors that lead to development of AML have been identified, including "pre-leukemic" blood disorders (e.g. myeloproliferative syndrome), chemotherapy, radiation exposure (e.g. survivors of the atomic bombings of Hiroshima and Nagasaki), other genetic alterations (e.g. Down syndrome) [185-188].

In recent years, the discovery of mutations in several genes and proteins like FLT3, NPM1, Wilms' tumor gene (WT1) and CEPTA has shown to play an important role in AML disorder. Mutations involving PTP non-receptor type II (PTPN11) have been also

found in AML patients and could be a risk factor for their survival but it is still not well verified [189].

The symptoms of AML can be similar to those of influenza including fever, fatigue, weight loss, shortness of breath, anemia, easy bleeding, bone and joint pain and frequent infections. Enlargement of the spleen can occur but it is usually asymptomatic [190].

Treatment of AML consists primarily of chemotherapy (two phases: induction and consolidation therapy). Despite aggressive therapy, there are still patients who relapse after treatment. For patients with relapsed AML, the stem cells transplantation is used as a therapy. In 1975 treatment of AML patients with stem cell transplantation was started in Sweden. It led to a better survival of patients under the age of 60 years [182]. Patients with relapsed AML after stem cell transplantation or who are not suitable for this kind of therapy are treated with cytotoxic drugs or various small molecular inhibitors (SMIs). Nowadays the combined therapies (e.g. non-cytotoxic agents and standard chemotherapy) are often used to treat AML patients [191].

10.1 "Two-hit" model of leukemogenesis

At least two mutations are required to initiate leukemogenesis: the mutations belonging to class I and class II. Class I mutations promote survival and proliferation of the cells, e.g. FLT3 and c-KIT activating mutations. The second class of mutations impairs transcription factors that are essential for the regulation of normal myeloid development, e.g. translocations of the genes targeting myeloid differentiation [192, 193]. Occurrence of class II mutations results in aberrant gene expression and abnormal development of hematopoietic cells. The translocations like t(8;21), inv(16), t(15;17) and t(9;11) impair myeloid differentiation and promote survival [192, 194]. The "two-hit" model is also supported by analyses of genetic alterations in AML patients with presence of both fusion genes and activating mutation of tyrosine kinases [192, 195]. Still, there are a lot of studies which show that FLT3 mutations alone can disrupt differentiation, proliferation and apoptosis [31, 196, 197].

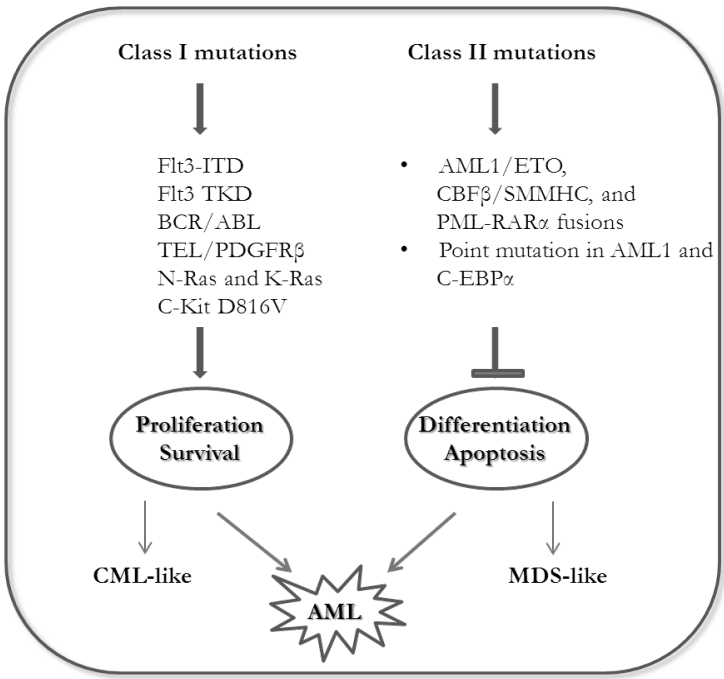

Figure 6.The two-hit-model of leukemogenesis. The two-hit-model suggests that development of Acute myeloid leukemia depends on at least two distinct mutations where class I mutations promote the proliferation and survival and another class of mutations, usually involving transcription factors, provide a block to differentiation and apoptosis**.**

11. FLT3 and Acute Myeloid Leukemia

The first report of AML [198] and the first description of mutations in FLT3 in hematopoietic malignancies was done by Nakao et al. in 1996 [199]. Since that the research on FLT3 has become extensive. High expression of FLT3 has been shown in around 70-100% of AML patients as well as in many ALL patients. FLT3 expression has been also detected in other hematopoietic malignancies such as myelodysplastic syndrome (MDS), CML and CLL [192, 200].

There are two distinct alterations in FLT3 receptor that can cause changes in its signaling. Overexpression of wild-type FLT3 is one of the abnormalities that can promote constitutive activation of the receptor in malignant cells and usually associates with a worse prognosis [201, 202]. Another reason for abnormal signaling of FLT3 is the

presence of mutations on this receptor which also lead to constitutive activation of receptor and worse prognosis of the patients carrying these mutations.

FLT3 ligand has been shown to have a survival-promoting effect on AML cells. It has also been reported that when FL synergizes with other growth factors it is capable to stimulate primary AML and ALL cell proliferation [192, 203, 204]. Studies have shown that FLT3 is constitutively active in primary AML samples expressing both FLT3 and its ligand [205]. Multiple studies on knock-out mice of FLT3 did not show any severe damage and mice were viable and fertile but contained a reduced number of B-cell precursors in the bone marrow [176]. FLT3 -/- mice also had abnormalities in the generation of dendritic cells [206].

11.1 FLT3-ITD mutations

The *FLT3* gene is one of the most commonly mutated genes in AML and the most common alteration in FLT3 is the internal tandem duplication (ITD) mutation which occurs in about 15% of pediatric and 30% of adult AML patients [207, 208]. ITD mutations were reported for the first time in 1996 by Nakao et al. [199]. The size of the insert can vary from 3 to more than 400 base pairs. ITDs are in-frame mutations caused by duplication of various in length fragments encoding the JM domain of FLT3 receptor (encoded by exons 14 and 15) (**Fig. 7)**. Interestingly, the length of ITD insert can influence the clinical outcome in AML patients. Several studies have shown that the treatment outcome, relapse-free survival and overall survival are worse in patients with the bigger size/length of ITD insert. That can probably be explained by the difference in inhibitory ability of JM domain by different ITDs. The small ITDs cannot completely disrupt the autoinhibitory activity of the JM domain compared to the large ones. Alternatively, different size of ITDs leads to variations in the length duplications of specific amino acid (AA) residues (within JM domain)which are involved in receptor activation thereby determining what downstream effectors can bind to the receptor [209, 210]. For example, arginine 595 (R595) has been found as the most frequently duplicated single AA in the majority of AML patients. R595 plays a critical role in the transforming potential of FLT3-ITD mutants and ligand-dependent wild-type FLT3 activation [211]. Another possible explanation could be that tyrosine phosphorylation sites that serve as docking sites for signal transduction molecules, such as Y589 and Y591 that are involved in activation of Src family kinases, could be duplicated in the long ITD forms, and thus mediate a stronger Src signal. This in turn could lead to stronger activation of the Ras/ERK pathway and the PI3-kinase/AKT pathway. It has been shown that FLT-ITD exists mainly in the immature, partially glycosylated, high mannose form (130 kDa) and is retained in an intracellular compartment in contrast to wild-type receptor which is expressed as a mature, glycosylated form (150 kDa) located in the cell membrane [212, 213]. Several studies suggest that intracellular localization contributes to the altered

signaling of mutant FLT3. The first study of biological significance of ITD mutations was done in Cos-7 cells transfected with FLT3-ITD showing the constitutive activation of the receptor and its downstream signals [201, 214-216]. These results were confirmed in growth factor dependent cell lines like Ba/F3 and 32D where ITD mutation induces constitutive activation of FLT3 receptor and its downstream signaling molecules as well as resulting in cytokine-independent cellular proliferation [216-218]. It has been shown that FLT3-ITD receptor can homodimerize with mutant receptors or heterodimerize with wild-type receptors independent of the ligand stimulation. This can lead to distinct signaling responses to ligand in cells with ITD mutation depending on ratio of the wild-type to the mutant receptors [192, 217, 219]. As it is in frame mutation a protein kinase domain remains always functional just containing an elongated JM region [199, 209, 210]. The cause of ITD mutation is not known but it is proposed that the duplication can result from a failure in mismatch repair mechanism during the replication of DNA [216]. ITD mutations usually involve at least one of the Y589, Y591, Y597 or Y599 tyrosine residues located in the juxtamembrane region [215]. Using a crystal structure of wild-type FLT3 receptor the mechanism regulating catalytic activity of the receptor can be studied. Normal phosphorylation of specific tyrosine residues causes the JM domain conformation into an active state revealing the biding sites for peptide substrates and ATP. This remains in a closed conformation when these tyrosines are not phosphorylated leading to induction of autoinhibition and restriction of ATP and protein substrates binding. In case of ITD mutation, it disrupts the autoinhibitory activity of the JM domain, resulting in a constitutive activation of the receptor [214, 220]. Vempati et al. has identified two main residues, Y589 and Y59 in the juxtamembrane domain, as a crucial for FL-dependent activation of wild-type FLT3 receptor and the transforming potential of oncogenic FLT3 mutants, FLT3-ITDs and FLT3-TKDs [221].

The first *in vivo* study on FLT3-ITD was done on syngeneic mice injected with 32D cells carrying FLT3-ITD and led to development of leukemia. However, the transplantation of BM cells carrying FLT3-ITD does not cause leukemia but only myeloproliferative disorder (MPD) which again indicates that at least 2 distinct mutations are required to induce an AML disorder [222-224].

FLT3-ITD is a common mutation in AML and correlates with a poor prognosis and higher risk of relapse of AML patients. ITDs have been also found in CML (5-10%), MDS (5-10%) and more rarely in ALL (< 1%) [225, 226]. In some cases FLT3-ITD mutations occur only at the relapse stage of AML patients but not present at the time of diagnosis [201].

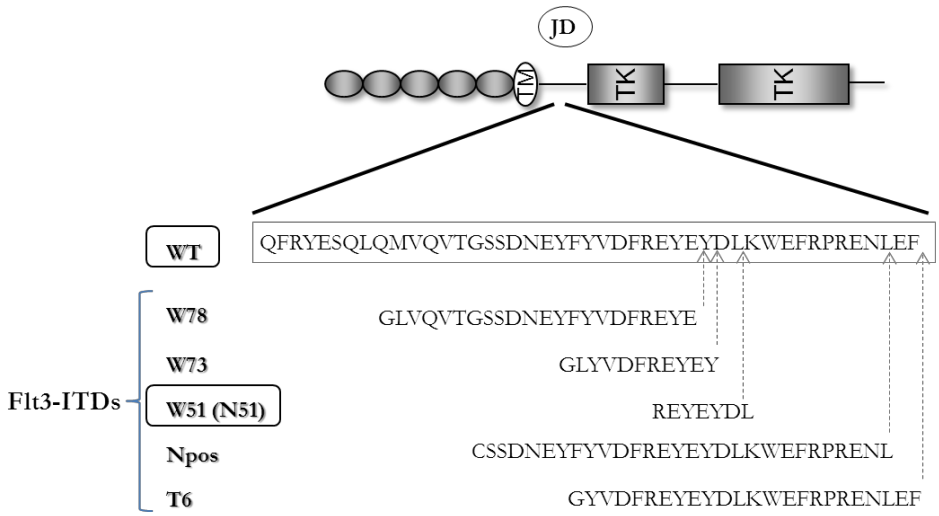

Figure 7. Some of the possible FLT3-ITD mutations. Wild-type FLT3 and W51 (N51) internal tandem duplication (ITD) mutation were used in our studies. The size of ITD insert can vary from 3-400 base pairs.

11.2 FLT3-TKD mutations

Since another member of the type III subfamily of RTKs, c-KIT has been found to have point mutations in the activation loop of the kinase domain in mast cell and associated with leukemia; FLT3 has been also screened for similar mutations [227, 228]. A point mutation similar to c-KIT has been identified in FLT3 receptor, D835 (Asp835). Among FLT3-TKD mutations, the aspartic acid at position 835 is the most frequent target. The D835 can be exchanged to Y (Tyr) or H (His) and less frequently to V (Val) or E (Glu) [229, 230]. D835 mutations are found in around 7% of AML patients [230-232]. Additionally, other FLT3 mutants containing a deletion or substitution of I836 and an insertion of glycine and serine (between S840 and N841) have been found in AML patients [233]. These mutations cause constitutive and ligand-independent phosphorylation of FLT3 receptor even without receptor dimerization. These results were confirmed in IL-3 dependent 32D cells [229]. TKD mutations have a similar function as ITD causing the disruption of the auto-inhibitory mechanism of FLT3 [214]. Interestingly, it has never been identified any AML case carrying both FLT3-ITD and D835 mutations suggesting that they result from a different underlying mechanisms and their downstream signaling is different [230]. The studies on point mutations suggest that they play a crucial role in activation loop of the kinase domain of subfamily III RTKs.

D835 mutations are also found in ALL (2.8%) and MDS (3.4%) [229]. The prognosis and overall survival is significantly higher in patients with TKD mutations compared to ITD mutations [234]. A novel AML-associated gain of function mutation, K663Q has been described by Schittenhelm et al. It is located in the activation loop of the N-terminal of TKD of FLT3 receptor [235]. It has been shown that mice harboring FLT3-TKD develop an oligoclonal lymphoid disorder in a mouse transplantation model but not MPD [224]. The prognostic relevance of FLT3-TKD mutations in AML patients still remains controversial [183].

11.3 FLT3-JM-PM

A third type of FLT3 mutations that has been found in AML patients, is a group of single point mutations in the juxtamembrane domain (FLT3-JM-PM). Several FLT3-JM-PMs have been expressed in IL-3-dependent Ba/F3 cell line. It led to factor-independent cell growth, hyperactivation of the receptors in response to the ligand, and resistance to apoptotic cell death. When active, FLT3-JM-PM receptors show a higher constitutive dimerization rate compared to the wild-type FLT3 receptor. FLT3-JM-PMs have been found to activate STAT5 and up-regulate Bcl-x in Ba/F3 cells [236].

The first report on JM-PMs of a subclass III RTK was published by Irusta et al. in 1998. They have shown that an introduced point mutation in cell line, valine to alanine at position 536 (V536A), in the murine PDGFRβ result in constitutive activation of the receptor. Other members of the same subclass have been analyzed at similar positions to V536. These mutations could activate human PDGFRβ(V568A), human PDGFRα(V561A), human CSF1-R (I552A), human c-KIT (V559A) in a similar manner [237-239].

Taken together, all these studies indicate that JM domain is important for autoinhibition of the kinase activity and mutations in this region lead to ligand-independent activation of the kinase. This type of mutations results in a decreased binding affinity to the N-terminal-lobe of the kinase domain and increased autophosphorylation and kinase activity of the receptor. The oncogenic role of FLT3-JM-PMs in AML patients has not yet been identified but based on multiple studies, it is suggested that these mutation might have important clinical implications [240].

11.4 FLT3 non-JM ITD mutations

Recently, FLT3-ITD mutation was also found in non-JM domain. This novel FLT3-ITD mutation, FLT3-ITD627E, was found in around 29% of unselected FLT3-ITD positive AML cases. FLT3-ITD627E mediates constitutive phosphorylation and activation of

FLT3 receptor and its downstream target STAT5. It also induces transformation of 32D cells and leads to a lethal myeloproliferative disease (MPD) in syngeneic mice. Further studies are needed to define the biological and clinical characteristics of the FLT3-ITDs in non-JM domain [241].

12. Oncogenic signaling of FLT3

Many studies have shown that mutations in FLT3 receptor cause alterations in downstream signaling of the receptor and lead to different cellular responses which can be advantageous for progression of cancer cells and development of hematopoietic malignancies. *FLT3* is recognized as one of the most frequently mutated genes in AML. FLT3 mutations trigger downstream signaling pathways including RAS-MAP/AKT kinases and signal transducer and activator of transcription-3 and 5 (STAT3; STAT5) [192].

Depending on ITD localization, different signaling pathways can be activated. It has been shown that FLT3-ITD can activate STAT3 and STAT5 as well as upregulate Pim1/2 (STAT5 targets) but not PI3K or MAPK signaling when localized at the endoplasmic reticulum (ER) [143, 144]. On the contrary, FLT3-ITD localized in the membrane has been found to strongly activate PI3K and MAPK pathways but fails to activate STAT5 [144].

The main deviation from wild-type FLT3 signaling lies in the ability to potently activate STAT5 by mutated FLT3 receptors [196, 218, 242]. STAT5 further activates downstream targets like Pim-1, Cdc25A and BAD (Bcl-2 antagonist of cell death). Bcl-2 is another target of STAT5. Overexpression of Bcl-2 is correlated with FLT3-ITD mutation with Y591 duplication [243]. The worse outcome of AML patients carrying FLT3-ITD expressing cells might be due to STAT5 activation which causes the enhanced anti-apoptotic signaling in these cells. Sallmyr et al. has demonstrated that FLT3-ITD cells show increased production of reactive oxygen species (ROS) via STAT5 activity, which in turn leads to increased DNA double-strand breaks (DSBs) and repair errors explaining the aggressiveness and poor prognosis of AML patients with FLT3-ITD mutations [244]. FLT3-ITDs have been found to cause a constitutive activation of AKT which in turn inactivates FOXO transcription factors leading to increased survival, proliferation, and leukemic transformation [111]. Constitutively activated FLT3 receptor has been found to upregulate certain genes including the proto-oncogenes serine/threonine-protein kinase *Pim1* and *Pim2*, suppressors of cytokine signaling *SOCS2* and *SOCS3*, myeloid cell leukemia sequence 1 (*MCL-1), MYC*, and cyclin D3 (*CCND3)*. These genes are involved in cell proliferation, survival, differentiation, apoptosis and cell cycle regulation [245-248]. Expression and function of additional transcription factors which are important for myeloid differentiation and leukemic transformation, such as PU.1 and C/EBPα

(CCAAT/enhancer-binding protein α) as well as regulator of G-protein signaling 2 (RGS2) are found to be suppressed by FLT3-ITD mutations [249, 250]. In addition, an increased activation of Wnt signaling induced by FLT3-ITDs in myeloid progenitor cells and in significant proportion of AML cases, suggesting the involvement of this pathway in pathogenesis of AML [251-253]. Okamoto, M. et al. has shown that Lyn is constitutively phosphorylated by FLT3-ITD signaling and thereby has been proposed as a specific target for AML patients carrying FLT3-ITD [254].

Mutations in TKD of FLT3 receptor cause distinct changes in FLT3 signaling, for example FLT3-D835V has been found to upregulate TSC-22 (transforming growth factor-β stimulated clone-22), a potential leukemia suppressor [255].

13. AML therapy and FLT3 Inhibitors

The common treatment of AML patients below 60 years of age is an induction therapy consisting of anthracyclines (daunorubicine or idarubicin) in combination with cytosine arabinoside (AraC). Consolidation treatment is initiated after a complete hematological remission (<5% of leukemic blasts in the BM) and is repeated 4 times [256]. An alternative therapy for patients with an increased risk for relapse is allogenic hematopoietic stem cell transplantation (HSCT), preferably for patients under 60 years of age. An alternative chemotherapy, without high dose of AraC is used for elderly patients due to a high risk of treatment-related toxicity [257]. It has been shown that patients with FLT3-ITD mutations had a lower risk of relapse and increased overall survival when received an allogeneic HCT instead of standard chemotherapy [201]. In general, the current treatments of patients with AML are considered to be unsuccessful because of a high incidence of disease relapse and therefore there is an increasing need for novel targeted therapy for AML patients.

The therapeutic strategy described above is applicable for all AML patients except those with acute promyelocytic leukemia (APL). APL patients contain fusion protein PML/RARα, a result of t(15;17) translocation. Such patients can be treated with all-trans retinoic acid (ATRA) in combination with chemotherapy or arsenic trioxide [258, 259].

High toxicity and relapse rate of current AML treatment underline the importance of finding novel therapeutic targets for treatment of AML patients. There have been already proposed and tested in clinical trials several new options for AML treatment. Depending on characteristic features of AML, different therapeutic targets are used. For example, the increased proliferation caused by constitutively activated MAPK pathway can be targeted by direct inhibition of oncogenic gain-of-function mutation in Ras protein (found in 15-25% of AML cases). Ras protein function can be inhibited by farnesyltransferase inhibitors (FTIs) which block the binding of Ras to the cell membrane [260]. Inhibitors of MEK have been also developed when an aberrant activation of MEK has been

detected in several human cancers. Inhibition of MEK has been found to effect the growth of AML cells as well as sensitize leukemic cells to chemotherapy [261]. Some AML patients have an overexpression of an important negative regulator of the p53 tumor suppressor, murine double minute 2 (Mdm2) which is correlated with a poor prognosis. A novel strategy to inhibit Mdm2 by potent and selective small-molecule antagonists of Mdm2, Nutlins, has been tested in AMLs with wild-type p53, resulting in induction of p-53-dependent apoptosis, cell cycle arrest and growth inhibition in these patients [262].

Another very important targeted therapy in AML is small molecule inhibitors (SMIs) for FLT3 receptor because of its overexpression in leukemic blasts and poor clinical prognosis in AML patients with FLT3 mutations. The SMIs can vary in selectivity, being either very specific or have a broad selectivity and either ATP competitive or non-competitive. FLT3 SMIs are molecules that competitively inhibit binding of ATP to the protein thereby inhibiting its activation and signaling. There are several FLT3 inhibitors that have been developed and many of them are currently at different stages of clinical trials. Among FLT3 inhibitors are PKC412 [263], CT53518 [264], SU11248 [265], SU5416 [266], SU5614 [267], sorafenib [268, 269], staurosporine and lestaurtinib (CEP-701) [270, 271], etc. Lestaurtinib (CEP-701) and tandutinib (MLN518) are considered to be more selective inhibitors compared to the others. Current clinical trials are mainly focused on the combined treatment of AML patients by FLT3 inhibitors with the conventional chemotherapy. The main problems with the inhibitors that researchers come across are high toxicity and low specificity. Most of FLT3 inhibitors are unspecific and can recognize other members of subclass III receptors leading to additional complications like high toxicity due to the multiple targets.

Recently, our group has shown that MEK5 inhibitor, BIX02188 blocks the MEK5/ERK5 signaling pathway leading to significant increase in apoptosis of AML cells, especially of those containing FLT3-ITD mutation [272].

In AML therapy, high risk of relapse is the main cause of treatment failure, usually due to therapy resistance. The ratio of the FLT3-ITD and wild-type receptors is different in every patient, therefore leading to distinct clinical implications. Patients carrying both wild-type FLT3 and FLT3-ITD mutations alleles have an increased risk of relapse and it is even worse survival than patients carrying only FLT3-ITD allele, because of the wild-type compensational ability for some of the oncogenic effects of FLT3-ITD mutations [232, 273]. Another type of kinase inhibitors, LS104, was tested in 2008 by Kasper, S. et al. This type of inhibitors competes out the substrate binding to the receptor instead of blocking the ATP binding site. It has been shown that LS104 target FLT3-ITD and its downstream signaling in primary AML blasts [274]. Use of anti-FLT3 antibodies as a potential therapy tool is under a current investigation. It has been already reported that anti-FLT3 antibodies give positive responses in leukemic mice models [275]. Using antibodies is considered to be a more specific and less toxic method with the best result

on cell surface expressed receptors. As this is not the case with FLT3-ITD mutations, it is impossible to use them in AML patients with these mutations. For AML patients containing at least one allele carrying FLT3 wild-type, anti-FLT3 antibodies might serve as a promising therapeutic tool for the treatment of patients [276].

The present Investigation

Aims of the Thesis

- Identification of novel phosphorylation sites and analysis the kinetics of the activation of wild-type FLT3 receptor versus mutants.

- Role of the protein tyrosine phosphatase, DEP-1 in FLT3 signaling.

- Investigation of novel tyrosine kinase inhibitors, 3,4-Diarylmaleimides and their mechanism of action in ITD-positive AML cells.

- Identification of novel signaling pathway activated by FLT3 receptor, MEK5/ERK5. Influence of MEK5 inhibitor, BIX02188 on ITD-positive AML cell survival.

Summary and discussions of Papers

Paper I. Oncogenic FLT3 receptors display different specificity and kinetics of autophosphorylation.

The first paper is focused on phosphorylation analysis of FLT3 wild type and its mutants, ITD and D835, which are found to be correlated with a poor prognosis in AML patients. FLT3 mutations cause constitutive activation of the receptor, leading to alteration in signal transduction. The aims of this study are to analyze the kinetics and specificity of FLT3 autophosphorylation in wild-type FLT3 and FLT3 mutants, to identify new phosphorylation sites in FLT3 receptor and to determine related signaling pathways. Using a panel of "homemade" phosphospecific antibodies directed against potential tyrosine phosphorylation sites in FLT3, we could identify novel phosphorylation sites and study the kinetics and specificity of phosphorylation in living cells. Our results show that activation of the wild-type FLT3 receptor is ligand- dependent and responding in a time dependent manner in contrast to FLT3-ITD and D835Y that are constitutive active and ligand-independent. Eight phosphorylated tyrosine residues (pY589, pY591, pY599, pY726, pY768, pY793, pY842 and pY955) were shown to have different phosphorylation characteristics in the wild-type receptor compared to the mutated receptors. Furthermore, we showed that Y726, Y793 and Y842 are novel phosphorylation sites of FLT3 receptor. From this study we could observe not only quantitative changes but also qualitative differences in the phosphorylation patterns of wild-type and mutated FLT3 receptors.

Compared to other members of subclass III RTK, there are very few phosphorylation sites have been identified on FLT3 receptor. Phosphospecific antibodies were used as a tool to identify phosphorylation sites - a method which enables rapid, sensitive and reproducible analysis of phosphorylation status. Seven of nine tested antibodies gave detectable phosphorylation in at least one of the three variants of FLT3 receptor. There is a difference in activation of corresponding phosphorylation sites in closely related receptor. Cell specificity is also present as a factor for phosphorylation ability. For example, we failed to detect phosphorylation of Y572 in Ba/F3 cells expressing FLT3 whereas Y572 has been identified as a phosphorylation site in another cell line, 32D expressing wild-type FLT3 [133]. After screening of all these phosphorylation sites we could clearly see that most sites were phosphorylated independently of FL stimulation in the mutants, FLT3-ITD and FLT3-D835Y. Interestingly, several studies have shown that

leukemic cell lines and AML blasts express FLT3 and FL simultaneously, suggesting autocrine stimulatory loops. It has also been noticed that there is an enhanced response after FL stimulation even with the mutant FLT3 receptor which we have also observed in our study [205, 277]. One of the newly identified phosphorylation sites is tyrosine 726 which is activated to a similar extent in wild-type as in the mutants. Still, potential interaction partners haven't been identified. The other two novel sites, Y793 and Y842 are located in kinase domain and have a significantly more prolonged phosphorylation in FLT3-D835Y compared to wild-type FLT3 and FLT3-ITD. So far, no interacting partners have been identified. Y842 is located in an activation loop and its function remains unknown. According to the crystal structure of FLT3, Y842 has a crucial role in keeping the activation loop in an inactive position [214]. The difference in activation of phosphorylation sites in wild-type, FLT3-ITD and D835Y receptors can be due to distinct mechanisms of acting in different mutants as well as FLT3-ITD mutation causes autoinhibition of FLT3 by the juxtamembrane region whereas D835Y mutation causes structural changes in the kinase domain and mimic its activated state which can alter the signaling specificity [214, 229]. Corresponding mutations to FLT3-D835Y in c-KIT (D816V) and MET (D1246N) have been demonstrated to lead to a shift in the substrate specificity of the kinase [278, 279]. Detailed study of every phosphorylation site of FLT3 receptor provide an important information about which sites are specific for the oncogenic signaling and which specific downstream proteins are activated. All this gives a huge input for future cancer therapy development to make it more specific and efficient.

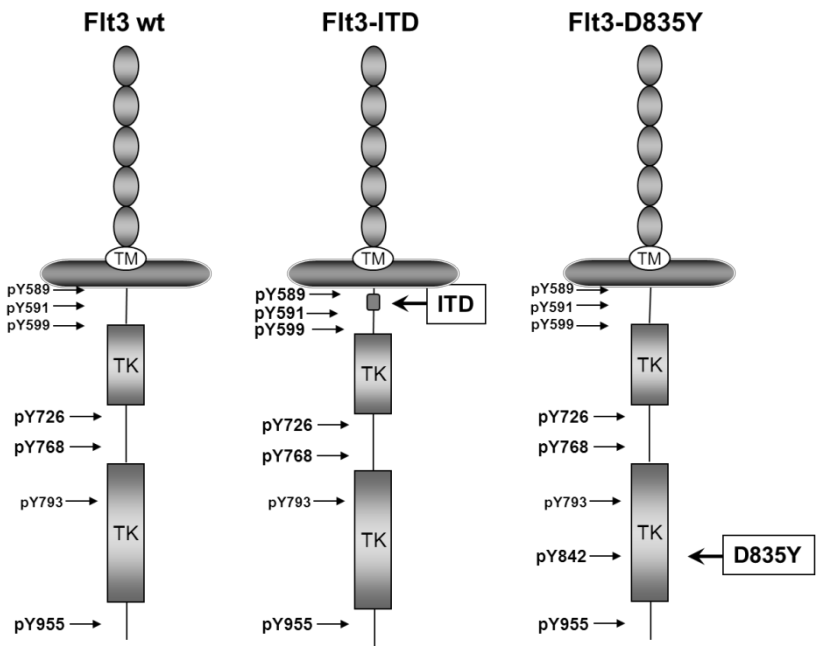

Figure 8.Schematic picture of the wild-type FLT3, FLT3-ITD and FLT3-D835Y and their phosphorylation sites. The different phosphorylation sites are summarized in the figure (the size indicates the strength of phosphorylation). TM, transmembrane domain; TK, tyrosine kinase domain.

Paper II. Protein tyrosine phosphatase DEP-1 controls receptor tyrosine kinase FLT3 signaling.

There is already rather much known about FLT3 activation and phosphorylation process but still very little known about the role of protein tyrosine phosphatases (PTPs) in FLT3 signaling. The aim of this study is to identify PTPs which can affect the signaling activity of FLT3 in 32D myeloid cell line expressing wild-type FLT3. These cells were infected with various lentiviral pseudotypes carrying shRNA targeting different PTPs. In total twenty PTPs were tested and DEP-1 (PTPRJ) was identified as a PTP which can negatively regulate activation and signaling of FLT3 receptor. The site-selective hyperphosphorylation of FLT3 was detected in stable 32D cells with reduced DEP-1 levels with the most effect on pY589, pY591 and pY842 ligand-stimulated activation of FLT3. Tyrosine residues Y589 and Y591 are known to be involved in wild-type FLT3 signaling as well as in oncogenic FLT3, especially FLT3-ITDand its downstream target, STAT5. Hyperphosphorylation of these sites cause activation of STAT5 in DEP-1 depleted cells. Similarly, the DEP-1 depleted human AML cell line, THP-1, cause enhanced FLT3 phosphorylation accompanied by FLT3-dependent activation of ERK and cell proliferation. Depletion of DEP-1 has been also found to cause an enhanced phosphorylation of PLC-γ. It is known that several PTPs may interact with unstimulated receptors. In case of FLT3 we could also see a weak elevation of basal phosphorylation of some sites (Y591 and Y842) on FLT3 receptor in cells with knockdown DEP-1. This result indicates that PTPs probably interact with inactivated receptors to prevent basal phosphorylation. It has been also demonstrated that the interaction between DEP-1 and FLT3 is direct. Moreover, activated FLT3 is dephosphorylated by recombinant DEP-1 *in vitro*. In case of stable DEP-1 overexpression in 32D cells and transient FLT3 overexpression in HEK293 cells the FL-mediated FLT3 signaling activity has been reduced. Furthermore, the FL-stimulated colony formation of 32D cells in response to DEP-1 knockdown was detected by methylcellulose colony formation assay. Remarkably, even DEP-1 depleted 32D cells expressing wild-type FLT3 could form colonies in a FL-dependent manner. This can probably be explained by the slight increase in activation of STAT5 upon FL-stimulation. Despite driving colony formation *in vitro,* DEP-1 knockdown could not induce formation of myeloproliferative disease in the 32D-C3H/HeJ mouse model. Thus, DEP-1 depletion contributes, but not sufficient to leukemic cell transformation via FLT3 activation.

In this study we can clearly see DEP-1 involvement in FLT3 activation but the effect on signaling and biological output by DEP-1 depletion is relatively weak. As there are several PTPs can dephosphorylate FLT3 [213], we suggest that other PTP can cooperate with DEP-1 in the process of FLT3 dephosphorylation and/or inactivation of downstream signaling pathways. Moreover, other PTPs may cooperate with DEP-1 and have the capacity to compensate DEP-1 loss-of-function in case of regulation of FLT3 signaling.

DEP-1 is the first PTP which negative role in FLT3 signaling has been demonstrated using a loss-of-function approach. Using DEP-1 depleted cell lines we could indicate a direct regulation of ERK and STAT5 phosphorylation by DEP-1 [280]. More specified, DEP-1 can regulate activation of various phosphorylation sites on FLT3 receptor in different manner. Differential effects of DEP-1 on those sites can be due to differential accessibility ability by steric reasons or its site-specificity, as we can see in case with PDGFβ receptor [281].

Identification of DEP-1 as a negative regulator for FLT3 contributes to further studies for analyzing possible role of DEP-1 in myeloid cells transformation.

Paper III. 3,4-Diarylmaleimides- a novel class of kinase inhibitors- effectively induce apoptosis in FLT3-ITD-dependent cells.

There are about 30% of AML patients harboring internal-tandem-duplication mutations in FLT3 receptor. ITD mutation is one of the aberrations that is correlated with a worse prognosis and overall survival of AML patients. Small molecule tyrosine kinase inhibitors are a relatively new class of therapeutic drugs. Several tyrosine kinase inhibitors (TKI) of FLT3 have already been designed and are currently being tested in clinical trials. These drugs have shown a remarkable efficiency. However, there are several problems like short remission time and high incidence of resistance when using these drugs as monotherapy. This imposes a strong need to develop novel "second-generation" inhibitors using different mechanisms of action.

In this study we have investigated two members of a novel class of tyrosine kinase inhibitors- 3,4-diarylmaleimides (DHF125 and DHF150) and their effect on oncogenic FLT3 signaling. The aim of the study has been to determine their mechanism of action and efficacy of the inhibitors on FLT3-ITD positive cell lines as well as in primary ITD-positive AML blasts.

3,4-Diarylmaleimides (DM) have been previously described as angiogenesis inhibitors [282]. It has been suggested that these compounds can bind at the ATP-binding site of kinase. 3,4-Diarylmaleimides have been screened for inhibitory activity in a panel of 12 selected protein kinases and revealed a high affinity for VEGF receptors but not to other kinases (e.g., IGF-R, EGF-R, ABL, PDGFR, CDK2, GSK2-b, and Aurora A and B),

indicating a narrow spectrum of targets [282, 283]. The majority of TKIs are known to inhibit mutated FLT3 kinase by competing for binding of ATP to the ATP-binding pocket. In this study we have found that DHF125 and DHF150 (DHFs) can inhibit FLT3-ITD kinase in 32D cells in an ATP-competitive manner and reduce phosphorylation of its downstream signaling molecules such as STAT5, ERK and AKT. Treatment with midostaurin (PKC412, an ATP-competitive inhibitor of FLT3 kinase) was used as a positive control. These findings were confirmed in the human leukemic cell line, MV4-11.

In order to determine the effects of DHF125 and DHF150 inhibitors on survival and proliferation, murine IL-3 dependent hematopoietic 32D cells transfected with either wild-type FLT3 or FLT3-ITD were used. Cells were incubated with inhibitors either in the presence or absence of the cytokine IL-3. The effective induction of apoptosis of 32D-FLT3-ITD positive cells was been detected at low concentrations but not in 32D cells expressing wild-type FLT3 when cultured in IL-3 containing media thereby indicating that there is no toxic off-target effect at the used concentrations (up to 5 μM). The effect of DM compounds on another kinase, JAK2 was also analyzed and no significant induction of apoptosis could be detected in Ba/F3 cells expressing the activating V617F mutant of JAK2. This suggests that the ability to induce apoptosis byDHF125 and DHF150 inhibitors is specific for FLT3-ITD-dependent murine cell lines. Primary AML cells were also checked for sensitivity to DHF compounds. These inhibitors could induce apoptosis in primary AML blasts harboring FLT3-ITD mutations *in vitro* while leaving colony formation of normal stem and progenitor cells unaffected. To summarize, these inhibitors induce apoptosis in FLT3-ITD dependent cells and patient blasts at low concentrations and with low toxicity which gives us a possibility to use them in the future as a therapeutic target in AML patients with FLT3-ITD mutations.

Several other important characteristics that these drugs possess are that they can be rapidly uptaken and retained in the cytoplasm of exposed cells (murine 32D-FLT3-ITD cells as well as primary patient blasts) which leads to a prolonged intracellular exposure of mutant FLT3 kinase, they synergize with chemotherapeutic drugs (anthracyclinedaunorubicin and cytarabine) and they have a distinct inhibition pattern of specific FLT3 phosphorylation sites. Interestingly, investigating inhibition by DHF-inhibitors of FL-mediated phosphorylation of tyrosine residues on FLT3 receptor, different patterns of inhibition were detected. Comparing the ability to inhibit different tyrosine residues on FLT3 receptor by DHF compounds and PKC412 we can suggest that some residues (Y842, Y726, Y7668, and Y955) do not play a crucial role in PKC412-mediated kinase inhibition as they were not affected by this compound, whereas DHF-inhibitors seem to carry out the inhibition of these tyrosines activation except Y589 and Y591. This novel group of compounds shows different inhibition patterns of distinct tyrosine residues of FLT3 receptor which can display a promising profile for further clinical studies.

There are several molecular markers, e.g. overexpression of the brain and acute leukemia gene (BAALC) [284], mutations of FLT3 receptor tyrosine kinase, nucleophosmin-1(NPM1) [285] and Wilms' tumor 1 gene [286] have shown the prognostic influence on AML patients. Therefore, the studies on their inhibition may play a crucial role in improvement of response rates to chemotherapy and overall survival of AML patients. Further studies investigating the efficiency of DHFs in cells resistant to other available FLT3-TKIs are also of big importance.

Paper IV. Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD.

The fourth study is focused on analysis of downstream signaling targets of oncogenic FLT3-ITD receptor. There are several downstream signaling pathways that are important for cell transformation, e.g. ERK kinase pathway, PI3K/AKT pathway and STAT5A. In this study we have shown that ligand stimulation of FLT3 induces phosphorylation/activation of its downstream target, ERK5. ERK5 belongs to a family of extracellular signal-regulated protein kinases (ERKs) and is a downstream of the MEK5. It has been found that ERK5 can get activated by other RTK, such as NGF, EGF and PDGF. ERK5 has been associated with cell survival, proliferation and migration. Important role of ERK5 has been shown in phosphorylation of BAD and AKT, thus explaining its anti-apoptotic function.

In the present study, we have focused on MEK5/ERK5 signaling and its role in FLT3-ITD mediated transformation. We have found that both wild-type FLT3 and FLT3-ITD activate ERK5 upon ligand stimulation. In order to assess the impact of ERK5 activation on signaling downstream of FLT3 as well as its role in proliferation and survival, we used the MEK5 selective inhibitor BIX02188. Since MEK5 is the kinase upstream ERK5, activation of both MEK5 and ERK5 can be inhibited by BOX02188. By inhibiting the activation of ERK5, we demonstrate a decreased phosphorylation of AKT in Ba/F3 cells expressing wild-type FLT3 as well as in cells expressing oncogenic FLT3-ITD, thus showing that the FLT3-mediated activation of AKT is partially dependent on ERK5. Interestingly, cells expressing the oncogenic FLT3-ITD were more sensitive to the inhibitor. Surprisingly, the phosphorylation of ERK1/2 was also partially reduced. Given the fact that neither MEK1/2 nor ERK1/2 can be inhibited by BIX02188 at the concentrations used (6-12 μM), this suggests that MEK5 could also act upstream of ERK1/2 through unknown mechanism. Despite the fact that FLT3-ITD is constitutively active, we could still see ligand-induced AKT and ERK phosphorylation which was partially inhibited by BIX02188. We have shown that inhibition of ERK5 activation can be achieved at the concentration of 12μM of BIX02188 inhibitor in Ba/F3 cells expressing wild-type FLT3 or FLT3-ITD. When treated with BIX02188 inhibitor the phosphorylation of AKT and ERK1/2 decreases in a dose-dependent manner, starting

from the concentration of 6μM with the better efficiency in cells expressing FLT3-ITD. In the closely related receptors, like PDGFβ, ligand-induced AKT activation is decreased upon ERK5 inhibition. Also in case with VEGF receptor, ERK5 regulates AKT phosphorylation which leads to an anti-apoptotic effect [83, 287, 288].

Ba/F3 cells expressing FLT3-ITD decrease in proliferation and survival upon MEK5/ERK5 inhibition. Furthermore, inhibition of MEK5/ERK5 induces apoptosis of both cells expressing wild-type FLT3 and FLT3-ITD with the most striking effect on FLT-ITD expressing Ba/F3 cells especially when stimulated with FL. In order to analyze the influence of BIX02188 in leukemic cell signaling, MV4-11 and MOLM-13 cell lines were used. Both cell lines derive from patients with acute myeloid leukemia and express FLT3-ITD endogenously. In these cells, activation of ERK5 was inhibited by BIX02188 and apoptosis could be induced at the concentration of 2μM of inhibitor with almost two times higher induction in MOLM-13 cells (16%) compared to MV4-11 cells (8.3%).

The role of ERK5 was found in several cancers, for example, ERK5 has been shown to be overexpressed in oral squamous cell carcinoma, where it is associated with the metastatic stages [289]. Furthermore, knockdown of ERK5 in leukemic T cells decreased nuclear accumulation of the NF-kB p65 subunit and suppressed the induction of tumours in mice [290]. It was recently demonstrated that ERK5 is involved in regulating the activity of the tumour suppressor PML by direct phosphorylation [291]. In this study we demonstrate that ERK5 is activated downstream of both the wild-type FLT3 and the oncogenic mutant FLT3-ITD, commonly found in patients with acute myeloid leukemia. By use of selective MEK5 inhibitor we have shown a role of ERK5 in FLT3-ITD mediated leukemogenesis which in turn can be used as an alternative therapeutic target in the treatment of FLT3-ITD positive AML patients.

Future Perspectives

During the past two decades the cell signaling process has become a dominant part of cell and molecular biology research, in part because of its main role in cell communication and because the alterations in this process can lead to a severe diseases like cancer. Today, when the proteomics and bioinformatics have been well developed we get solved several main research tasks like mapping of the whole human genome. Nowadays, we have a good proteomic tools which help us to provide much insight into proteins, their structures and signaling processes. The more we discover the more complex the signaling system appears to be, with multiple variants arising from splicing forms, post-translational modifications of proteins and cross-talks between different receptors and their signaling pathways. In the coming years, it will become clearer how the complex proteins interact to form signaling networks, mostly through success in important application development like mass spectrometry, flow cytometry, hybridization and protein expression techniques.

The importance of FLT3 signaling in normal hematopoiesis and especially in transforming cells and AML is evident and many researchers have been already focused on finding the details of these processes. Nowadays, most of the FLT3 studies are focused on finding specific markers in transforming cells and thereafter synthesizing small molecular inhibitors against them. There are already several FLT3 inhibitors have been identified and they are currently in different phases of clinical trials. The development of novel specific drugs is still required due to high relapse rate among AML patients and high toxicity of existing medicines which are in use nowadays for treatment of these patients.

This thesis includes the articles which are also focused on detailed analysis of phosphorylation of each tyrosine residue on FLT3 receptor by a powerful technique - usage of phosphospecific antibodies. These antibodies could be of help in analysis of patient samples giving the information on FLT3 expression and

thereby giving a more specific diagnosis of AML patients. Our group has identified three novel phosphorylation sites on FLT3 by using phosphospecific FLT3 antibodies. Two of these sites get activated in FLT3-D835Y mutant that is highly expressed in AML patients. Therefore it is important to further identify potential binding partners of these sites as well as to determine the biological importance of their activation. Protein tyrosine phosphatase DEP-1 has been found to negatively regulate FLT3 signaling and its depletion contributes to leukemic cell transformation via FLT3 activation. It is important to further analyze in which pathway DEP-1 is involved and if loss of DEP-1 functionally occurs in AMLs. The last two studies are focused on novel inhibitors which can possibly alter oncogenic FLT3 signaling and induce apoptosis in FLT3-ITD-positive AML cells.

Populärvetenskaplig Sammanfattning

Vår kropp består av olika celler som kommunicerar med varandra genom signalering. Cellerna tar emot signaler från omgivningen med hjälp av proteiner som sätter sig fast på deras yta genom mottagare, så kallade receptorer. När receptorerna får signaler skickas de vidare in i cellkärnan där det bestäms hur cellen ska agera.

Tyrosinkinasreceptorer är proteiner som sitter på cellens yta och är involverade i många processer i celler, till exempel tillväxt, celldelning, mognad, metabolism, överlevnad och rörelseförmåga. Tyrosinkinasreceptorer brukar aktiveras av en typ av ligander som kallas tillväxtfaktorer. När liganden binder till en receptor leder detta till att receptorerna slås ihop två och två, vilket i syn tur leder till fosforylering- ett sätt att skicka signaler inom celler.

FLT3 tillhör familjen tyrosinkinasreceptorer och uttrycks på omogna blodceller och har en viktig roll i nybildningen av blodceller, cellernas överlevnad och celldelning. Receptorn aktiveras av en tillväxtfaktor, FLT3 ligand (FL). Det finns flera sjukdomsalstrande former av FLT3 (mutation ITD och D835Y) som leder till ständig aktivering av FLT3 dvs. utan tillväxtfaktor-stimulering, vilket leder till förändringar i signalvägarna och kan bidra till akut myeloisk leukemi (blodcancer). Dessa förändringar förekommer hos cirka 30% av patienterna med akut myeloisk leukemi (AML) och korrelerar även till en sämre prognos. AML drabbar ungefär 360 personer per år i Sverige. AML är den vanligaste formen av leukemi hos vuxna och orsakar en onormalt stor produktion av omogna blodceller vilket orsakar sjukdom. Eftersom dessa mutationer är vanligt förekommande är det viktigt att studera den här receptorn för att försöka framställa läkemedel som fungerar hämmande endast mot receptorerna med dessa mutationer men inte mot normal FLT3 eller andra receptorer.

Syftet med det första arbetet var att undersöka och jämföra fosforylering/aktivering av FLT3 receptorn i både normala celler och leukemiska celler. Vi har identifierat flera nya fosforyleringsställen på FLT3 receptorn med hjälp av hemgjorda fosfospecifika antikroppar. Vi jämförde aktiveringen av specifika tyrosiner mellan vildtypsreceptorn och respektive muterade receptorer. Studien har visat att det finns såväl kvalitativa som kvantitativa skillnader i hur receptorerna fosforyleras. Det visar sig att alla tyrosinerfosforylerades olika i de tre FLT3-varianterna. Med detta har vi visat att de

muterande FLT3 har specifika signaleringsvägar som är olika från den normala s.k. vildtypsreceptorn. Det är en viktig information för att kunna skapa nya behandlingsmöjligheter för AML patienter.

Det andra arbetet handlar om ett proteintyrosinfosfatas (PTP), DEP-1 och hur det kontrollerar FLT3 signaling. PTP är en grupp av enzymer som tar bort fosfatgrupper från fosforylerade tyrosiner på proteiner. Vi har identifierat ett enzym, DEP-1 och visat att det är involverat i negativ reglering på fosforylering och signalering genom FLT3. Vi har också visat att DEP-1 interagerar direkt med FLT3. Akut brist av DEP-1 i human cell linje med AML orsakar förhöjd fosforylering av såväl FLT3 som MAP-kinaset ERK. DEP-1 visade sig också öka cellernas tillväxt och överlevnad. Dessa data tyder på att DEP-1 kan negativt reglera FLT3s signareringsaktivitet och kan bidra till leukemogenisk celltransformation.

I det tredje arbetet undersökte vi 3,4-Diarylmaleimider, en typ av kinashämmare, som nedreglerar fosforyleringen av signaleringsmolekyler nedströms om Flt3 receptorn (t ex STAT5, AKT and ERK). Denna nya klass av hämmare framkallar celldöd (apoptos) i FLT3-ITD celler redan vid låg koncentration hämmare. Eftersom dessa hämmare är så effektiva mot FLT3-ITD skulle de i framtiden kunna användas i terapi för AML patienter i kombination med kemoterapi. Det unika med denna studie är att den visar inte bara hur generell fosforylering av FLT3 påverkas av hämmare, utan en mer detaljerad undersökning har också gjorts för att undersöka hur varje fosforyleringsställe influeras.

I det fjärde projektet studerade vi hur ERK5 är involverat i Flt3-medierad signalering och transformation med hjälp av en specifik hämmare av Mek5, BIX02188. Eftersom MEK5 är uppströms om ERK5, leder hämning av MEK5 till hämning av ERK5. Det visar sig att cellens överlevnad och tillväxt hämmas när de behandlas med hämmaren, speciellt celler som uttrycker FLT3-ITD. Vi undersökte även vilka signaleringsvägar som är inblandade i dessa processer och visade att BIX02188 hämmar fosforylering av AKT och ERK1/2. Denna studie visar att ERK5 otvetydigt är involverat i FLT3-medierad signalering speciellt genom FLT3-ITD receptorn och därför kan denna hämmare troligtvis också bidra till potentiella cancerterapier.

Sammantaget har vi visat att FLT3 receptorn signalerar olika beroende på vilken mutation som den bär. Vidare har vi studerat olika nya hämmare av olika signaleringsmolekyler och deras roll i FLT3 signalering. Allt detta visar att det finns fortfarande mycket att studera för att hitta rätt typ av terapi för de olika AML patienterna.

Краткое содержание диссертационной работы

Клетка как и весь организм в целом обладает "органами чувств" и способна воспринимать внеклеточные факторы и реагировать на них. Механизм получения сигналов из окружающей среды и передачи их в клетку осуществляется с помощью специализированных белковых субстратов, образующих в мембранах клеток активные участки- рецепторы, специфически реагирующие на определенные виды стимулов. Специфическое взаимодействие внешних факторов и мембранных рецепторов инициирует процесс передачи сигналов. При этом взаимодействии стимул, возникающий на клеточной мембране, усиливается и передается внутрь клетки по определенным сигнальным цепям. Некоторые из сигналов направляются в клеточное ядро и запускают экспрессию определенных генов, определяя, таким образом, специфическую реакцию данной клетки. Механизм передачи сигналов в клетке представляет собой прямое взаимодействие специфических белков в строго определенной последовательности. Основными молекулярными принципами, на которых базируется механизм передачи сигналов в клетке, являются специфическая ассоциация белков и их фосфорилирование-дефосфорилирование. Баланс между фосфорилированием и дефосфорилированием удерживает передачу внутриклеточных сигналов в норме. Из белков, вовлеченных в процессы фосфорилирования и регулирующих клеточный рост и дифференцировку, наиболее подробно исследованы тирозин-специфичные протеинкиназы.

Семейство тирозин-специфических протеинкиназ относится к каталитическим белкам-рецепторам, однократно пронизывающим мембрану. Рецепторы тирозинкиназы находятся на поверхности клеток и участвуют во многих видах жизнедеятельности клеток, таких как: рост, деление, созревание, обмен веществ и выживаемость клеток. Рецептор тирозинкиназы, как правило, активируется лигандом, например, фактором роста. Взаимодействие лиганда с рецептором вызывает димеризацию рецепторов и, тем самым, их активацию, фосфорилируя множественные тирозиновые остатки, находящиеся на внутриклеточной части белка. Фосфорилирование рецептора ведет к мгновенному изменению его конфигурации, формируя зоны связывания с другими внутриклеточными белками. Это, в свою очередь, приводит к запуску каскада различных реакций, стимулируя

экспрессию различных генов, в том числе отвечающих за пролиферацию и рост клеток.

Рецептор FLT3-тирозинкиназа относится к III классу семейства рецепторов тирозин-специфических протеинкиназ. Экспрессия FLT3-тирозинкиназы обнаружена на гемопоэтических клетках- предшественниках и стволовых кроветворных клетках в костном мозге. FLT3-тирозинкиназа играет важную роль в формировании новых клеток крови, их выживаемости и клеточном делении. В неактивном состоянии рецептор представлен в виде одиночной молекулы. Активация его происходит при воздействии цитокина- лиганда для FLT3 (FL). FL является фактором роста для стволовых клеток и миелоидных предшественников, способствуя их пролиферации. Когда лиганд взаимодействует с рецептором FLT3-тирозинкиназы, вызывая его димеризацию и фосфорилирование, происходит активация рецептора. Молекула FLT3 состоит из рецепторной части, расположенной на клеточной мембране и внутриклеточной части, которая состоит из подмембанного домена (JM) и каталитического киназного домена [292, 293].

Существует несколько форм патогенных FLT3 (ITD и D835Y мутации), которые приводят к независимой от лиганда активации FLT3 и неограниченному росту клеток. Эти мутации ведут к активации различных сигнальных путей и могут участвовать в развитии острой миелоидной лейкемии (ОМЛ). Внутреннее тандемное удвоение нуклеотидов различной длины (ITD) является наиболее частой мутацией у больных с ОМЛ, возникая примерно у 30% пациентов и способствует менее благоприятному прогнозу лечения. В А-петле киназного домена FLT3 также была найдена точечная мутация- замена нуклеотида в нуклеотидном триплете 835, веедущая к замене аминокислоты аспарагин, Asp (D), на тирозин, Tyr (Y)- D835Y. Частота D835Y мутации в киназном домене FLT3 составляет около 7% у пациентов с ОМЛ. ОМЛ достаточно редкое заболевание, но тем не менее количество больньных ОМЛ насчитывает около 300 случаев в год в Швеции и около 10500 случаев в год в США. Острый миелоидный лейкоз- это злокачественное заболевание миелоидных ростков кроветворных клеток, вызываемое быстрым делением (усиленной пролиферацией) бластных клеток в костном мозге, которое приводит к вытеснению нормального процесса кроветворения, уменьшая число зрелых клеток крови. У больных с ОМЛ проявляются слеющие симптомы: слабость, одышка, сердцебиение, предрасположенность к инфекционным заболеваниям, кровотечения и другие.

За последние годы в терапии острых лейкозов были достигнуты значительные успехи. В настоящее время основным методом лечения ОМЛ является полихимиотерапия, включающая в себя две основных фазы: индукционную и консолидационную. Индукционная терапия вкючает в себя антрациклин-цитарабиновую комбинацию, при применении которой достигается практически полная ремиссия (< 5% бластных клеток в костном мозге). Консолидационная фаза

направлена на уничтожение оставшихся лейкемических клеток. В случаях, когда классическая схема лечения ОМЛ невозможна (пациенты с высоким риском развития рецидива), может применяться альтернативная терапия- аллогенная трансплантация гемопоэтических стволовых клеток (ГСК) в комбинации с химиотерапией. Следовательно, все виды терапии, используемые на сегодняшний день высоко токсичны и имеют высокий риск развития рецидива, особенно у пациентов старше 60 лет. Вследствие этого, в настоящее время уделяется большое внимание поиску эффективных видов терапии, изучению биологических особенностей этого заболевания и поиску новых факторов (маркеров) для лучшей диагностики и оценки клинического прогноза, учитывающих особенности заболевания каждого пациента.

Неблагоприятными факторами, влияющими на течение терапии у пациентов с ОМЛ, являются вторичный характер заболевания, т.е. развившийся после прохождения курса химиотерапии и/или лучевой терапии, а так же, облучение вследствие взрыва атомной бомбы или наличие других дополнительных генетических аномалий (например, синдром Дауна). Мутации рецептора FLT3, особенно внутренние тандемные удвоения нуклеотидов (ITD), также имеют негативное влияние на течение, результаты терапии и на достижение полной ремиссии заболевания. Поскольку FLT3 мутации встечаются часто у пациентов с ОМЛ, важно детально изучить этот рецептор, чтобы можно было производить новые более специфичные препараты, которые бы действовали только на рецепторы FLT3 с мутациями, но не на неизмененные рецепторы FLT3 или другие рецепторы.

Ниже приводятся результаты четырех самостоятельных частей проведенного диссертационного исследования.

Целью первой части научно-исследовательской работы (НИР) было изучение и сравнение процесса фосфорилирования (активации) рецептов FLT3 в здоровых и пораженных лейкозом клетках. При этом мы выявили несколько новых тирозиновых остатков, способных к фосфорилированию на рецепторе FLT3, используя антитела, произведенные в нашей лаборатории (Departement of Laboratory Medicine, Lund University, Sweden) специально против каждого тирозинового остатка на рецепторе FLT3. Кроме того, нами было проведено сравнение активации конкретных тирозиновых остатков в клетках с экспрессией неизмененногорецептора FLT3 и FLT3 рецептора с мутациями (ITD и D835Y). Исследование показало, что существуют качественные и количественные различия в фосфорилировании различных рецепторов FLT3. Оказалось, что все тирозиновые остатки фосфорилируются по-разному в зависимости от варианта рецептора FLT3. Таким образом, мы показали, что рецептор FLT3, содержащий мутацию, генерирует сигнал с помощью специфических, отличающихся от неизмененого рецептора, сигнальных путей. Эта информация имеет чрезвычайно

важное значение для последующего создания новых препаратов для более эффективной терапии больных ОМЛ.

Вторая часть НИР была посвящена тирозиновой протеинфосфатазе DEP-1, и выяснению ее роли в управлении сигнальными путями рецептора FLT3. Протеинфосфатазы- это ферменты, которые способствуют процессу отщепления остатка фосфорной кислоты от молекулы фосфорсодержащего соединения, инактивируя при этом рецептор. Нам удалось выделить фермент DEP-1 и показать его участие в негативной регуляции фосфорилирования рецептора FLT3 а, следовательно, и в процессе передачи сигналов через FLT3. Полное блокирование экспрессии протеина DEP-1 в клетках, пораженных патологическим процессом при ОМЛ, приводит к гиперфосфорилированию как самого рецептора FLT3, так и дополнительного фермента, относящегося к группе MAP киназ- ERK1/2. Кроме того, было установлено, что DEP-1 стимулирует рост и выживаемость клеток. Эти данные позволили нам предположить, что DEP-1 негативно влияет на активацию рецептора FLT3 и может способствовать лейкемической трансформации клеток.

Третья часть работы была посвящена исследованию группы диарилмалеймидов (3,4-Diarylmaleimides), ингибиторов тирозинкиназ, которые ингибируют фосфорилирование сигнальных молекул рецептора FLT3 таких, как STAT5, AKT и ERK1/2. Этот новый ряд ингибиторов даже в низких концентрациях вызывает апоптоз клеток с экспрессией FLT3-ITD мутации. В связи с этим указанные ингибиторы являются достаточно эффективными при уничтожении клеток с FLT3-ITD мутацией. Можно также предположить, что они могут быть использованны при лечение больных ОМЛ в комбинации с химиотерапией. Следует отметить, что уникальность данного исследования состоит в том, что оно раскрывает механизм действия этих ингибиторов не только на общее фосфорилирование FLT3 рецептора, но и на фосфорилирование каждого тирозинового остатка в отдельности.

В четвертой заключительной части работы было исследовано участие ERK5 в каскаде реакций, запущенных рецептором FLT3, с помощью специфического MEK5 ингибитора- BIX02188. MEK5 является предшественником ERK5 в цепочке реакций, следовательно, ингибирование MEK5 ведет к блокированию ERK5. Наши исследования показали, что выживание и рост неизмененных клеток подавляется при использовании данного ингибитора. При этом особенно чувствительными к его действию являются клетки, экспрессирующие FLT3-ITD мутацию. Кроме того, нам удалось изучить сигнальные молекулы, участвующие в сигнальной цепочке рецептора FLT3 и показать, что BIX02188 также ингибирует фосфорилирование белков AKT и ERK1/2. Данное исследование доказывает, что ERK5 участвует в каскаде реакций, запускаемых при активации рецептора FLT3, и особенно рецептора с ITD мутацией. Таким образом, данный ингибитор может рассматриваться как один из потенциальных методов лечения рака крови.

В итоге проделанной НИР можно заключить, что сигнальные каскады рецептора FLT3 различны в зависимости от его мутаций. Мы изучили механизм действия новых ингибиторов сигнальных молекул и оценили их роль в сигнальных цепочках FLT3. Однако научные исследования в этом направлении должны быть продолжены с целью нахождения путей индивидуальной эффективной терапии пациентов с ОМЛ.

Acknowledgments

This work was carried out at the Department of Laboratory medicine, Experimental Clinical Chemistry, Malmö University Hospital, Lund University, Sweden.

First of all I would like to thank my supervisor, Lars Rönnstrand, who had always been a great teacher supporting me throughout all these years that I spent in this lab. Thank you for sharing with me your endless knowledge, participating in the process of making me a grown-up person in all the possible aspects and for being an open and positive person.

Håkan Axelsson: Thank you, Håkan, for all the help and support you have provided me. You are a great mentor. Without your help I would have never been able to finish my PhD and feel so self-confident.

Helen Petersson: I am very glad to meet such a nice person in my life. Thank you very much for teaching me how to work with mice and helping me overcome the struggle with my "fears".

I would also like to thank my co-supervisor, Jianmin Sun, and all other past and present co-workers in the lab: Kristina, Malin, Rasheed, Bengt, Susanne, Oscar, Kazi, Shruti and Tine. Without all of your support I could have never been able to do this work. I really appreciate everybody's help, collaboration and encouragement.

Jianmin, thank you for sharing your skills in molecular biology and for a good supervision.

Kristina, I will always be an admirer of you for how you study, work and dance! You are the best, thank you for everything!

Rasheed, thank you for being my office mate for the first 2 years. We had a lot of fun together talking on all possible subjects. I am very thankful for your support during all these years and for being my co-author, co-worker and friend.

Susanne, as I always say, you are my "second mother"! Thank you for helping me both inside and outside the lab. You are the person in the lab who is always ready to help with a positive mind. Dear Per, Thank you for all the help and especially for all the wonderful books that you gave me. You have always been a very positive and kind hearted person.

Bengt, you are the best office mate. Thank you for all the scientific discussions, support, nice lunches together and, of course for helping me with my driving license! Wish you all the best with research. Just continue loving science!

Malin, Oscar, Kazi, Shruti and Tine. I want to thank all of you for great help in the lab, scientific discussions and nice time working together.

Collaborators: Jörg Müller, Florian Heidel and Thomas Fischer. Thank you for good collaboration and successful publications.

I also want to thank all the people in the Wallenberg Laboratory. I would especially like to thank my former supervisor during my master project, Björn Dahlbäck. Thank you for teaching me science and for being a positive and encouraging person all the time. I also want to thank Carl Ekman (Calle), who taught me everything in the lab and forced me to speak Swedish. Tack så mycket, Calle! Det är tack vare dig jag kan prata svenska! Sofia, we became very good friends and I am thankful for that. Myriam, thank you very much for helping me with the FACS machine and for all our scientific discussions.

Anki, Monica and Ulla, thank you for helping me with the administrative issues. Anki, thank you for your presence in the lab, especially on our "Friday breakfast".

My endless amount of friends:
From St. Petersburg: Lena and Lena, Yana, Yula and Kostia, Ljuba, Katja and Lilia
From Sweden: Alla and Stefan, Diana, Cara and Henrik, Neivis and Bengt, Maria,

Sayeh, Lilia, Regina, Eka and Kalender, Yulia and Viktor, Ilona and Phil, Olga, Yana and Magnus, Ira and Jura, Santhosh, Alex, Arun and Anila, Raja and Anita, Sumesh and Sudha, Ravi, Umit, Yulia and Jura, Tamara, Yaron, and many others. I love you all and I am very proud to have you as my friends! Endless thanks to all of you for the support, fun and friendship.

My teachers in Russia and Sweden: I had a great opportunity in my life to learn from fantastic teachers from two different countries. I have learned a lot from them and would like to thank them all for being my teachers. I also want to thank Lotta Persmark who was my "studievägledare" at Lund University. I appreciate your support a lot and the care you always showed to me.

My big Family: For loving me as I am. For supporting me in all my decisions and for just being in my life. Special thanks to Vishnu's family: Amma, Chetan and Chechi. Огромное спасибо моей семье, которая всегда со мной, всегда поддерживает в любую минуту. Мамочка, Юра и Лешка- я вас обожаю. Вы мои самые близкие люди. Бабушка Маша и дедушка Миша: спасибо за поддержку, любовь и помощь во всем! Бабуля Гита, спасибо за большой вклад в мое становление, за наши проведенные на даче летние каникулы. Сережка, Анька, Женька и Димочка: я вас очень люблю, спасибо за то, что вы у меня есть. Желаю чтоб вас сопровождала удача и сопудствовал успех во всем. Спасибо огромное всем остальным родственникам за все, что вы для меня делаете и за прекрасное чувство, что мы одна большая сплоченная семья.

My darling, Vishnu, every day I thank God I met you! You are my life, my soul and my love!

References

1. Blume-Jensen, P. and T. Hunter, *Oncogenic kinase signalling.* Nature, 2001. **411**(6835): p. 355-65.
2. Ullrich, A. and J. Schlessinger, *Signal transduction by receptors with tyrosine kinase activity.* Cell, 1990. **61**(2): p. 203-12.
3. Hunter, T., *The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease.* Philos Trans R Soc Lond B Biol Sci, 1998. **353**(1368): p. 583-605.
4. Schlessinger, J., *Cell signaling by receptor tyrosine kinases.* Cell, 2000. **103**(2): p. 211-25.
5. Pawson, T. and G.D. Gish, *SH2 and SH3 domains: from structure to function.* Cell, 1992. **71**(3): p. 359-62.
6. Ostman, A., Q. Yang, and N.K. Tonks, *Expression of DEP-1, a receptor-like protein-tyrosine-phosphatase, is enhanced with increasing cell density.* Proc Natl Acad Sci U S A, 1994. **91**(21): p. 9680-4.
7. Ostman, A., C. Hellberg, and F.D. Bohmer, *Protein-tyrosine phosphatases and cancer.* Nat Rev Cancer, 2006. **6**(4): p. 307-20.
8. den Hertog, J., A. Ostman, and F.D. Bohmer, *Protein tyrosine phosphatases: regulatory mechanisms.* FEBS J, 2008. **275**(5): p. 831-47.
9. Neel, B.G. and N.K. Tonks, *Protein tyrosine phosphatases in signal transduction.* Curr Opin Cell Biol, 1997. **9**(2): p. 193-204.
10. Neel, B.G., H. Gu, and L. Pao, *The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling.* Trends Biochem Sci, 2003. **28**(6): p. 284-93.
11. Van Vactor, D., A.M. O'Reilly, and B.G. Neel, *Genetic analysis of protein tyrosine phosphatases.* Curr Opin Genet Dev, 1998. **8**(1): p. 112-26.
12. Liu, F. and J. Chernoff, *Protein tyrosine phosphatase 1B interacts with and is tyrosine phosphorylated by the epidermal growth factor receptor.* Biochem J, 1997. **327 (Pt 1)**: p. 139-45.
13. Bandyopadhyay, D., et al., *Protein-tyrosine phosphatase 1B complexes with the insulin receptor in vivo and is tyrosine-phosphorylated in the presence of insulin.* J Biol Chem, 1997. **272**(3): p. 1639-45.

14. Uchida, T., et al., *Insulin stimulates the phosphorylation of Tyr538 and the catalytic activity of PTP1C, a protein tyrosine phosphatase with Src homology-2 domains.* J Biol Chem, 1994. **269**(16): p. 12220-8.

15. Berset, T.A., E.F. Hoier, and A. Hajnal, *The C. elegans homolog of the mammalian tumor suppressor Dep-1/Scc1 inhibits EGFR signaling to regulate binary cell fate decisions.* Genes Dev, 2005. **19**(11): p. 1328-40.

16. Tarcic, G., et al., *An unbiased screen identifies DEP-1 tumor suppressor as a phosphatase controlling EGFR endocytosis.* Curr Biol, 2009. **19**(21): p. 1788-98.

17. Kappert, K., et al., *Dynamic changes in the expression of DEP-1 and other PDGF receptor-antagonizing PTPs during onset and termination of neointima formation.* FASEB J, 2007. **21**(2): p. 523-34.

18. Chabot, C., et al., *New role for the protein tyrosine phosphatase DEP-1 in Akt activation and endothelial cell survival.* Mol Cell Biol, 2009. **29**(1): p. 241-53.

19. Grazia Lampugnani, M., et al., *Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, beta-catenin, and the phosphatase DEP-1/CD148.* J Cell Biol, 2003. **161**(4): p. 793-804.

20. Palka, H.L., M. Park, and N.K. Tonks, *Hepatocyte growth factor receptor tyrosine kinase met is a substrate of the receptor protein-tyrosine phosphatase DEP-1.* J Biol Chem, 2003. **278**(8): p. 5728-35.

21. Arora, D., et al., *Protein-tyrosine phosphatase DEP-1 controls receptor tyrosine kinase FLT3 signaling.* J Biol Chem, 2011. **286**(13): p. 10918-29.

22. Cantley, L.C., et al., *Oncogenes and signal transduction.* Cell, 1991. **64**(2): p. 281-302.

23. Rosnet, O., et al., *Isolation and chromosomal localization of a novel FMS-like tyrosine kinase gene.* Genomics, 1991. **9**(2): p. 380-5.

24. Rosnet, O., et al., *Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family.* Oncogene, 1991. **6**(9): p. 1641-50.

25. Rosnet, O., et al., *Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells.* Blood, 1993. **82**(4): p. 1110-9.

26. Matthews, W., et al., *A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations.* Cell, 1991. **65**(7): p. 1143-52.

27. Maroc, N., et al., *Biochemical characterization and analysis of the transforming potential of the FLT3/FLK2 receptor tyrosine kinase.* Oncogene, 1993. **8**(4): p. 909-18.

28. Brasel, K., et al., *Expression of the flt3 receptor and its ligand on hematopoietic cells.* Leukemia, 1995. **9**(7): p. 1212-8.

29. Turner, A.M., et al., *FLT3 receptor expression on the surface of normal and malignant human hematopoietic cells.* Blood, 1996. **88**(9): p. 3383-90.

30. Kikushige, Y., et al., *Human Flt3 is expressed at the hematopoietic stem cell and the granulocyte/macrophage progenitor stages to maintain cell survival.* J Immunol, 2008. **180**(11): p. 7358-67.

31. Stirewalt, D.L. and J.P. Radich, *The role of FLT3 in haematopoietic malignancies.* Nat Rev Cancer, 2003. **3**(9): p. 650-65.

32. Lyman, S.D., et al., *Characterization of the protein encoded by the flt3 (flk2) receptor-like tyrosine kinase gene.* Oncogene, 1993. **8**(4): p. 815-22.

33. Agnes, F., et al., *Genomic structure of the downstream part of the human FLT3 gene: exon/intron structure conservation among genes encoding receptor tyrosine kinases (RTK) of subclass III.* Gene, 1994. **145**(2): p. 283-8.

34. Dosil, M., S. Wang, and I.R. Lemischka, *Mitogenic signalling and substrate specificity of the Flk2/Flt3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells.* Mol Cell Biol, 1993. **13**(10): p. 6572-85.

35. Zhang, S. and H.E. Broxmeyer, *Flt3 ligand induces tyrosine phosphorylation of gab1 and gab2 and their association with shp-2, grb2, and PI3 kinase.* Biochem Biophys Res Commun, 2000. **277**(1): p. 195-9.

36. Lyman, S.D. and S.E. Jacobsen, *c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities.* Blood, 1998. **91**(4): p. 1101-34.

37. Lyman, S.D., et al., *Identification of soluble and membrane-bound isoforms of the murine flt3 ligand generated by alternative splicing of mRNAs.* Oncogene, 1995. **10**(1): p. 149-57.

38. Hannum, C., et al., *Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs.* Nature, 1994. **368**(6472): p. 643-8.

39. Lyman, S.D., et al., *Cloning of the human homologue of the murine flt3 ligand: a growth factor for early hematopoietic progenitor cells.* Blood, 1994. **83**(10): p. 2795-801.

40. Lyman, S.D., et al., *Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells.* Cell, 1993. **75**(6): p. 1157-67.

41. Jacobsen, S.E., et al., *The FLT3 ligand potently and directly stimulates the growth and expansion of primitive murine bone marrow progenitor cells in vitro: synergistic interactions with interleukin (IL) 11, IL-12, and other hematopoietic growth factors.* J Exp Med, 1995. **181**(4): p. 1357-63.

42. Sitnicka, E., et al., *Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool.* Immunity, 2002. **17**(4): p. 463-72.

43. Maraskovsky, E., et al., *Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified.* J Exp Med, 1996. **184**(5): p. 1953-62.

44. Marchetto, S., et al., *SHC and SHIP phosphorylation and interaction in response to activation of the FLT3 receptor.* Leukemia, 1999. **13**(9): p. 1374-82.

45. Drexler, H.G. and H. Quentmeier, *FLT3: receptor and ligand.* Growth Factors, 2004. **22**(2): p. 71-3.

46. Tanoue, T. and E. Nishida, *Molecular recognitions in the MAP kinase cascades.* Cell Signal, 2003. **15**(5): p. 455-62.

47. Kyriakis, J.M., et al., *Raf-1 activates MAP kinase-kinase.* Nature, 1992. **358**(6385): p. 417-21.

48. Yoon, S. and R. Seger, *The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions.* Growth Factors, 2006. **24**(1): p. 21-44.

49. Murphy, L.O. and J. Blenis, *MAPK signal specificity: the right place at the right time.* Trends Biochem Sci, 2006. **31**(5): p. 268-75.

50. Seger, R. and E.G. Krebs, *The MAPK signaling cascade.* FASEB J, 1995. **9**(9): p. 726-35.

51. Jelinek, T., et al., *Ras-induced activation of Raf-1 is dependent on tyrosine phosphorylation.* Mol Cell Biol, 1996. **16**(3): p. 1027-34.

52. Choy, E., et al., *Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi.* Cell, 1999. **98**(1): p. 69-80.

53. McKay, M.M. and D.K. Morrison, *Integrating signals from RTKs to ERK/MAPK.* Oncogene, 2007. **26**(22): p. 3113-21.

54. Campbell, S.L., et al., *Increasing complexity of Ras signaling.* Oncogene, 1998. **17**(11 Reviews): p. 1395-413.

55. Eychene, A., et al., *Expression and activation of B-Raf kinase isoforms in human and murine leukemia cell lines.* Oncogene, 1995. **10**(6): p. 1159-65.

56. Dhillon, A.S. and W. Kolch, *Untying the regulation of the Raf-1 kinase.* Arch Biochem Biophys, 2002. **404**(1): p. 3-9.

57. Wellbrock, C., M. Karasarides, and R. Marais, *The RAF proteins take centre stage.* Nat Rev Mol Cell Biol, 2004. **5**(11): p. 875-85.

58. Traverse, S., et al., *Specific association of activated MAP kinase kinase kinase (Raf) with the plasma membranes of ras-transformed retinal cells.* Oncogene, 1993. **8**(11): p. 3175-81.

59. Zhang, X.F., et al., *Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1.* Nature, 1993. **364**(6435): p. 308-13.

60. Roy, S., et al., *Activity of plasma membrane-recruited Raf-1 is regulated by Ras via the Raf zinc finger.* J Biol Chem, 1997. **272**(32): p. 20139-45.

61. Jaumot, M. and J.F. Hancock, *Protein phosphatases 1 and 2A promote Raf-1 activation by regulating 14-3-3 interactions.* Oncogene, 2001. **20**(30): p. 3949-58.

62. Rodriguez-Viciana, P., et al., *A phosphatase holoenzyme comprised of Shoc2/Sur8 and the catalytic subunit of PP1 functions as an M-Ras effector to modulate Raf activity.* Mol Cell, 2006. **22**(2): p. 217-30.

63. Mansour, S.J., et al., *Mitogen-activated protein (MAP) kinase phosphorylation of MAP kinase kinase: determination of phosphorylation sites by mass spectrometry and site-directed mutagenesis.* J Biochem, 1994. **116**(2): p. 304-14.

64. Zheng, C.F. and K.L. Guan, *Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues.* EMBO J, 1994. **13**(5): p. 1123-31.

65. Zhou, G., Z.Q. Bao, and J.E. Dixon, *Components of a new human protein kinase signal transduction pathway.* J Biol Chem, 1995. **270**(21): p. 12665-9.

66. Lee, J.D., R.J. Ulevitch, and J. Han, *Primary structure of BMK1: a new mammalian map kinase.* Biochem Biophys Res Commun, 1995. **213**(2): p. 715-24.

67. Nishimoto, S. and E. Nishida, *MAPK signalling: ERK5 versus ERK1/2.* EMBO Rep, 2006. **7**(8): p. 782-6.

68. Wang, X. and C. Tournier, *Regulation of cellular functions by the ERK5 signalling pathway.* Cell Signal, 2006. **18**(6): p. 753-60.

69. Chao, T.H., et al., *MEKK3 directly regulates MEK5 activity as part of the big mitogen-activated protein kinase 1 (BMK1) signaling pathway.* J Biol Chem, 1999. **274**(51): p. 36035-8.

70. Sun, W., et al., *MEKK2 associates with the adapter protein Lad/RIBP and regulates the MEK5-BMK1/ERK5 pathway.* J Biol Chem, 2001. **276**(7): p. 5093-100.

71. Chang, L. and M. Karin, *Mammalian MAP kinase signalling cascades.* Nature, 2001. **410**(6824): p. 37-40.

72. Buschbeck, M. and A. Ullrich, *The unique C-terminal tail of the mitogen-activated protein kinase ERK5 regulates its activation and nuclear shuttling.* J Biol Chem, 2005. **280**(4): p. 2659-67.

73. Kato, Y., et al., *BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C.* EMBO J, 1997. **16**(23): p. 7054-66.

74. Yan, C., et al., *Molecular cloning of mouse ERK5/BMK1 splice variants and characterization of ERK5 functional domains.* J Biol Chem, 2001. **276**(14): p. 10870-8.

75. Morimoto, H., et al., *Activation of a C-terminal transcriptional activation domain of ERK5 by autophosphorylation.* J Biol Chem, 2007. **282**(49): p. 35449-56.

76. Abe, J., et al., *Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase.* J Biol Chem, 1996. **271**(28): p. 16586-90.

77. Kato, Y., et al., *Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor.* Nature, 1998. **395**(6703): p. 713-6.

78. Kamakura, S., T. Moriguchi, and E. Nishida, *Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus.* J Biol Chem, 1999. **274**(37): p. 26563-71.

79. Watson, F.L., et al., *Neurotrophins use the Erk5 pathway to mediate a retrograde survival response.* Nat Neurosci, 2001. **4**(10): p. 981-8.

80. Obara, Y., et al., *ERK5 activity is required for nerve growth factor-induced neurite outgrowth and stabilization of tyrosine hydroxylase in PC12 cells.* J Biol Chem, 2009. **284**(35): p. 23564-73.

81. Izawa, Y., et al., *Big mitogen-activated protein kinase 1 (BMK1)/extracellular signal regulated kinase 5 (ERK5) is involved in platelet-derived growth factor (PDGF)-induced vascular smooth muscle cell migration.* Hypertens Res, 2007. **30**(11): p. 1107-17.

82. Rovida, E., et al., *ERK5 differentially regulates PDGF-induced proliferation and migration of hepatic stellate cells.* J Hepatol, 2008. **48**(1): p. 107-15.

83. Lennartsson, J., et al., *Erk 5 is necessary for sustained PDGF-induced Akt phosphorylation and inhibition of apoptosis.* Cell Signal. **22**(6): p. 955-60.

84. Mody, N., et al., *An analysis of the phosphorylation and activation of extracellular-signal-regulated protein kinase 5 (ERK5) by mitogen-activated protein kinase kinase 5 (MKK5) in vitro.* Biochem J, 2003. **372**(Pt 2): p. 567-75.

85. Cavanaugh, J.E., *Role of extracellular signal regulated kinase 5 in neuronal survival.* Eur J Biochem, 2004. **271**(11): p. 2056-9.

86. Finegan, K.G., et al., *Regulation of neuronal survival by the extracellular signal-regulated protein kinase 5.* Cell Death Differ, 2009. **16**(5): p. 674-83.

87. Wang, X., et al., *Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress.* Cell Death Differ, 2006. **13**(12): p. 2099-108.

88. Drew, B.A., M.E. Burow, and B.S. Beckman, *MEK5/ERK5 pathway: The first fifteen years.* Biochim Biophys Acta, 2011.

89. Masson, K., et al., *A role of Gab2 association in Flt3 ITD mediated Stat5 phosphorylation and cell survival.* Br J Haematol, 2009. **146**(2): p. 193-202.

90. Carpenter, C.L. and L.C. Cantley, *Phosphoinositide 3-kinase and the regulation of cell growth.* Biochim Biophys Acta, 1996. **1288**(1): p. M11-6.

91. Carpenter, C.L. and L.C. Cantley, *Phosphoinositide kinases.* Curr Opin Cell Biol, 1996. **8**(2): p. 153-8.

92. Dhand, R., et al., *PI 3-kinase: structural and functional analysis of intersubunit interactions.* EMBO J, 1994. **13**(3): p. 511-21.

93. Dhand, R., et al., *PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity.* EMBO J, 1994. **13**(3): p. 522-33.

94. Chan, T.O., et al., *Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit.* Cancer Cell, 2002. **1**(2): p. 181-91.

95. Jimenez, C., et al., *The p85 regulatory subunit controls sequential activation of phosphoinositide 3-kinase by Tyr kinases and Ras.* J Biol Chem, 2002. **277**(44): p. 41556-62.

96. Franke, T.F., et al., *The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase.* Cell, 1995. **81**(5): p. 727-36.

97. Franke, T.F., D.R. Kaplan, and L.C. Cantley, *PI3K: downstream AKTion blocks apoptosis.* Cell, 1997. **88**(4): p. 435-7.

98. Klippel, A., et al., *Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways.* Mol Cell Biol, 1996. **16**(8): p. 4117-27.

99. Klippel, A., et al., *A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain.* Mol Cell Biol, 1997. **17**(1): p. 338-44.

100. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: navigating downstream.* Cell, 2007. **129**(7): p. 1261-74.

101. Franke, T.F., *PI3K/Akt: getting it right matters.* Oncogene, 2008. **27**(50): p. 6473-88.

102. Franke, T.F., *Intracellular signaling by Akt: bound to be specific.* Sci Signal, 2008. **1**(24): p. pe29.

103. Facchinetti, V., et al., *The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C.* EMBO J, 2008. **27**(14): p. 1932-43.

104. Stambolic, V., et al., *Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN.* Cell, 1998. **95**(1): p. 29-39.

105. Carpten, J.D., et al., *A transforming mutation in the pleckstrin homology domain of AKT1 in cancer.* Nature, 2007. **448**(7152): p. 439-44.

106. Kalesnikoff, J., et al., *The role of SHIP in cytokine-induced signaling.* Rev Physiol Biochem Pharmacol, 2003. **149**: p. 87-103.

107. Muraille, E., et al., *Distribution of the src-homology-2-domain-containing inositol 5-phosphatase SHIP-2 in both non-haemopoietic and haemopoietic cells and possible involvement of SHIP-2 in negative signalling of B-cells.* Biochem J, 1999. **342 Pt 3**: p. 697-705.

108. Perez, L.E., et al., *SH2-inositol phosphatase 1 negatively influences early megakaryocyte progenitors.* PLoS One, 2008. **3**(10): p. e3565.

109. Lawlor, M.A. and D.R. Alessi, *PKB/Akt: a key mediator of cell proliferation, survival and insulin responses?* J Cell Sci, 2001. **114**(Pt 16): p. 2903-10.

110. Scheijen, B., et al., *FLT3 receptors with internal tandem duplications promote cell viability and proliferation by signaling through Foxo proteins.* Oncogene, 2004. **23**(19): p. 3338-49.

111. Brandts, C.H., et al., *Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation.* Cancer Res, 2005. **65**(21): p. 9643-50.

112. Rottapel, R., et al., *Substrate specificities and identification of a putative binding site for PI3K in the carboxy tail of the murine Flt3 receptor tyrosine kinase.* Oncogene, 1994. **9**(6): p. 1755-65.

113. Serve, H., Y.C. Hsu, and P. Besmer, *Tyrosine residue 719 of the c-kit receptor is essential for binding of the P85 subunit of phosphatidylinositol (PI) 3-kinase and for c-kit-associated PI 3-kinase activity in COS-1 cells.* J Biol Chem, 1994. **269**(8): p. 6026-30.

114. Sun, J., M. Pedersen, and L. Ronnstrand, *Gab2 is involved in differential phosphoinositide 3-kinase signaling by two splice forms of c-Kit.* J Biol Chem, 2008. **283**(41): p. 27444-51.

115. Karlsson, R., et al., *Phosphatidylinositol 3-kinase is essential for kit ligand-mediated survival, whereas interleukin-3 and flt3 ligand induce expression of antiapoptotic Bcl-2 family genes.* J Leukoc Biol, 2003. **74**(5): p. 923-31.

116. Yuan, T.L. and L.C. Cantley, *PI3K pathway alterations in cancer: variations on a theme.* Oncogene, 2008. **27**(41): p. 5497-510.

117. Hunter, T. and B.M. Sefton, *Transforming gene product of Rous sarcoma virus phosphorylates tyrosine.* Proc Natl Acad Sci U S A, 1980. **77**(3): p. 1311-5.

118. Martin, G.S., *The road to Src.* Oncogene, 2004. **23**(48): p. 7910-7.

119. Collett, M.S. and R.L. Erikson, *Protein kinase activity associated with the avian sarcoma virus src gene product.* Proc Natl Acad Sci U S A, 1978. **75**(4): p. 2021-4.

120. Stein, P.L., H. Vogel, and P. Soriano, *Combined deficiencies of Src, Fyn, and Yes tyrosine kinases in mutant mice.* Genes Dev, 1994. **8**(17): p. 1999-2007.

121. Lowell, C.A., et al., *Deficiency of the Hck and Src tyrosine kinases results in extreme levels of extramedullary hematopoiesis.* Blood, 1996. **87**(5): p. 1780-92.

122. Thomas, S.M. and J.S. Brugge, *Cellular functions regulated by Src family kinases.* Annu Rev Cell Dev Biol, 1997. **13**: p. 513-609.

123. Wadhawan, A., et al., *Src-mediated regulation of homotypic cell adhesion: implications for cancer progression and opportunities for therapeutic intervention.* Cancer Treat Rev, 2011. **37**(3): p. 234-41.

124. Brown, M.T. and J.A. Cooper, *Regulation, substrates and functions of src.* Biochim Biophys Acta, 1996. **1287**(2-3): p. 121-49.

125. Pawson, T., *New impressions of Src and Hck.* Nature, 1997. **385**(6617): p. 582-3, 585.

126. Sicheri, F. and J. Kuriyan, *Structures of Src-family tyrosine kinases.* Curr Opin Struct Biol, 1997. **7**(6): p. 777-85.

127. Yeatman, T.J., *A renaissance for SRC.* Nat Rev Cancer, 2004. **4**(6): p. 470-80.

128. Broudy, V.C., et al., *Signaling via Src family kinases is required for normal internalization of the receptor c-Kit.* Blood, 1999. **94**(6): p. 1979-86.

129. Masson, K., et al., *Direct binding of Cbl to Tyr568 and Tyr936 of the stem cell factor receptor/c-Kit is required for ligand-induced ubiquitination, internalization and degradation.* Biochem J, 2006. **399**(1): p. 59-67.

130. Biscardi, J.S., et al., *c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function.* J Biol Chem, 1999. **274**(12): p. 8335-43.

131. Hansen, K., et al., *Mutation of a Src phosphorylation site in the PDGF beta-receptor leads to increased PDGF-stimulated chemotaxis but decreased mitogenesis.* EMBO J, 1996. **15**(19): p. 5299-313.

132. Mitina, O., et al., *Src family tyrosine kinases phosphorylate Flt3 on juxtamembrane tyrosines and interfere with receptor maturation in a kinase-dependent manner.* Ann Hematol, 2007. **86**(11): p. 777-85.

133. Heiss, E., et al., *Identification of Y589 and Y599 in the juxtamembrane domain of Flt3 as ligand-induced autophosphorylation sites involved in binding of Src family kinases and the protein tyrosine phosphatase SHP2.* Blood, 2006. **108**(5): p. 1542-50.

134. Schittenhelm, M.M., et al., *Dasatinib (BMS-354825), a dual SRC/ABL kinase inhibitor, inhibits the kinase activity of wild-type, juxtamembrane, and activation loop mutant KIT isoforms associated with human malignancies.* Cancer Res, 2006. **66**(1): p. 473-81.

135. Horvath, C.M. and J.E. Darnell, *The state of the STATs: recent developments in the study of signal transduction to the nucleus.* Curr Opin Cell Biol, 1997. **9**(2): p. 233-9.

136. Leonard, W.J. and J.J. O'Shea, *Jaks and STATs: biological implications.* Annu Rev Immunol, 1998. **16**: p. 293-322.

137. Imada, K. and W.J. Leonard, *The Jak-STAT pathway.* Mol Immunol, 2000. **37**(1-2): p. 1-11.

138. Vinkemeier, U., et al., *DNA binding of in vitro activated Stat1 alpha, Stat1 beta and truncated Stat1: interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites.* EMBO J, 1996. **15**(20): p. 5616-26.

139. Xu, X., Y.L. Sun, and T. Hoey, *Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain.* Science, 1996. **273**(5276): p. 794-7.

140. Zhang, X., et al., *Requirement of serine phosphorylation for formation of STAT-promoter complexes.* Science, 1995. **267**(5206): p. 1990-4.

141. Choudhary, C., et al., *Activation mechanisms of STAT5 by oncogenic Flt3-ITD.* Blood, 2007. **110**(1): p. 370-4.

142. Choudhary, C., et al., *AML-associated Flt3 kinase domain mutations show signal transduction differences compared with Flt3 ITD mutations.* Blood, 2005. **106**(1): p. 265-73.

143. Schmidt-Arras, D., et al., *Anchoring of FLT3 in the endoplasmic reticulum alters signaling quality.* Blood, 2009. **113**(15): p. 3568-76.

144. Choudhary, C., et al., *Mislocalized activation of oncogenic RTKs switches downstream signaling outcomes.* Mol Cell, 2009. **36**(2): p. 326-39.

145. Rocnik, J.L., et al., *Roles of tyrosine 589 and 591 in STAT5 activation and transformation mediated by FLT3-ITD.* Blood, 2006. **108**(4): p. 1339-45.

146. Nishizuka, Y., *Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C.* Science, 1992. **258**(5082): p. 607-14.

147. Carpenter, G. and Q. Ji, *Phospholipase C-gamma as a signal-transducing element.* Exp Cell Res, 1999. **253**(1): p. 15-24.

148. Wilde, J.I. and S.P. Watson, *Regulation of phospholipase C gamma isoforms in haematopoietic cells: why one, not the other?* Cell Signal, 2001. **13**(10): p. 691-701.

149. Choi, J.H., S.H. Ryu, and P.G. Suh, *On/off-regulation of phospholipase C-gamma 1-mediated signal transduction.* Adv Enzyme Regul, 2007. **47**: p. 104-16.

150. Margolis, B., et al., *The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-gamma.* EMBO J, 1990. **9**(13): p. 4375-80.

151. Mohammadi, M., et al., *A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1.* Mol Cell Biol, 1991. **11**(10): p. 5068-78.

152. Ronnstrand, L., et al., *Identification of two C-terminal autophosphorylation sites in the PDGF beta-receptor: involvement in the interaction with phospholipase C-gamma.* EMBO J, 1992. **11**(11): p. 3911-9.

153. Herbst, R., et al., *Substrate phosphorylation specificity of the human c-kit receptor tyrosine kinase.* J Biol Chem, 1991. **266**(30): p. 19908-16.

154. Herbst, R., et al., *Formation of signal transfer complexes between stem cell and platelet-derived growth factor receptors and SH2 domain proteins in vitro.* Biochemistry, 1995. **34**(17): p. 5971-9.

155. Gommerman, J.L., et al., *Differential stimulation of c-Kit mutants by membrane-bound and soluble Steel Factor correlates with leukemic potential.* Blood, 2000. **96**(12): p. 3734-42.

156. Rodriguez-Viciana, P., et al., *Phosphatidylinositol-3-OH kinase as a direct target of Ras.* Nature, 1994. **370**(6490): p. 527-32.

157. Rodriguez-Viciana, P., et al., *Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras.* Cell, 1997. **89**(3): p. 457-67.

158. Zimmermann, S. and K. Moelling, *Phosphorylation and regulation of Raf by Akt (protein kinase B).* Science, 1999. **286**(5445): p. 1741-4.

159. Ramjaun, A.R. and J. Downward, *Ras and phosphoinositide 3-kinase: partners in development and tumorigenesis.* Cell Cycle, 2007. **6**(23): p. 2902-5.

160. Ogawa, M., *Differentiation and proliferation of hematopoietic stem cells.* Blood, 1993. **81**(11): p. 2844-53.

161. Till, J.E. and C.E. Mc, *A direct measurement of the radiation sensitivity of normal mouse bone marrow cells.* Radiat Res, 1961. **14**: p. 213-22.

162. Weissman, I.L. and J.A. Shizuru, *The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases.* Blood, 2008. **112**(9): p. 3543-53.

163. Giebel, B. and M. Punzel, *Lineage development of hematopoietic stem and progenitor cells.* Biol Chem, 2008. **389**(7): p. 813-24.

164. Sigvardsson, M., *New light on the biology and developmental potential of haematopoietic stem cells and progenitor cells.* J Intern Med, 2009. **266**(4): p. 311-24.

165. Reya, T., et al., *Stem cells, cancer, and cancer stem cells.* Nature, 2001. **414**(6859): p. 105-11.

166. Adolfsson, J., et al., *Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment.* Cell, 2005. **121**(2): p. 295-306.

167. Lai, A.Y., S.M. Lin, and M. Kondo, *Heterogeneity of Flt3-expressing multipotent progenitors in mouse bone marrow.* J Immunol, 2005. **175**(8): p. 5016-23.

168. Krause, D.S., *Regulation of hematopoietic stem cell fate.* Oncogene, 2002. **21**(21): p. 3262-9.

169. Rizo, A., et al., *Signaling pathways in self-renewing hematopoietic and leukemic stem cells: do all stem cells need a niche?* Hum Mol Genet, 2006. **15 Spec No 2**: p. R210-9.

170. Cantor, A.B. and S.H. Orkin, *Hematopoietic development: a balancing act.* Curr Opin Genet Dev, 2001. **11**(5): p. 513-9.

171. Georgopoulos, K., *Haematopoietic cell-fate decisions, chromatin regulation and ikaros.* Nat Rev Immunol, 2002. **2**(3): p. 162-74.

172. Kaushansky, K., *Lineage-specific hematopoietic growth factors.* N Engl J Med, 2006. **354**(19): p. 2034-45.

173. Metcalf, D., *Hematopoietic regulators: redundancy or subtlety?* Blood, 1993. **82**(12): p. 3515-23.

174. Miller, C.L. and C.J. Eaves, *Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability.* Proc Natl Acad Sci U S A, 1997. **94**(25): p. 13648-53.

175. Sauvageau, G., N.N. Iscove, and R.K. Humphries, *In vitro and in vivo expansion of hematopoietic stem cells.* Oncogene, 2004. **23**(43): p. 7223-32.

176. Mackarehtschian, K., et al., *Targeted disruption of the flk2/flt3 gene leads to deficiencies in primitive hematopoietic progenitors.* Immunity, 1995. **3**(1): p. 147-61.

177. Vogelstein, B. and K.W. Kinzler, *Cancer genes and the pathways they control.* Nat Med, 2004. **10**(8): p. 789-99.

178. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.

179. Al-Hajj, M. and M.F. Clarke, *Self-renewal and solid tumor stem cells.* Oncogene, 2004. **23**(43): p. 7274-82.

180. Schwarz-Cruz-y-Celis, A. and J. Melendez-Zajgla, *Cancer stem cells.* Rev Invest Clin, 2011. **63**(2): p. 179-86.

181. Jemal, A., et al., *Cancer statistics, 2002.* CA Cancer J Clin, 2002. **52**(1): p. 23-47.

182. Prigorowsky, M., *Cancerfondsrapporten 2011*. 2011. p. 114-117.

183. Dohner, H., et al., *Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet.* Blood, 2010. **115**(3): p. 453-74.

184. Larson, R.A., *Is secondary leukemia an independent poor prognostic factor in acute myeloid leukemia?* Best Pract Res Clin Haematol, 2007. **20**(1): p. 29-37.

185. Gunz, F.W. and A.M. Veale, *Leukemia in close relatives--accident or predisposition?* J Natl Cancer Inst, 1969. **42**(3): p. 517-24.

186. Leone, G., et al., *The incidence of secondary leukemias.* Haematologica, 1999. **84**(10): p. 937-45.

187. Bizzozero, O.J., Jr., K.G. Johnson, and A. Ciocco, *Radiation-related leukemia in Hiroshima and Nagasaki, 1946-1964. I. Distribution, incidence and appearance time.* N Engl J Med, 1966. **274**(20): p. 1095-101.

188. Sanz, G.F., et al., *Two regression models and a scoring system for predicting survival and planning treatment in myelodysplastic syndromes: a multivariate analysis of prognostic factors in 370 patients.* Blood, 1989. **74**(1): p. 395-408.

189. de Jonge, H.J., G. Huls, and E.S. de Bont, *Gene expression profiling in acute myeloid leukaemia.* Neth J Med, 2011. **69**(4): p. 167-76.

190. Hoffman, R., *Hematology: Basic principles and practice*. 4 ed. 2005.

191. Estey, E. and H. Dohner, *Acute myeloid leukaemia.* Lancet, 2006. **368**(9550): p. 1894-907.

192. Gilliland, D.G. and J.D. Griffin, *The roles of FLT3 in hematopoiesis and leukemia.* Blood, 2002. **100**(5): p. 1532-42.

193. Kelly, L.M. and D.G. Gilliland, *Genetics of myeloid leukemias.* Annu Rev Genomics Hum Genet, 2002. **3**: p. 179-98.

194. Renneville, A., et al., *Cooperating gene mutations in acute myeloid leukemia: a review of the literature.* Leukemia, 2008. **22**(5): p. 915-31.

195. Schnittger, S., et al., *Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease.* Blood, 2002. **100**(1): p. 59-66.

196. Mizuki, M., et al., *Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways.* Blood, 2000. **96**(12): p. 3907-14.

197. Zheng, R., A.D. Friedman, and D. Small, *Targeted inhibition of FLT3 overcomes the block to myeloid differentiation in 32Dcl3 cells caused by expression of FLT3/ITD mutations.* Blood, 2002. **100**(12): p. 4154-61.

198. Birg, F., et al., *Expression of the FMS/KIT-like gene FLT3 in human acute leukemias of the myeloid and lymphoid lineages.* Blood, 1992. **80**(10): p. 2584-93.

199. Nakao, M., et al., *Internal tandem duplication of the flt3 gene found in acute myeloid leukemia.* Leukemia, 1996. **10**(12): p. 1911-8.

200. Drexler, H.G., *Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells.* Leukemia, 1996. **10**(4): p. 588-99.

201. Meshinchi, S. and F.R. Appelbaum, *Structural and functional alterations of FLT3 in acute myeloid leukemia.* Clin Cancer Res, 2009. **15**(13): p. 4263-9.

202. Ozeki, K., et al., *Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia.* Blood, 2004. **103**(5): p. 1901-8.

203. Drexler, H.G., C. Meyer, and H. Quentmeier, *Effects of FLT3 ligand on proliferation and survival of myeloid leukemia cells.* Leuk Lymphoma, 1999. **33**(1-2): p. 83-91.

204. Piacibello, W., et al., *Effects of human FLT3 ligand on myeloid leukemia cell growth: heterogeneity in response and synergy with other hematopoietic growth factors.* Blood, 1995. **86**(11): p. 4105-14.

205. Zheng, R., et al., *FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells.* Blood, 2004. **103**(1): p. 267-74.

206. Waskow, C., et al., *The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues.* Nat Immunol, 2008. **9**(6): p. 676-83.

207. Stirewalt, D.L., et al., *FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia.* Blood, 2001. **97**(11): p. 3589-95.

208. Meshinchi, S., et al., *Activating mutations of RTK/ras signal transduction pathway in pediatric acute myeloid leukemia.* Blood, 2003. **102**(4): p. 1474-9.

209. Stirewalt, D.L., et al., *Size of FLT3 internal tandem duplication has prognostic significance in patients with acute myeloid leukemia.* Blood, 2006. **107**(9): p. 3724-6.

210. Meshinchi, S., et al., *Structural and numerical variation of FLT3/ITD in pediatric AML.* Blood, 2008. **111**(10): p. 4930-3.

211. Vempati, S., et al., *Arginine 595 is duplicated in patients with acute leukemias carrying internal tandem duplications of FLT3 and modulates its transforming potential.* Blood, 2007. **110**(2): p. 686-94.

212. Koch, S., et al., *Abnormal localization and accumulation of FLT3-ITD, a mutant receptor tyrosine kinase involved in leukemogenesis.* Cells Tissues Organs, 2008. **188**(1-2): p. 225-35.

213. Schmidt-Arras, D.E., et al., *Tyrosine phosphorylation regulates maturation of receptor tyrosine kinases.* Mol Cell Biol, 2005. **25**(9): p. 3690-703.

214. Griffith, J., et al., *The structural basis for autoinhibition of FLT3 by the juxtamembrane domain.* Mol Cell, 2004. **13**(2): p. 169-78.

215. Naoe, T. and H. Kiyoi, *Normal and oncogenic FLT3.* Cell Mol Life Sci, 2004. **61**(23): p. 2932-8.

216. Kiyoi, H., et al., *Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product.* Leukemia, 1998. **12**(9): p. 1333-7.

217. Kiyoi, H., et al., *Mechanism of constitutive activation of FLT3 with internal tandem duplication in the juxtamembrane domain.* Oncogene, 2002. **21**(16): p. 2555-63.

218. Hayakawa, F., et al., *Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines.* Oncogene, 2000. **19**(5): p. 624-31.

219. Bruserud, O., et al., *Flt3-mediated signaling in human acute myelogenous leukemia (AML) blasts: a functional characterization of Flt3-ligand effects in AML cell populations with and without genetic Flt3 abnormalities.* Haematologica, 2003. **88**(4): p. 416-28.

220. Schlessinger, J., *Signal transduction. Autoinhibition control.* Science, 2003. **300**(5620): p. 750-2.

221. Vempati, S., et al., *Transformation by oncogenic mutants and ligand-dependent activation of FLT3 wild-type requires the tyrosine residues 589 and 591.* Clin Cancer Res, 2008. **14**(14): p. 4437-45.

222. Zhao, M., et al., *In vivo treatment of mutant FLT3-transformed murine leukemia with a tyrosine kinase inhibitor.* Leukemia, 2000. **14**(3): p. 374-8.

223. Kelly, L.M., et al., *FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model.* Blood, 2002. **99**(1): p. 310-8.

224. Grundler, R., et al., *FLT3-ITD and tyrosine kinase domain mutants induce 2 distinct phenotypes in a murine bone marrow transplantation model.* Blood, 2005. **105**(12): p. 4792-9.

225. Yokota, S., et al., *Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines.* Leukemia, 1997. **11**(10): p. 1605-9.

226. Xu, F., et al., *Tandem duplication of the FLT3 gene is found in acute lymphoblastic leukaemia as well as acute myeloid leukaemia but not in myelodysplastic syndrome or juvenile chronic myelogenous leukaemia in children.* Br J Haematol, 1999. **105**(1): p. 155-62.

227. Furitsu, T., et al., *Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product.* J Clin Invest, 1993. **92**(4): p. 1736-44.

228. Longley, B.J., Jr., et al., *Activating and dominant inactivating c-KIT catalytic domain mutations in distinct clinical forms of human mastocytosis.* Proc Natl Acad Sci U S A, 1999. **96**(4): p. 1609-14.

229. Yamamoto, Y., et al., *Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies.* Blood, 2001. **97**(8): p. 2434-9.

230. Abu-Duhier, F.M., et al., *Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia.* Br J Haematol, 2001. **113**(4): p. 983-8.

231. Moreno, I., et al., *Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia.* Haematologica, 2003. **88**(1): p. 19-24.

232. Thiede, C., et al., *Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis.* Blood, 2002. **99**(12): p. 4326-35.

233. Spiekermann, K., et al., *A new and recurrent activating length mutation in exon 20 of the FLT3 gene in acute myeloid leukemia.* Blood, 2002. **100**(9): p. 3423-5.

234. Mead, A.J., et al., *FLT3 tyrosine kinase domain mutations are biologically distinct from and have a significantly more favorable prognosis than FLT3 internal tandem duplications in patients with acute myeloid leukemia.* Blood, 2007. **110**(4): p. 1262-70.

235. Schittenhelm, M.M., et al., *FLT3 K663Q is a novel AML-associated oncogenic kinase: Determination of biochemical properties and sensitivity to Sunitinib (SU11248).* Leukemia, 2006. **20**(11): p. 2008-14.

236. Reindl, C., et al., *Point mutations in the juxtamembrane domain of FLT3 define a new class of activating mutations in AML.* Blood, 2006. **107**(9): p. 3700-7.

237. Irusta, P.M. and D. DiMaio, *A single amino acid substitution in a WW-like domain of diverse members of the PDGF receptor subfamily of tyrosine kinases causes constitutive receptor activation.* EMBO J, 1998. **17**(23): p. 6912-23.

238. Ma, Y., et al., *Inhibition of spontaneous receptor phosphorylation by residues in a putative alpha-helix in the KIT intracellular juxtamembrane region.* J Biol Chem, 1999. **274**(19): p. 13399-402.

239. Chan, P.M., et al., *Autoinhibition of the kit receptor tyrosine kinase by the cytosolic juxtamembrane region.* Mol Cell Biol, 2003. **23**(9): p. 3067-78.

240. Reindl, C. and K. Spiekermann, *From kinases to cancer: leakiness, loss of autoinhibition and leukemia.* Cell Cycle, 2006. **5**(6): p. 599-602.

241. Breitenbuecher, F., et al., *Identification of a novel type of ITD mutations located in nonjuxtamembrane domains of the FLT3 tyrosine kinase receptor.* Blood, 2009. **113**(17): p. 4074-7.

242. Zhang, S., et al., *Essential role of signal transducer and activator of transcription (Stat)5a but not Stat5b for Flt3-dependent signaling.* J Exp Med, 2000. **192**(5): p. 719-28.

243. Irish, J.M., et al., *Flt3 Y591 duplication and Bcl-2 overexpression are detected in acute myeloid leukemia cells with high levels of phosphorylated wild-type p53.* Blood, 2007. **109**(6): p. 2589-96.

244. Sallmyr, A., et al., *Internal tandem duplication of FLT3 (FLT3/ITD) induces increased ROS production, DNA damage, and misrepair: implications for poor prognosis in AML.* Blood, 2008. **111**(6): p. 3173-82.

245. Choudhary, C., et al., *Signal transduction of oncogenic Flt3.* Int J Hematol, 2005. **82**(2): p. 93-9.

246. Kim, K.T., et al., *Constitutive Fms-like tyrosine kinase 3 activation results in specific changes in gene expression in myeloid leukaemic cells.* Br J Haematol, 2007. **138**(5): p. 603-15.

247. van Lohuizen, M., et al., *Predisposition to lymphomagenesis in pim-1 transgenic mice: cooperation with c-myc and N-myc in murine leukemia virus-induced tumors.* Cell, 1989. **56**(4): p. 673-82.

248. Yoshimoto, G., et al., *FLT3-ITD up-regulates MCL-1 to promote survival of stem cells in acute myeloid leukemia via FLT3-ITD-specific STAT5 activation.* Blood, 2009. **114**(24): p. 5034-43.

249. Mizuki, M., et al., *Suppression of myeloid transcription factors and induction of STAT response genes by AML-specific Flt3 mutations.* Blood, 2003. **101**(8): p. 3164-73.

250. Schwable, J., et al., *RGS2 is an important target gene of Flt3-ITD mutations in AML and functions in myeloid differentiation and leukemic transformation.* Blood, 2005. **105**(5): p. 2107-14.

251. Tickenbrock, L., et al., *Flt3 tandem duplication mutations cooperate with Wnt signaling in leukemic signal transduction.* Blood, 2005. **105**(9): p. 3699-706.

252. Simon, M., et al., *Constitutive activation of the Wnt/beta-catenin signalling pathway in acute myeloid leukaemia.* Oncogene, 2005. **24**(14): p. 2410-20.

253. Mikesch, J.H., et al., *The emerging role of Wnt signaling in the pathogenesis of acute myeloid leukemia.* Leukemia, 2007. **21**(8): p. 1638-47.

254. Okamoto, M., et al., *Lyn is an important component of the signal transduction pathway specific to FLT3/ITD and can be a therapeutic target in the treatment of AML with FLT3/ITD*. Leukemia, 2007. **21**(3): p. 403-10.

255. Lu, Y., et al., *Identification of TSC-22 as a potential tumor suppressor that is upregulated by Flt3-D835V but not Flt3-ITD*. Leukemia, 2007. **21**(11): p. 2246-57.

256. Mayer, R.J., et al., *Intensive postremission chemotherapy in adults with acute myeloid leukemia. Cancer and Leukemia Group B*. N Engl J Med, 1994. **331**(14): p. 896-903.

257. Cornelissen, J.J. and B. Lowenberg, *Role of allogeneic stem cell transplantation in current treatment of acute myeloid leukemia*. Hematology Am Soc Hematol Educ Program, 2005: p. 151-5.

258. Castaigne, S., et al., *All-trans retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results*. Blood, 1990. **76**(9): p. 1704-9.

259. Huang, M.E., et al., *Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia*. Blood, 1988. **72**(2): p. 567-72.

260. Lancet, J.E. and J.E. Karp, *Farnesyltransferase inhibitors in hematologic malignancies: new horizons in therapy*. Blood, 2003. **102**(12): p. 3880-9.

261. Lunghi, P., et al., MEK1 inhibition sensitizes primary acute myelogenous leukemia to arsenic trioxide-induced apoptosis. Blood, 2006. **107**(11): p. 4549-53.

262. Kojima, K., et al., *MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy*. Blood, 2005. **106**(9): p. 3150-9.

263. Fabbro, D., et al., *PKC412--a protein kinase inhibitor with a broad therapeutic potential*. Anticancer Drug Des, 2000. **15**(1): p. 17-28.

264. Kelly, L.M., et al., *CT53518, a novel selective FLT3 antagonist for the treatment of acute myelogenous leukemia (AML)*. Cancer Cell, 2002. **1**(5): p. 421-32.

265. O'Farrell, A.M., et al., *SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo*. Blood, 2003. **101**(9): p. 3597-605.

266. Giles, F.J., et al., *SU5416, a small molecule tyrosine kinase receptor inhibitor, has biologic activity in patients with refractory acute myeloid leukemia or myelodysplastic syndromes*. Blood, 2003. **102**(3): p. 795-801.

267. Spiekermann, K., et al., *The protein tyrosine kinase inhibitor SU5614 inhibits FLT3 and induces growth arrest and apoptosis in AML-derived cell lines expressing a constitutively activated FLT3*. Blood, 2003. **101**(4): p. 1494-504.

268. Zhang, W., et al., *Mutant FLT3: a direct target of sorafenib in acute myelogenous leukemia*. J Natl Cancer Inst, 2008. **100**(3): p. 184-98.

269. Zhang, W., et al., *Sorafenib induces apoptosis of AML cells via Bim-mediated activation of the intrinsic apoptotic pathway*. Leukemia, 2008. **22**(4): p. 808-18.

270. Smith, B.D., et al., *Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia.* Blood, 2004. **103**(10): p. 3669-76.

271. Knapper, S., et al., *A phase 2 trial of the FLT3 inhibitor lestaurtinib (CEP701) as first-line treatment for older patients with acute myeloid leukemia not considered fit for intensive chemotherapy.* Blood, 2006. **108**(10): p. 3262-70.

272. Razumovskaya, E., J. Sun, and L. Ronnstrand, *Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD.* Biochem Biophys Res Commun, 2011. **412**(2): p. 307-12.

273. Whitman, S.P., et al., *Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: a cancer and leukemia group B study.* Cancer Res, 2001. **61**(19): p. 7233-9.

274. Kasper, S., et al., *The kinase inhibitor LS104 induces apoptosis, enhances cytotoxic effects of chemotherapeutic drugs and is targeting the receptor tyrosine kinase FLT3 in acute myeloid leukemia.* Leuk Res, 2008. **32**(11): p. 1698-708.

275. Li, Y., et al., *Suppression of leukemia expressing wild-type or ITD-mutant FLT3 receptor by a fully human anti-FLT3 neutralizing antibody.* Blood, 2004. **104**(4): p. 1137-44.

276. Small, D., *FLT3 mutations: biology and treatment.* Hematology Am Soc Hematol Educ Program, 2006: p. 178-84.

277. Quentmeier, H., et al., *FLT3 mutations in acute myeloid leukemia cell lines.* Leukemia, 2003. **17**(1): p. 120-4.

278. Miller, M., et al., *Structural basis of oncogenic activation caused by point mutations in the kinase domain of the MET proto-oncogene: modeling studies.* Proteins, 2001. **44**(1): p. 32-43.

279. Sun, J., M. Pedersen, and L. Ronnstrand, *The D816V mutation of c-Kit circumvents a requirement for Src family kinases in c-Kit signal transduction.* J Biol Chem, 2009. **284**(17): p. 11039-47.

280. Sacco, F., et al., *Tumor suppressor density-enhanced phosphatase-1 (DEP-1) inhibits the RAS pathway by direct dephosphorylation of ERK1/2 kinases.* J Biol Chem, 2009. **284**(33): p. 22048-58.

281. Persson, C., et al., *Site-selective regulation of platelet-derived growth factor beta receptor tyrosine phosphorylation by T-cell protein tyrosine phosphatase.* Mol Cell Biol, 2004. **24**(5): p. 2190-201.

282. Peifer, C., et al., *Design, synthesis, and biological evaluation of 3,4-diarylmaleimides as angiogenesis inhibitors.* J Med Chem, 2006. **49**(4): p. 1271-81.

283. Peifer, C., et al., *Profile and molecular modeling of 3-(indole-3-yl)-4-(3,4,5-trimethoxyphenyl)-1 H-pyrrole-2,5-dione (1) as a highly selective VEGF-R2/3 inhibitor.* J Med Chem, 2006. **49**(25): p. 7549-53.

284. Langer, C., et al., *High BAALC expression associates with other molecular prognostic markers, poor outcome, and a distinct gene-expression signature in cytogenetically normal patients younger than 60 years with acute myeloid leukemia: a Cancer and Leukemia Group B (CALGB) study.* Blood, 2008. **111**(11): p. 5371-9.

285. Dohner, K., et al., *Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations.* Blood, 2005. **106**(12): p. 3740-6.

286. Paschka, P., et al., *Wilms' tumor 1 gene mutations independently predict poor outcome in adults with cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study.* J Clin Oncol, 2008. **26**(28): p. 4595-602.

287. Lennartsson, J., et al., *Erk 5 is necessary for sustained PDGF-induced Akt phosphorylation and inhibition of apoptosis.* Cell Signal, 2010. **22**(6): p. 955-60.

288. Roberts, O.L., et al., *ERK5 is required for VEGF-mediated survival and tubular morphogenesis of primary human microvascular endothelial cells.* J Cell Sci, 2010. **123**(Pt 18): p. 3189-200.

289. Sticht, C., et al., *Activation of MAP kinase signaling through ERK5 but not ERK1 expression is associated with lymph node metastases in oral squamous cell carcinoma (OSCC).* Neoplasia, 2008. **10**(5): p. 462-70.

290. Garaude, J., et al., *ERK5 activates NF-kappaB in leukemic T cells and is essential for their growth in vivo.* J Immunol, 2006. **177**(11): p. 7607-17.

291. Yang, Q., et al., *Pharmacological inhibition of BMK1 suppresses tumor growth through promyelocytic leukemia protein.* Cancer Cell, 2010. **18**(3): p. 258-67.

292. Бавыкин, А.С., Волкова, М.А. , *FLT3-тирозинкиназа при острых нелимфобластных лейкозах.* Онкогематология, 2006. **1-2**: p. 15-24.

293. Баранова, О.Ю., Ширин, А.Д., *Лечение острых лейкозов у взрослых.* ГУ РОНЦ им. Н.Н. Блохина, 2006.

Paper I

ELSEVIER

Experimental Hematology

Experimental Hematology 2009;37:979–989

Oncogenic Flt3 receptors display different specificity and kinetics of autophosphorylation

Elena Razumovskaya*, Kristina Masson*, Rasheed Khan, Susanne Bengtsson, and Lars Rönnstrand

Experimental Clinical Chemistry, Department of Laboratory Medicine, Lund University, Malmö University Hospital, Malmö, Sweden

(Received 19 December 2008; revised 20 April 2009; accepted 29 May 2009)

Objective. **Fms-like tyrosine kinase-3 (Flt3), a growth factor receptor normally expressed in hematopoietic progenitor cells, has been shown to have an important role in development of acute myeloid leukemia (AML) due to activating mutations. Flt3 mutations are found in approximately one-third of AML patients and correlate with a poor prognosis, thus making the Flt3 receptor a potential therapeutic target. The aim of the investigation was to analyze the kinetics and specificity of Flt3 autophosphorylation in wild-type Flt3 as well as in oncogenic Flt3 mutants.**

Materials and methods. **We have used Ba/F3 cells stably expressing either wild-type, internal tandem duplication, or D835Y mutants of Flt3 in order to compare the site selectivity of tyrosine phosphorylation sites. By the use of a panel of phosphospecific antibodies directed against potential tyrosine phosphorylation sites in Flt3, we identified several novel phosphorylation sites in Flt3 and studied the kinetics and specificity of ligand-induced phosphorylation in living cells.**

Results. **Eight phosphorylated tyrosines (pY589, pY591, pY599, pY726, pY768, pY793, pY842, and pY955) were investigated and shown to be differentially phosphorylated in the wild-type versus the mutated receptors. Furthermore, we show that tyrosines 726, 793, and 842 are novel phosphorylation sites of Flt3 in intact cells.**

Conclusion. **In this study, we have looked at the site-specific phosphorylation in the wild-type Flt3 in comparison to the mutants found in AML. We observed not only quantitative changes but, more importantly, qualitative differences in the phosphorylation patterns of the wild-type and the mutated Flt3 receptors, which might enhance the understanding of the mechanisms by which Flt3 contributes to AML in patients with mutations in Flt3.**

In the context of acute myeloid leukemia (AML), Fms-like tyrosine kinase-3 (Flt3) is one of the most frequently altered genes, resulting in constitutive activation in approximately 30% of all patients, and correlating to a worse prognosis as compared with patients carrying the wild-type gene [1,2]. Human Flt3 (also known as fetal liver kinase-2 or human stem cell kinase-1) [3] was cloned in 1991 [4,5] and identified as a member of the class III receptor tyrosine kinase family. Flt3 is expressed on the cell surface of immature/progenitor cells of the myeloid and B-lymphoid lineages and the expression is lost upon differentiation, with the exception of dendritic cells [6,7]. Recent studies on the Flt3 expression have indicated distinct differences between the human and murine hematopoietic systems. While expression of murine Flt3 is restricted to multipotent and lymphoid progenitor cells incapable of self-renewal, the human Flt3 is expressed on primitive hematopoietic stem cells and on progenitors along both the lymphoid and granulocyte/macrophage pathway [8]. The main functions of Flt3 include induction of proliferation, differentiation, and survival of normal hematopoietic progenitor cells. The Flt3 receptor also plays a role in the immune system by promoting dendritic cell development when activated by Flt3 ligand (FL) [9–11]. FL is expressed by bone marrow stroma cells and in many other tissues [2,12]. Activation of the wild-type receptor occurs by FL binding, causing dimerization and autophosphorylation of specific tyrosine residues that, in turn, trigger signal transduction further into the cell [13,14].

*E. Razumovskaya and K. Masson contributed equally to this work.

Offprint requests to: Lars Rönnstrand, Ph.D., Experimental Clinical Chemistry, Wallenberg Laboratory, Malmö University Hospital, SE-20502 Malmö, Sweden; E-mail: Lars.Ronnstrand@med.lu.se

0301-472X/09 $–see front matter.
doi: 10.1016/j.exphem.2009.05.008

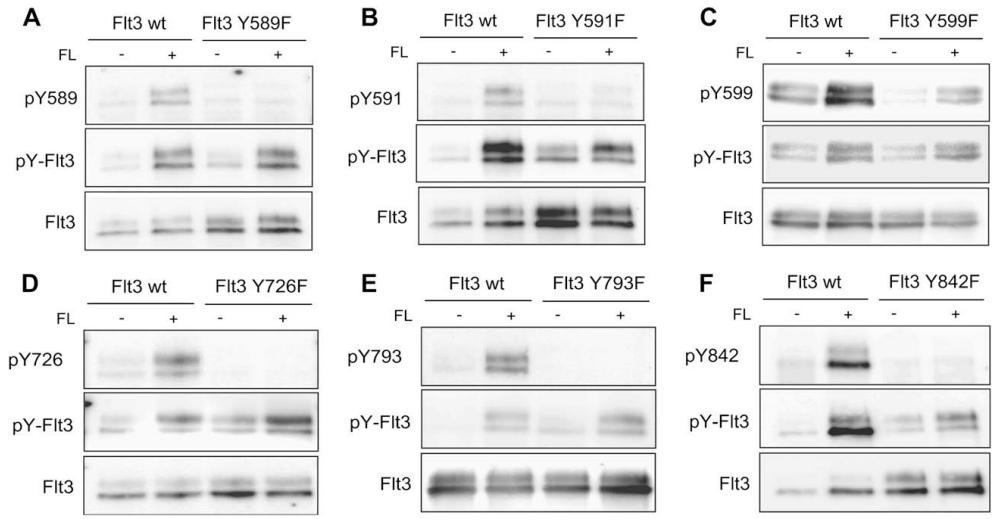

Figure 1. Verification of the phosphospecific Fms-like tyrosine kinase 3 (Flt3) antibodies in COS-1 cells. COS-1 cells transiently transfected with either the wild-type Flt3 or each of the corresponding Y-to-F mutants, were starved overnight and stimulated with Flt3 ligand (FL) for 5 minutes, lysed, and subjected to immunoprecipitation with anti-Flt3. Samples were analyzed by Western blotting and the membranes were probed with pY589 (**A**), pY591 (**B**), pY599 (**C**), pY726 (**D**), pY793 (**E**), and pY842 (**F**) antibodies. All membranes were reprobed with antibodies against phospho-tyrosine (4G10, shown as pY-Flt3 in the figure) and total FLT3, as controls. The figure shows one representative experiment out of three.

The first described activating mutation in Flt3 was the so-called internal tandem duplication (ITD). The ITD occurs as an in-frame duplication of a sequence of varying length, leading to release of negative regulatory constraint that the juxtamembrane domain poses on the kinase domain of Flt3 [15,16]. This is found in about 17% to 26% of patients with AML, and is the most common mutation in Flt3 [1,2]. Another mutation shown to be of much importance is a missense point mutation of D835 (substitution to Y, V, H, E, or N) in the activation loop of the tyrosine kinase domain of Flt3, which is found in approximately 7% of AML patients [17]. Both the ITD and D835Y mutations cause constitutive activation and ligand-independent phosphorylation of the receptor [18].

Mutations analogous to D835Y have been found in the closely related receptor tyrosine kinase c-KIT at aspartic acid 816, which is mutated in a subset of AML patients [19]. The corresponding site in c-MET receptor (D1264 N) has been found to be mutated in papillary renal carcinoma, leading not only to activation of the kinase activity of the receptor, but also altering its substrate specificity [20]. Furthermore, the oncogenic M918T mutant of the receptor tyrosine kinase c-RET (found in multiple endocrine neoplasia type 2B) showed both altered substrate specificity toward exogenous substrates as well as altered pattern of autophosphorylation [21]. This prompted us to investigate whether the oncogenic mutants of Flt3 showed altered pattern of autophosphorylation as compared with wild-type Flt3 and whether this could explain the phenotypic differences of cells expressing the respective mutant.

In the undertaken study, we have identified the tyrosine residues Y726, Y793, and Y842 as novel phosphorylation sites of Flt3 in intact cells. Furthermore, in total, eight phosphorylation sites in Flt3 were analyzed and shown to be phosphorylated upon ligand stimulation. Kinetics of the phosphorylation of these sites was compared in Ba/F3 cells stably transfected with either wild-type Flt3, ITD, or D835Y mutants. Tyrosine residues Y589, Y591, Y599, Y726, Y768, Y793, Y842, and Y955 were shown to be differentially activated in the wild-type receptor and the mutant receptors, respectively. These data form the basis for additional exploration of the function of the individual tyrosine phosphorylation sites in downstream signaling through the wild-type Flt3 as well as the oncogenic mutants of Flt3 in order to understand the mechanism by which Flt3-ITD and Flt3-D835Y functions in pathological conditions.

Materials and methods

Plasmids, peptides, and antibodies

The pMSCV-puro vector containing human Flt3 cDNA was a kind gift from Dr. D. Gary Gilliland. The anti-Flt3 antibody was raised against synthetic peptides corresponding to amino acids 740–757 of human Flt3 and purified as described [22]. Phosphospecific antibodies

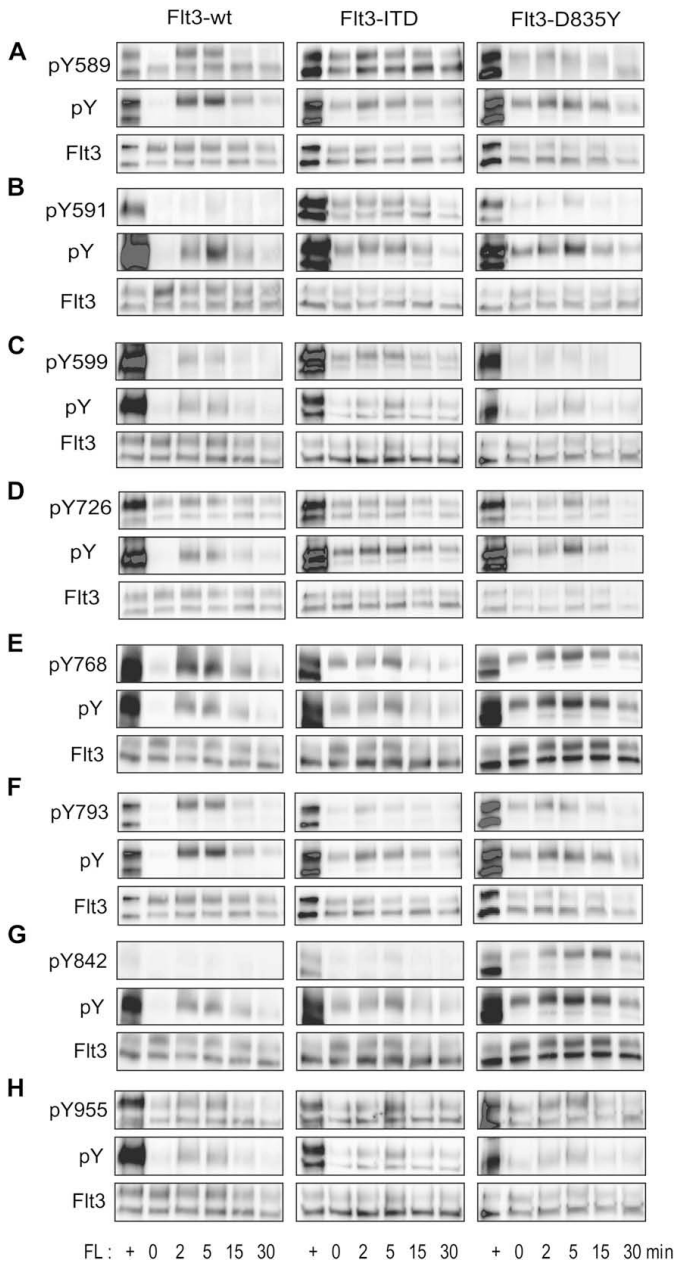

Figure 2. Kinetic studies on the site-specific phosphorylation in Ba/F3 cells. Ba/F3 cells stably transfected with either Fms-like tyrosine kinase 3 (Flt3) wild-type, Flt3-internal tandem duplication (ITD), or Flt3-D835Y, were starved for 4 hours prior to stimulation with Flt3 ligand (FL) for the indicated periods of time. In parallel as a positive control, cells were incubated with sodium pervanadate for 15 minutes at 37°C and stimulated for 5 minutes before lysis (denoted "+" in the figures). The analysis was performed using Western blotting and the membranes were probed with pY589 (**A**), pY591 (**B**), pY599 (**C**), pY726 (**D**), pY768 (**E**), pY793 (**F**), pY842 (**G**), and pY955 (**H**) antibodies. As controls, all membranes were reprobed with antibodies against phosphotyrosine (4G10, shown as pY in the figure) and total Flt3. The figure shows one representative experiment out of three.

against individual tyrosine phosphorylation sites in Flt3 were raised by immunizing rabbits with synthetic peptides (JPT Peptides Technology, Berlin, Germany) corresponding to the peptide sequence surrounding the individual tyrosine residues conjugated to keyhole limpet hemocyanin (KLH) (pY589: CGSSDNEpYFYVDFREY; pY591:CTGSSDNEYFpYVDFREY,pY599:CYVDFREYEpYDLK WEF;pY726:CEHNFSFpYPTFQSHP;pY768:CSEDEIEpYENQKR LEE;pY793:CDLLCFApYQVAKGMEF;pY842:CIMSDSNpYVV RGNAR;pY955:CDAEEAMpYQNVDGRVS;pY969:CSESPHTpY QNRRPFSR). All antibodies were affinity purified as described previously [23]. 4G10 antiphosphotyrosine antibody was from Upstate Biotechnology and the horseradish peroxidase–coupled secondary anti-mouse and anti-rabbit antibodies were from Pierce (Rockford, IL, USA).

Site-directed mutagenesis

To mutate specific tyrosine residues to phenylalanine residues, the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used and all mutations were confirmed by sequencing.

Cell culture

Murine pro-B cell line Ba/F3 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), was cultured in RPMI-1640 medium with L-glutamine and HEPES (PAA Laboratories GmbH, Pasching, Austria). Medium was supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria), 10 ng/mL recombinant murine interleukin-3 (Prospec Tany, Rehovot, Israel), 100 U/mL penicillin, and streptomycin (PAA Laboratories GmbH, Pasching, Austria). COS-1 and EcoPack cells were cultured in Dulbecco's modified essential medium (PAA Laboratories GmbH), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human AML cell lines MV4-11 and THP-1 were maintained in RPMI-1640 medium with 20% fetal bovine serum and 100 U/mL penicillin and streptomycin.

Transient and stable transfection

COS-1 cells were transiently transfected using JetPEI (PolyPlus-transfection, Illkirch, France) according to manufacturer's instructions. To establish cells stably expressing wild-type, ITD, or D835Y of Flt3, packaging EcoPack cells were transfected with the corresponding pMSCV-Flt3 constructs and virus-containing supernatants were collected 72 hours after transfection. Retroviral infection of Ba/F3 cells was followed by a 2-week selection in 2 μg/mL puromycin (Sigma-Aldrich, Steinheim, Germany) and Flt3 expression was confirmed using flow cytometry.

Cell stimulation, immunoprecipitation, and Western blotting

Before stimulation Ba/F3 cells were starved for 4 hours in RPMI-1640 medium without serum and cytokines. Treatment of cells with sodium pervanadate for 15 minutes was used as positive control for full kinase activation [24]. Cells were stimulated with 100 ng/mL FL (Prospec Tany) for the indicated periods of time at 37°C, washed once with cold phosphate-buffered saline and lysed. Lysates were subjected to immunoprecipitation and Western blotting as described elsewhere [23,25]. Immunodetection was performed by enhanced chemoluminescence using Immobilon Western chemoluminescent horseradish peroxidase substrate (Millipore Corporation, Milford, MA, USA) and a CCD camera (LAS-3000, Fujifilm, Tokyo, Japan). Signal intensity was quantified by MultiGauge software (Fujifilm).

Statistical analysis

Nonparametric Kruskal-Wallis test was performed to observe the difference in each time points between wild-type Flt3, Flt3-ITD, and Flt3-D835Y mutated Ba/F3 cell lines. Post-hoc analysis using nonparametric unpaired *t*-test, Mann-Whitney test, was performed when the *p*-value were significant ($p \leq 0.05$) or close to significant ($p < 0.067$) level.

Results

Assessment of functionality and specificity of the phosphospecific antibodies against Flt3 and identification of the novel autophosphorylation sites Y726, Y793, and Y842 in living cells

COS-1 cells were used as a model for assessing the functionality and specificity of the phosphospecific antibodies. Cells were transfected with wild-type Flt3 and each corresponding Y-to-F mutant. Following starvation, cells were stimulated for 5 minutes with FL. Samples were immunoprecipitated with Flt3 antibody and subsequently analyzed by Western blotting with the corresponding affinity purified antibody. As controls for receptor activation and expression, the membranes were stripped and reprobed with antibodies against phosphotyrosine (4G10) and Flt3. Thus, the kinase activity of Flt3 was intact in the individual mutants. Results using the Flt3 antibodies against pY589, pY591, pY599, pY726, pY793, and pY842 are shown in Figure 1. In addition to the previously identified phosphorylation sites pY589; pY591; pY599; pY768; pY955; and pY969 [26,27], we demonstrate three novel phosphorylation sites pY726; pY793; and pY842.

Comparison of the site-specific activation kinetics between the wild-type Flt3, ITD, and D835Y receptors

Depending on several factors, such as ligand, signal strength, and duration of activation, receptor tyrosine kinases often show different patterns of autophosphorylation and activation of downstream targets [28–30]. Correspondingly transfected Ba/F3 cells were used in order to study the kinetic response for the different phosphorylation sites in Flt3 between wild-type, ITD, and D835Y receptors. After starvation, cells were stimulated with FL for 0, 2, 5, 15, and 30 minutes. Western blot analysis with phosphospecific antibodies was used for comparison between the receptors (Fig. 2). As positive control for the tyrosine phosphorylation and functionality of the antibodies, cells were treated with sodium pervanadate in parallel, which induces global tyrosine phosphorylation in a nonspecific manner [31]. For most of the tyrosine phosphorylation sites in the wild-type receptor, there was a rapid response to FL and the signal decreased after 5 to 15 minutes, also reflected by the analysis of total tyrosine

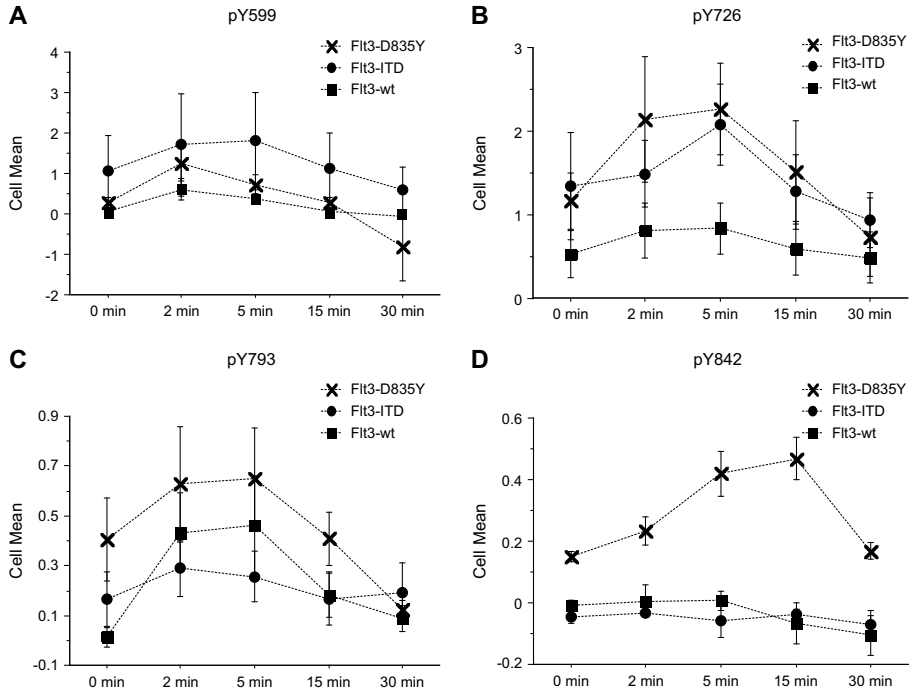

Figure 3. Statistical analysis of the kinetics and specificity of phosphorylation between wild-type Fms-like tyrosine kinase 3 (Flt3), Flt3-internal tandem duplication (ITD), and FLT3-D835Y. Ba/F3 cells expressing wild-type Flt3, Flt3-ITD, and Flt3-D835Y were stimulated with Flt3 ligand (FL) for 0, 2, 5, 15, and 30 minutes. Western blotting analysis was performed with phosphospecific antibodies against individual sites of Flt3, and with anti-phosphotyrosine antibody and anti-Flt3 antibody. Signals were quantified and normalized to the total Flt3 expression level; data obtained were analyzed statistically. Representative graphs shows results obtained for pY599 (**A**), pY726 (**B**), pY793 (**C**), and pY842 (**D**).

phosphorylation. The signals were prolonged and enhanced in both Flt3-ITD and D835Y, however, with somewhat different site specificity. Y589, Y591, Y599, and Y726 were highly phosphorylated in the Flt3-ITD receptor and Y768, Y793, and Y842 in the Flt3-D835Y receptor. In order to investigate the different phosphorylation patterns for each specific site of Flt3, signals were quantified and normalized to the total Flt3 expression levels and acquired data were statistically analyzed. Compared to wild-type Flt3, Y591 showed significant phosphorylation in Flt3-ITD ($p \leq 0.05$) and Flt3-D835Y ($p \leq 0.05$) in the absence of ligand stimulation. However, this significant difference was only present up to 2 and 5 minutes of stimulation with FL, and not present after 15 and 30 minutes of stimulation (data not shown). Y599 (Fig. 3A) and Y726 (Fig. 3B) showed no significant difference in phosphorylation between these three receptor versions at any time point. Y589 also showed no significant difference in phosphorylation except after 30 minutes of stimulation with FL, where ITD was significantly phosphorylated ($p \leq 0.05$) as compared to wild-type and D835Y (data not shown). Moreover, similar to Y591, Y768 showed significant phosphorylation in Flt3-ITD ($p \leq 0.05$) and D835Y ($p \leq 0.05$) in the absence of ligand stimulation as compared to wild-type Flt3, but no significant difference was observed at any time point within the groups after stimulation with FL (data not shown). Y793 showed significant phosphorylation in Flt3-D835Y ($p \leq 0.05$) compared to wild-type Flt3 only, but not to ITD when unstimulated (Fig. 3C). In addition, Y842 showed significant phosphorylation both in the absence or presence of FL and at all time points in Flt3-D835Y ($p \leq 0.05$) compared both to wild-type Flt3 and ITD (Fig. 3D). For a summary of our findings on site-specific phosphorylation in Flt3 wild-type, ITD, and D835Y, see Fig. 4.

Verification of the functionality of the phosphospecific antibodies in human AML cell lines

Because Flt3 is the most commonly mutated gene in AML, it is of high relevance to investigate the pattern of site-specific phosphorylation of Flt3 and its downstream signaling in human AML cell lines. For this purpose,

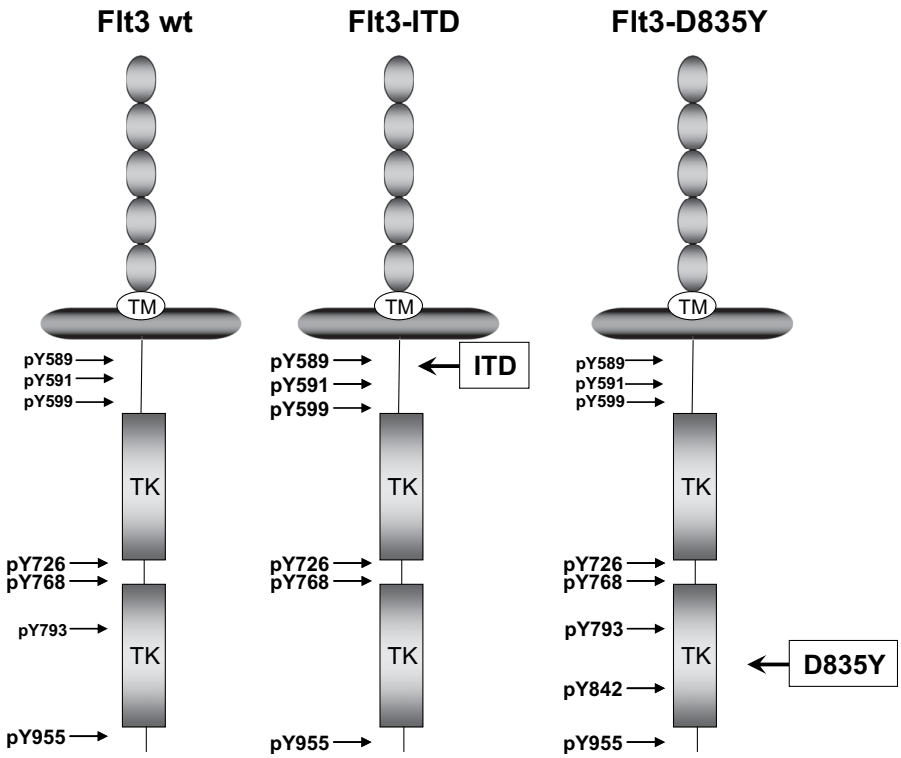

Figure 4. Schematic picture of the wild-type Fms-like tyrosine kinase 3 (Flt3), Flt3-internal tandem duplication (ITD), and FLT3-D835Y and their phosphorylation sites. Wild-type Flt3, Flt3-ITD, and Flt3-D835Y are pointed out as shown, and the different phosphorylation sites are indicated in the figure (the size indicates the phosphorylation ability). TM (transmembrane domain), TK (tyrosine kinase domain).

THP-1 and MV4-11, which have endogenous expression of wild-type Flt3 and Flt3-ITD, respectively, were used. Cells were stimulated for 5 minutes with FL followed by Western blot analysis using the different phosphospecific Flt3 antibodies. The total tyrosine phosphorylation levels as well as the total protein levels were detected using 4G10 and anti-Flt3 antibodies, respectively. THP-1 and MV4-11 cells show similar results with phosphospecific antibodies against tyrosine residues 589, 591, 599, 768, 726, 793, 842, and 955, as observed in Ba/F3 cells expressing wild-type Flt3 and Flt3-ITD, respectively (Fig. 5).

Discussion

Only a few phosphorylation sites of Flt3 have been identified to date, but many remain to be investigated. Single Y-to-F mutations of the cytoplasmic tyrosine residues of murine Flt3 have been analyzed in the context of wild-type versus corresponding D838 V kinase domain mutant of Flt3 [32]. In that study, tyrosines 845, 892, and 922 of the murine Flt3 proved to be critical for constitutive activation of the mutant receptor, suggesting that these residues are involved in stabilizing the active structure. However, they were not demonstrated to be phosphorylation sites. In addition, it has been shown that mutation of the critical aspartic acid residue to a valine residue is not equivalent to a tyrosine residue in terms of ability to confer cytokine-independent growth of transfected Ba/F3 cells and in terms of sensitivity to kinase inhibitors [33].

In the present study, we used phosphospecific antibodies to identify Y726, Y793, and Y842 as novel Flt3 phosphorylation sites. In addition, our group recently identified Y768, Y955, and Y969 as phosphorylation sites in living cells by use of phosphospecific Flt3 antibodies [27]. Previously, we also identified Y572, Y589, Y591, and Y599 of Flt3 to be phosphorylated in 32D cells transfected with wild-type Flt3 using two-dimensional phosphopeptide mapping combined with radio-Edman degradation [26]. In a study using a recombinant intracellular fragment of Flt3, several autophosphorylation site were identified in vitro by mass spectrometry, including Y589, Y591, Y726, Y842, Y955, and Y969 [34] (Table 1). However, in order

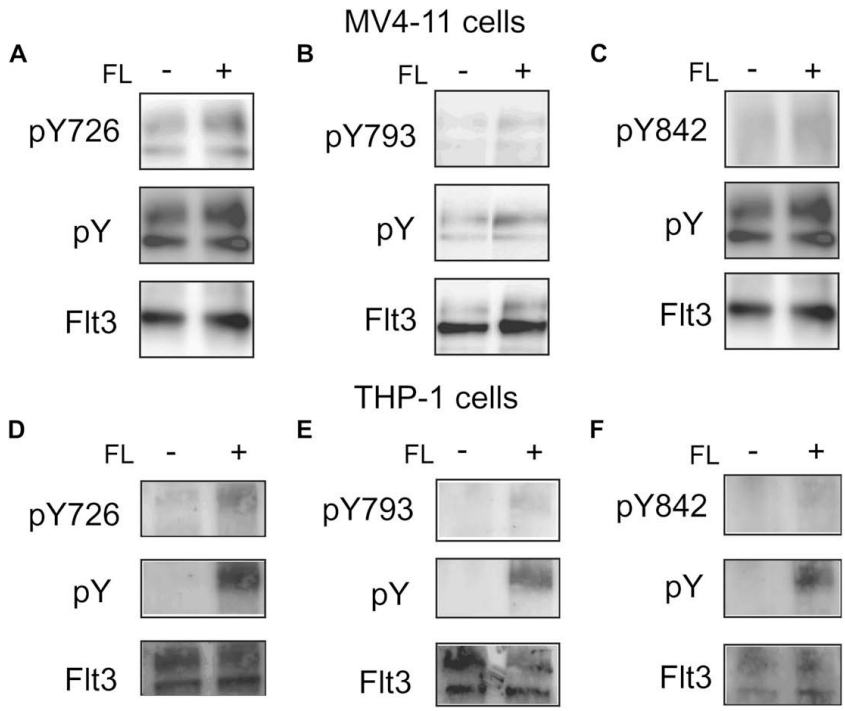

Figure 5. Verification of the phosphorylated tyrosine residues of Fms-like tyrosine kinase 3 (Flt3) in the acute myeloid leukemia cell lines THP-1 and MV4-11. MV4-11 and THP-1 cells were starved overnight and stimulated with Flt3 ligand (FL) for 5 minutes, lysed, and subjected to immunoprecipitation with anti-Flt3. Samples were analyzed by Western blotting and the membranes were probed with pY726 (**A**, **D**), pY793 (**B**, **E**), and pY842 (**C**, **F**). All membranes were reprobed with antibodies against phosphotyrosine (4G10, shown as pY in the figure) and total Flt3, as controls. The figure shows one representative experiment out of three.

to draw conclusions from these studies, one should consider that the different methods used to identify phosphorylation sites have their individual drawbacks as well as advantages. The receptor in its physiological context can use accessory tyrosine kinases both for its own phosphorylation and for downstream signaling, and thus in vitro approaches might miss some physiologically relevant sites. Use of phospho-specific antibodies is a relatively biased approach, but enables fast and reproducible detection of phosphorylation status. It allows rapid and sensitive analysis of cellular samples treated under different conditions. We have produced a panel of site-specific antibodies in our laboratory based on the fact that most of the known tyrosine phosphorylation sites in receptor tyrosine kinases reside outside the kinase domains (with exception of the activation loop tyrosines and some other rare cases) (for reviews see [35,36]). Thus, we generated phosphospecific antibodies against phosphorylated Y572, Y589, Y591, Y599, Y726, Y768, Y842, Y919, and Y955. Of these, all gave detectable phosphorylation in at least one of the three variants of Flt3, with the exception of Y572 and Y919 (data now shown). Y572 was identified as a phosphorylation site in 32D cells expressing wild-type Flt3 [26], but we failed to detect any signal in Ba/F3 cells expressing Flt3. This might be due to the presence or absence of a tyrosine kinase or protein tyrosine phosphatase in either cell type. Y919 is homologous to Y900 in c-KIT [37] and Y934 in the platelet-derived growth factor−α-receptor [38], which was identified as low stoichiometry phosphorylation sites whose phosphorylation was mediated by Src. However, we failed to detect phosphorylation of Y919 in Flt3, but we do not know whether this is due to a poor antibody or lack of surface exposure of the tyrosine residue (data not shown).

We have screened through wild-type Flt3 as well as the Flt3-ITD and Flt3-D835Y mutants, with regards to site-specific phosphorylation and kinetics. As expected, most sites were phosphorylated independently of FL in the mutants. Nevertheless, an enhanced response after stimulation with FL was observed with the mutants. Interestingly, several reports have shown that leukemic cell lines express Flt3 and FL simultaneously, which indicates

Table 1. Phosphorylation sites in Fms-like tyrosine kinase 3

Identified phosphorylation sites	In vitro	Intact cells
Y572	–	+ [26]
Y589	+ [34]	+ [26]
Y591	+ [34]	+ [26]
Y599	–	+ [26]
Y726	+ [34]	+*
Y768	–	+ [27]
Y793	–	+*
Y842	+ [34]	+*
Y955	+ [34]	+ [27]
Y969	+ [34]	+ [27]

Identified phosphorylation sites in vitro or in intact cells are indicated by "+" and the reference is given within brackets.
*Phosphorylation sites identified in this work.

a potential role for the ligand even when the receptor is constitutively activated [39]. In a study by Zheng et al. [40], primary AML blasts were screened for Flt3 and FL protein coexpression, suggesting autocrine or paracrine stimulatory loops, and further supporting our results.

The juxtamembrane tyrosines (Y589, Y591, and Y599) show a stronger and more prolonged phosphorylation in the ITD receptor, which at least in part might be a result of a duplication of these sites (the Flt3-ITD used in this study has a duplication containing Y589 and Y591; [41]). Y589 and Y591 have been identified as Src association sites [26] and involved in the activation of signal transducers and activators of transcription 5 (STAT5) [34]. Moreover, these two residues were shown to be crucial for the transforming potential of Flt3-ITD and D835Y as well as for the ligand-dependent activation of wild-type Flt3 [42]. Y599 was demonstrated to be involved in binding of the protein tyrosine phosphatase SHP2 [26]. Evidently, in addition to a quantitative, a qualitative difference in downstream signaling exists between wild-type and the Flt3-ITD receptors. Furthermore, in a microarray experiment, several genes were found to be selectively regulated by the ITD and not by the wild-type Flt3, e.g., Pim-2 and the STAT5 target genes Socs2 and Socs3. Additionally, Flt3-ITD repressed the expression of C/EBPα, a transcription factor essential for granulocytic differentiation and important roles in downregulating proteins such as CDK2/4 and c-Myc [43].

Tyrosine 726 is phosphorylated to a similar extent in wild-type Flt3 as in the mutants. However, potential interaction partners for this site have not yet been reported. When performing a database search, no characteristic binding motif was found from the surrounding sequence, and additional studies are required. We have previously shown that the autophosphorylation sites Y768, Y955, and Y969 are GRB2 binding sites [27]. Interestingly, these sites demonstrate a similar phosphorylation pattern in response to FL in both wild-type and mutant Flt3.

Strikingly, tyrosines 793 and 842 have a significantly more pronounced phosphorylation in the Flt3-D835Y, as compared to both the wild-type and ITD receptors. Both of these sites are located within the kinase domain and no interacting proteins have so far been identified.

The differences in phosphorylation sites between wild-type Flt3, Flt3-ITD, and Flt3-D835Y can have several explanations. The two activating mutations act through two distinctively different mechanisms. The Flt3-ITD mutation activates the kinase activity through releasing the negative constraint that the juxtamembrane region poses on the kinase domain of Flt3 [15]. Thus, the mutation does not involve any structural changes in the kinase domain. In contrast, the Flt3-D835Y mutation resides in the activation loop of the kinase domain. This mutation is thought to mimic the activated state of the kinase domain after ligand-induced activation. From the study of several other receptor tyrosine kinases, it is known that mutations in this region not only activate kinase activity, but also alter its specificity. Thus, in the case of RET, the M918 T mutation in the kinase domain of patients with multiple endocrine neoplasia 2B leads to constitutive activation of the kinase and a shift in specificity, which in turn leads to an altered pattern of autophosphorylation [44]. Likewise, the activating mutations D1246 N and D816 V in the kinase domain of MET and KIT (corresponding to D835 in Flt3) have also been demonstrated to lead to a shift in substrate specificity of the kinase [45,46].

Phosphorylation sites seen in living cells could be due to true autophosphorylation, i.e., a receptor phosphorylates another receptor in a cross-wise manner, but it could also be due, in part, to the activity of other tyrosine kinases activated downstream of the receptor (such as Src family kinases, Fes, etc.). This could explain the discrepancy between the sites found using recombinant Flt3 in vitro and the findings in living cells. Yet another factor that could contribute to the differences in phosphorylation sites seen in these experiments is the subcellular localization of the receptor. It is known that the Flt3-ITD, in contrast to wild-type Flt3 and Flt3-D835Y, is trapped in the endoplasmic reticulum and does not reach the cell surface to any appreciable extent [47]. Thus, the signal transduction molecules (including kinases and phosphatases) it encounters are likely to be different from those at the cell surface, and this could contribute to differences in phosphorylation seen in the different forms of the receptor.

Tyrosine kinase domain mutations of Flt3, such as D835Y, give rise to similar transforming abilities as Flt3-ITD, but the downstream signaling and its phenotype more resembles wild-type Flt3. This is also supported by the finding of clonogenic growth of 32D cells when transfected with Flt3-ITD, but not with wild-type Flt3 or Flt3-D835Y mutation [48]. Given the fact that both receptors are constitutively activated but give rise to different outcomes in terms of signaling and biological phenotype, these observations argue for qualitatively different downstream signaling patterns. One well-studied example is

STAT5, which is activated by the ITD but not the wild-type Flt3 or Flt3-D835Y [49]. Furthermore, the Flt3-ITD causes myeloproliferative disease in mouse models, while the Flt3-D835Y mutant does not [50]. Several clinical studies show that AML patients with ITD belong to a worse prognostic group than those with wild-type Flt3 or D835Y mutations [51]. In line with our findings, this indicates signaling differences between ITDs and D835Y of Flt3. As observed in the whole population of AML patients, about 7% of AML patients with Flt3-ITD also carry the D835Y mutation, indicating that the ITD and the D835Y mutant receptors are not functionally redundant [48].

Receptor tyrosine kinases commonly contain one or more tyrosine residues in the kinase activation loop, and the biological function of several receptor tyrosine kinases, including insulin-like growth factor−1 receptor [52], fibroblast growth factor receptor [53], and hepatocyte growth factor receptor (Met) [54], depends on the autophosphorylation of these residues. However, similar to our findings that the activation loop tyrosine 842 does not have a critical role in wild-type Flt3 receptor signaling, it has been reported that the corresponding Y823 site of the closely related c-KIT receptor was not required for the kinase activity of the receptor. Phosphorylation of this residue was secondary to phosphorylation of tyrosines 568 and 570 in the juxtamembrane region [55].

No potential interacting partners to Y842 of Flt3 have been suggested to this point, and there are a few examples from other studies where direct binding partners of an activation loop tyrosine have been found. GRB10 was found to interact with tyrosines 1162 and 1163 of the insulin receptor [56]. It was also shown that the adapter protein APS associates with the same phosphotyrosine residues within the activation loop of insulin receptor [57]. Another example of an SH2-domain protein interacting directly with the activation loop is the closely related SH2B, also identified as a substrate for insulin receptor, where mutation of the three activation loop tyrosines 1158, 1162, and 1163 abolished the observed interaction [58]. Moreover, SOCS-1 was shown to interact with JAK2 through binding of the SH2 domain to Y1007 on the activation loop, a process required for proteasomal degradation of JAK2 [59].

In conclusion, we have compared the site-specific phosphorylation patterns of wild-type Flt3, Flt3-ITD, and Flt3-D835Y in Ba/F3 cells using a panel of phosphospecific Flt3 antibodies. Interestingly, there were not only quantitative, but also qualitative differences between the receptors. Some sites were activated in similar fashion, whereas some sites were activated selectively either by the Flt3-ITD or the Flt3-D835Y mutant. As mutations in the Flt3 receptor are found to be a major cause of transformation in AML cells, several different Flt3 inhibitors have been tested in clinical trials [60], but more efficient and specific therapy is required. Future work will focus on the detailed downstream signaling from the newly identified sites presented here. The current findings contribute to the overall understanding of oncogenic Flt3 signaling and enable more specific approaches for targeting mutant receptors and their downstream signal transduction.

Acknowledgments

This work was supported by grants from the Swedish Cancer Society (Stockholm, Sweden), the Swedish Children's Cancer Society (Stockholm, Sweden), the Swedish Research Council (Stockholm, Sweden), the Knut and Alice Wallenberg Foundation (Stockholm, Sweden), Malmö University Hospital Foundation (Malmö, Sweden), Malmö University Hospital Cancer Foundation (Malmö, Sweden) and the Royal Physiographical Society (Lund, Sweden).

Conflict of Interest

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

References

1. Nakao M, Yokota S, Iwai T, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia. 1996;10:1911–1918.
2. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood. 2002;100:1532–1542.
3. Small D, Levenstein M, Kim E, et al. STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in CD34+ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. Proc Natl Acad Sci U S A. 1994;91:459–463.
4. Rosnet O, Marchetto S, deLapeyriere O, Birnbaum D. Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. Oncogene. 1991;6:1641–1650.
5. Rosnet O, Mattei MG, Marchetto S, Birnbaum D. Isolation and chromosomal localization of a novel FMS-like tyrosine kinase gene. Genomics. 1991;9:380–385.
6. Turner AM, Lin NL, Issarachai S, Lyman SD, Broudy VC. FLT3 receptor expression on the surface of normal and malignant human hematopoietic cells. Blood. 1996;88:3383–3390.
7. Brasel K, Escobar S, Anderberg R, de Vries P, Gruss HJ, Lyman SD. Expression of the flt3 receptor and its ligand on hematopoietic cells. Leukemia. 1995;9:1212–1218.
8. Kikushige Y, Yoshimoto G, Miyamoto T, et al. Human Flt3 is expressed at the hematopoietic stem cell and the granulocyte/macrophage progenitor stages to maintain cell survival. J Immunol. 2008; 180:7358–7367.
9. Maroc N, Rottapel R, Rosnet O, et al. Biochemical characterization and analysis of the transforming potential of the FLT3/FLK2 receptor tyrosine kinase. Oncogene. 1993;8:909–918.
10. Maraskovsky E, Brasel K, Teepe M, et al. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. J Exp Med. 1996;184:1953–1962.
11. Gilliland DG, Griffin JD. Role of FLT3 in leukemia. Curr Opin Hematol. 2002;9:274–281.
12. Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer. 2003;3:650–665.
13. Dosil M, Wang S, Lemischka IR. Mitogenic signalling and substrate specificity of the Flk2/Flt3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells. Mol Cell Biol. 1993;13:6572–6585.
14. Rosnet O, Bühring HJ, deLapeyriere O, et al. Expression and signal transduction of the FLT3 tyrosine kinase receptor. Acta Haematol. 1996;95:218–223.

15. Griffith J, Black J, Faerman C, et al. The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. Mol Cell. 2004;13:169–178.
16. Kiyoi H, Ohno R, Ueda R, Saito H, Naoe T. Mechanism of constitutive activation of FLT3 with internal tandem duplication in the juxtamembrane domain. Oncogene. 2002;21:2555–2563.
17. Yamamoto Y, Kiyoi H, Nakano Y, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001;97:2434–2439.
18. Kiyoi H, Towatari M, Yokota S, et al. Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. Leukemia. 1998;12:1333–1337.
19. Beghini A, Peterlongo P, Ripamonti CB, et al. C-kit mutations in core binding factor leukemias. Blood. 2000;95:726–727.
20. Jeffers M, Schmidt L, Nakaigawa N, et al. Activating mutations for the met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci U S A. 1997;94:11445–11450.
21. Liu X, Vega QC, Decker RA, Pandey A, Worby CA, Dixon JE. Oncogenic RET receptors display different autophosphorylation sites and substrate binding specificities. J Biol Chem. 1996;271: 5309–5312.
22. Blume-Jensen P, Siegbahn A, Stabel S, Heldin CH, Rönnstrand L. Increased Kit/SCF receptor induced mitogenicity but abolished cell motility after inhibition of protein kinase C. EMBO J. 1993;12: 4199–4209.
23. Voytyuk O, Lennartsson J, Mogi A, et al. Src family kinases are involved in the differential signaling from two splice forms of c-Kit. J Biol Chem. 2003;278:9159–9166.
24. Pumiglia KM, Lau LF, Huang CK, Burroughs S, Feinstein MB. Activation of signal transduction in platelets by the tyrosine phosphatase inhibitor pervanadate (vanadyl hydroperoxide). Biochem J. 1992; 286(Pt 2):441–449.
25. Lennartsson J, Blume-Jensen P, Hermanson M, Pontén E, Carlberg M, Rönnstrand L. Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and c-fos induction. Oncogene. 1999;18:5546–5553.
26. Heiss E, Masson K, Sundberg C, et al. Identification of Y589 and Y599 in the juxtamembrane domain of Flt3 as ligand-induced autophosphorylation sites involved in binding of Src family kinases and the protein tyrosine phosphatase SHP2. Blood. 2006;108: 1542–1550.
27. Masson K, Liu T, Khan R, Sun J, Rönnstrand L. A role of Gab2 association in Flt3 ITD mediated STAT5 phosphorylation and cell survival. Br J Haematol. In press (2009).
28. Sweeney C, Lai C, Riese DJ 2nd, Diamonti AJ, Cantley LC, Carraway KL 3rd. Ligand discrimination in signaling through an ErbB4 receptor homodimer. J Biol Chem. 2000;275:19803–19807.
29. Schmidt MH, Furnari FB, Cavenee WK, Bogler O. Epidermal growth factor receptor signaling intensity determines intracellular protein interactions, ubiquitination, and internalization. Proc Natl Acad Sci U S A. 2003;100:6505–6510.
30. Lundin L, Rönnstrand L, Cross M, Hellberg C, Lindahl U, Claesson-Welsh L. Differential tyrosine phosphorylation of fibroblast growth factor (FGF) receptor-1 and receptor proximal signal transduction in response to FGF-2 and heparin. Exp Cell Res. 2003;287:190–198.
31. Horion J, Gloire G, El Mjiyad N, et al. Histone deacetylase inhibitor trichostatin A sustains sodium pervanadate-induced NF-kappaB activation by delaying IkappaBalpha mRNA resynthesis: comparison with tumor necrosis factor alpha. J Biol Chem. 2007;282:15383–15393.
32. Ishiko J, Mizuki M, Matsumura I, et al. Roles of tyrosine residues 845, 892 and 922 in constitutive activation of murine FLT3 kinase domain mutant. Oncogene. 2005;24:8144–8153.
33. Clark JJ, Cools J, Curley DP, et al. Variable sensitivity of FLT3 activation loop mutations to the small molecule tyrosine kinase inhibitor MLN518. Blood. 2004;104:2867–2872.
34. Rocnik JL, Okabe R, Yu JC, et al. Roles of tyrosine 589 and 591 in STAT5 activation and transformation mediated by FLT3-ITD. Blood. 2006;108:1339–1345.
35. Heldin CH, Östman A, Rönnstrand L. Signal transduction via platelet-derived growth factor receptors. Biochim Biophys Acta. 1998;1378: F79–F113.
36. Ronnstrand L. Signal transduction via the stem cell factor receptor/c-Kit. Cell Mol Life Sci. 2004;61:2535–2548.
37. Lennartsson J, Wernstedt C, Engström U, Hellman U, Rönnstrand L. Identification of Tyr900 in the kinase domain of c-Kit as a Src-dependent phosphorylation site mediating interaction with c-Crk. Exp Cell Res. 2003;288:110–118.
38. Hansen K, Johnell M, Siegbahn A, et al. Mutation of a Src phosphorylation site in the PDGF beta-receptor leads to increased PDGF-stimulated chemotaxis but decreased mitogenesis. EMBO J. 1996; 15:5299–5313.
39. Quentmeier H, Reinhardt J, Zaborski M, Drexler HG. FLT3 mutations in acute myeloid leukemia cell lines. Leukemia. 2003;17:120–124.
40. Zheng R, Levis M, Piloto O, et al. FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. Blood. 2004;103: 267–274.
41. Kelly LM, Liu Q, Kutok JL, Williams IR, Boulton CL, Gilliland DG. FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. Blood. 2002;99:310–318.
42. Vempati S, Reindl C, Wolf U, et al. Transformation by oncogenic mutants and ligand-dependent activation of FLT3 wild-type requires the tyrosine residues 589 and 591. Clin Cancer Res. 2008;14:4437–4445.
43. Mizuki M, Schwäble J, Steur C, et al. Suppression of myeloid transcription factors and induction of STAT response genes by AML-specific Flt3 mutations. Blood. 2003;101:3164–3173.
44. Santoro M, Carlomagno F, Romano A, et al. Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. Science. 1995;267:381–383.
45. Miller M, Ginalski K, Lesyng B, Nakaigawa N, Schmidt L, Zbar B. Structural basis of oncogenic activation caused by point mutations in the kinase domain of the MET proto-oncogene: modeling studies. Proteins. 2001;44:32–43.
46. Sun J, Pedersen M, Rönnstrand L. The D816V mutation of c-Kit circumvents a requirement for Src family kinases in c-Kit signal transduction. J Biol Chem. 2009;24(284):11039–11047.
47. Schmidt-Arras D, Böhmer SA, Koch S, et al. Anchoring of FLT3 in the endoplasmic reticulum alters signaling quality. Blood. 2009;113: 3568–3576.
48. Choudhary C, Schwäble J, Brandts C, et al. AML-associated Flt3 kinase domain mutations show signal transduction differences compared with Flt3 ITD mutations. Blood. 2005;106:265–273.
49. Mizuki M, Fenski R, Halfter H, et al. Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. Blood. 2000;96: 3907–3914.
50. Grundler R, Miething C, Thiede C, Peschel C, Duyster J. FLT3-ITD and tyrosine kinase domain mutants induce 2 distinct phenotypes in a murine bone marrow transplantation model. Blood. 2005;105: 4792–4799.
51. Levis M, Small D. FLT3: ITDoes matter in leukemia. Leukemia. 2003; 17:1738–1752.
52. Kato H, Faria TN, Stannard B, Roberts CT Jr, LeRoith D. Essential role of tyrosine residues 1131, 1135, and 1136 of the insulin-like growth factor-I (IGF-I) receptor in IGF-I action. Mol Endocrinol. 1994;8:40–50.
53. Mohammadi M, Schlessinger J, Hubbard SR. Structure of the FGF receptor tyrosine kinase domain reveals a novel autoinhibitory mechanism. Cell. 1996;86:577–587.

54. Longati P, Bardelli A, Ponzetto C, Naldini L, Comoglio PM. Tyrosines1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). Oncogene. 1994;9: 49–57.
55. Mol CD, Lim KB, Sridhar V, et al. Structure of a c-kit product complex reveals the basis for kinase transactivation. J Biol Chem. 2003;278:31461–31464.
56. Dong LQ, Farris S, Christal J, Liu F. Site-directed mutagenesis and yeast two-hybrid studies of the insulin and insulin-like growth factor-1 receptors: the Src homology-2 domain-containing protein hGrb10 binds to the autophosphorylated tyrosine residues in the kinase domain of the insulin receptor. Mol Endocrinol. 1997;11: 1757–1765.
57. Moodie SA, Alleman-Sposeto J, Gustafson TA. Identification of the APS protein as a novel insulin receptor substrate. J Biol Chem. 1999;274:11186–11193.
58. Kotani K, Wilden P, Pillay TS. SH2-Balpha is an insulin-receptor adapter protein and substrate that interacts with the activation loop of the insulin-receptor kinase. Biochem J. 1998;335(Pt 1): 103–109.
59. Ungureanu D, Saharinen P, Junttila I, Hilton DJ, Silvennoinen O. Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1. Mol Cell Biol. 2002;22: 3316–3326.
60. Small D. FLT3 mutations: biology and treatment. Hematology Am Soc Hematol Educ Program. 2006;178–184.

Paper II

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 13, pp. 10918–10929, April 1, 2011

Protein-tyrosine Phosphatase DEP-1 Controls Receptor Tyrosine Kinase FLT3 Signaling*[S]

Received for publication, November 22, 2010, and in revised form, January 21, 2011 Published, JBC Papers in Press, January 24, 2011, DOI 10.1074/jbc.M110.205021

Deepika Arora[‡], Sabine Stopp[‡], Sylvia-Annette Böhmer[‡], Julia Schons[‡], Rinesh Godfrey[‡], Kristina Masson[§], Elena Razumovskaya[§], Lars Rönnstrand[§], Simone Tänzer[¶], Reinhard Bauer[‡], Frank-D. Böhmer[‡], and Jörg P. Müller[‡1]

From the [‡]Institute of Molecular Cell Biology, Center for Molecular Biomedicine, Friedrich Schiller University, D-07745 Jena, Germany, the [§]Experimental Clinical Chemistry, Department of Laboratory Medicine, Lund University, Malmö University Hospital, SE-20502 Malmö, Sweden, and the [¶]Research Group Immunology, Leibniz-Institute for Age Research-Fritz-Lipmann-Institute, D-07745 Jena, Germany

Fms-like tyrosine kinase 3 (FLT3) plays an important role in hematopoietic differentiation, and constitutively active FLT3 mutant proteins contribute to the development of acute myeloid leukemia. Little is known about the protein-tyrosine phosphatases (PTP) affecting the signaling activity of FLT3. To identify such PTP, myeloid cells expressing wild type FLT3 were infected with a panel of lentiviral pseudotypes carrying shRNA expression cassettes targeting different PTP. Out of 20 PTP tested, expressed in hematopoietic cells, or presumed to be involved in oncogenesis or tumor suppression, DEP-1 (PTPRJ) was identified as a PTP negatively regulating FLT3 phosphorylation and signaling. Stable 32D myeloid cell lines with strongly reduced DEP-1 levels showed site-selective hyperphosphorylation of FLT3. In particular, the sites pTyr-589, pTyr-591, and pTyr-842 involved in the FLT3 ligand (FL)-mediated activation of FLT3 were hyperphosphorylated the most. Similarly, acute depletion of DEP-1 in the human AML cell line THP-1 caused elevated FLT3 phosphorylation. Direct interaction of DEP-1 and FLT3 was demonstrated by "substrate trapping" experiments showing association of DEP-1 D1205A or C1239S mutant proteins with FLT3 by co-immunoprecipitation. Moreover, activated FLT3 could be dephosphorylated by recombinant DEP-1 *in vitro*. Enhanced FLT3 phosphorylation in DEP-1-depleted cells was accompanied by enhanced FLT3-dependent activation of ERK and cell proliferation. Stable overexpression of DEP-1 in 32D cells and transient overexpression with FLT3 in HEK293 cells resulted in reduction of FL-mediated FLT3 signaling activity. Furthermore, FL-stimulated colony formation of 32D cells expressing FLT3 in methylcellulose was induced in response to shRNA-mediated DEP-1 knockdown. This transforming effect of DEP-1 knockdown was consistent with a moderately increased activation of STAT5 upon FL stimulation but did not translate into myeloproliferative disease formation in the 32D-C3H/HeJ mouse model. The data indicate that DEP-1 is negatively regulating FLT3 signaling activity and that its loss may contribute to but is not sufficient for leukemogenic cell transformation.

Fms-like tyrosine kinase 3 (FLT3) is a class III receptor tyrosine kinase and plays a role in proliferation and differentiation of B-cell progenitors, myelomonocytic and dendritic cells, as well as in the maintenance of pluripotent hematopoietic stem cells (reviewed in Refs. 1, 2). Recently, FLT3 has received much attention as an important oncoprotein. Mutations in FLT3 that lead to constitutive activation are among the most common molecular lesions found in acute myeloid leukemia (AML)[2] (3–5). Prevalent mutations result in internal tandem duplications (ITD) of amino acid stretches in the juxtamembrane domain of FLT3 (6) and in the N-terminal region of the kinase domain (7, 8). FLT3-ITD is constitutively active, can transform myeloid cell lines *in vitro* (9), and can induce a myeloproliferative disease when retrovirally transduced into primary murine bone marrow cells (10). Wild type FLT3 and FLT3-ITD exhibit qualitative differences in signal transduction. The wild type receptor signals via the PI3K/AKT and the RAS/ERK pathways, whereas FLT3-ITD can activate additional pathways, notably phosphorylation of STAT5 (reviewed in Ref. 11). Altered signaling quality is at least partially mediated by retention of the constitutive active receptor in an intracellular compartment (12–14).

Phosphorylation of wild type FLT3 and AML-associated mutant FLT3 was recently analyzed using site-specific phosphotyrosine antibodies (15). Interestingly, the phosphorylation pattern of the different FLT3 variants showed quantitative and also qualitative differences. Although FLT3-ITD or mutations in the kinase domain resulted in ligand-independent FLT3 autophosphorylation and signaling activity, the wild type receptor is only autophosphorylated in response to stimulation with its cytokine FL.

Signaling of receptor tyrosine kinases is modulated by protein-tyrosine phosphatases (PTP) (16), and aberrations in PTP function play a role in carcinogenesis (17). Some PTP, notably SHP-2, have been found to positively influence growth-stimulatory signaling pathways, and mutations leading to gain-of-function of these PTP can potentially be oncogenic. It has been demonstrated that SHP-2 directly interacts with FLT3 in a phosphorylation-dependent manner via phosphotyrosine 599.

* This work was supported by European Community Grant "PTP-NET" Marie Curie Network MRTN-CT-2006-035830 and Deutsche Krebshilfe Collaborative Grant 108401, TP2 (to F. D. B.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S7 and Table S1.

[1] To whom correspondence should be addressed. Tel.: 49-3641-9395634; Fax: 49-3641-9395602; E-mail: joerg.mueller2@med.uni-jena.de.

[2] The abbreviations used are: AML, acute myeloid leukemia; PTP, protein-tyrosine phosphatase; FL, FLT3 ligand; PLCγ, phospholipase Cγ; ITD, internal tandem duplication; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

SHP-2 contributes to FL-mediated ERK activation and proliferation (18, 19), but it appears dispensable for cell transformation by the FLT3-ITD mutant receptor (18). Little is known about PTP, which negatively regulate FLT3. Loss of such PTP could potentially also contribute to transformation of AML cells. Initial studies demonstrated that co-expression of FLT3 with the PTP SHP-1, PTP1B, and PTP-PEST leads to FLT3 dephosphorylation, suggesting an inhibitory function of these PTP for FLT3 signaling (14).

To further elucidate the function of PTP in FLT3 signaling, we have analyzed a panel of relevant PTP by using shRNA-mediated down-regulation in myeloid 32D cells expressing wild type FLT3 as a model system. Stable down-regulation of the transmembrane PTP DEP-1/PTPRJ positively affected signaling of FLT3. In addition, we found that autophosphorylation of FLT3 as well as FL-stimulated cell proliferation were enhanced in response to DEP-1 depletion. Overexpression of DEP-1 inhibited FLT3 phosphorylation and signaling. Direct interaction studies using DEP-1 "trapping mutants" and dephosphorylation *in vitro* further supported that FLT3 is a *bona fide* substrate of DEP-1. Identification of DEP-1 as a negatively regulating PTP for FLT3 will enable analyzing a possible role in transformation of myeloid cells.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Antisera—The IL-3-dependent murine myeloid cell line 32D clone 3 (32D) (German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany) was maintained in RPMI 1640 medium supplemented with sodium pyruvate (5 mg/ml), 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mM), and 1 ng/ml IL-3 or conditioned medium obtained from murine IL-3 producing BPV cells (20). Cells were cultured in a humidified incubator at 37 °C with 5% CO_2. 32D cells stably expressing murine FLT3 wild type were kindly provided by Drs. R. Grundler and J. Duyster (Technical University Munich, Germany). Recombinant human FL and murine IL-3 were purchased from PeproTech Ltd., London, UK. Human THP-1 cells (DSMZ, Braunschweig, Germany) endogenously expressing wild type FLT3 were grown in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% heat-inactivated FCS. HEK293 cells were cultivated in DMEM/F-12 medium (1:1) (Invitrogen), supplemented with stabilized glutamine and 10% FCS.

Anti-P-AKT (Ser-473) (193H12), anti-AKT (catalog no. 9272), anti-P-p44/42 MAPK (Tyr-202/Tyr-204), anti-P-STAT5 (Tyr-694, EPITOMICS catalog no. 1208-1), and anti-STAT5 (catalog no. 9310) antibodies were from Cell Signaling Technology (Frankfurt, Germany). Anti-ERK1 (catalog no. M12320) was purchased from BD Transduction Laboratories. Polyclonal anti-FLT3 antibody (from goat, AF768) recognizing the extracellular domain of the murine protein, anti-DEP-1 antibody (from goat, AF1934) recognizing murine DEP-1 and cross-reacting with human DEP-1, and anti-CD45 antibody (from goat, AF114) were obtained from R&D Systems (Wiesbaden, Germany). Monoclonal antibody 143-41 against the human DEP-1 (CD148) and polyclonal antibodies recognizing Thr-202 of ERK1/2 (sc-101760) were from Santa Cruz Biotechnology. Human FLT3 was detected with polyclonal rabbit antibody C-20 obtained from Santa Cruz Biotechnology. Polyclonal rabbit ubiquitin antibody (U5379) was from Sigma.

For immunoprecipitation of human FLT3, S-18 polyclonal rabbit antibody from Santa Cruz Biotechnology was used, and for immunoprecipitation of murine FLT3, R&D antibody (AF768) was used. Phosphorylation-specific antibodies against individual tyrosine phosphorylation sites in FLT3 were raised by immunizing rabbits and purified as described earlier (15). 4G10 or pTyr-100 (I-9411) anti-phosphotyrosine antibodies were from Upstate Biotechnology, Inc. (Milton Keynes, UK) or Cell Signaling Technology (Frankfurt, Germany), respectively. Immunoprecipitation of DEP-1 was done with monoclonal mouse antibody 143-41 (sc-21761, Santa Cruz Biotechnology).

Antibodies recognizing β-actin or vinculin were obtained from Sigma. HRP-coupled secondary anti-mouse IgG and anti-rabbit IgG antibodies were from KPL (Gaithersburg, MD). HRP-coupled secondary anti-goat IgG (sc2056) was from Santa Cruz Biotechnology.

DNA Expression Vectors—Plasmid pLKO.1 vectors encoding shRNA constructs targeting murine PTP and plasmid pLKO.1 encoding a nontargeting control shRNA were obtained from Sigma (MISSION® shRNA lentivirus-mediated transduction system, Taufkirchen, Germany). If necessary, the puromycin resistance gene was replaced by a neomycin resistance-conferring gene using standard cloning techniques.

pMSCV-huFLT3-IRES-gfp plasmid encoding wild type human FLT3 was constructed by replacing SHP-2 (kindly provided by Drs. Golam Mohi and Benjamin Neel (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston)) by human FLT3 (9), kindly provided by Hubert Serve (Frankfurt, Germany). For expression of DEP-1, the sequence of human DEP-1 was cut out from pNRTIS33-DEP-1-WT (21) and inserted into the retroviral vector pMEX-IRES-puro (22).

Constructs for expression of GST-DEP-1 (wild type or C1239S cytoplasmic domain) fusion proteins in *E. coli* were described earlier (23). Expression in bacteria (strain *Escherichia coli* BL21) and purification of GST fusion proteins were performed by standard techniques. Constructs for transient expression of wild type DEP-1 or DEP-1 C1239S mutant were described previously (24). An expression construct for the DEP-1 D1205A trapping mutant was kindly provided by Dr. Tencho Tenev (The Breakthrough Breast Cancer Centre, Institute of Cancer Research, London, UK).

Small Interfering RNA (siRNA)-mediated Knockdown of Dep-1—Dep-1 siRNA duplex oligonucleotides (5′-UACUGUGUCUUGGAAUCUAdGdC-3′ (sense) and 5′-UAGAUUCCAAGACACAGUAdGdG-3′ (antisense)) or control siRNA (target DNA sequence AATTCTCCGAACGTGTCACGT; SI03650325) used in this study were obtained from Qiagen (Hilden, Germany). siRNA was transfected into THP-1 cells using AMAXA Nucleofector system, program version 01. 1.5×10^6 cells were transfected with 2.1 μg of siRNA using Nucleofector buffer V.

Production of Pseudoviral Particles and Cell Transduction—For generation of retroviral particles, Phoenix amphotropic packaging cells (kindly provided by Dr. Gary Nolan, Stanford, CA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FCS. The cells

were transiently transfected with the pMSCV-derivative plasmids using polyethyleneimine, and retrovirus-containing media were collected 48 h after transfection. 10^4 32D cells were infected three times with the pseudotyped particles in the presence of 8 μg/ml Polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide, AL-118, catalog no. 10,768-9, Sigma). The transduction efficiency was in the range of 10–30% as assessed by parallel transduction with GFP-expressing viral particles. Selecting the population of the transduced cell pool with 2 μg/ml puromycin was started 48 h after transduction. Sufficiently propagated cell pools ($5–10 \times 10^6$ cells) were subjected for phenotypic characterization immediately after establishment. In some cases, cells were sorted for equal FLT3 levels before analysis.

Production of lentiviral particles and transduction of 32D cells with corresponding cell supernatants was carried out essentially following the above protocol, except that Phoenix gp cells were used for virus particle production by transfecting them with pLKO.1 derivative plasmids in combination with pRev, pEnv-VSV-G, and pMDLg (kindly provided by Dr. Carol Stocking, Hamburg, Germany) (25, 26). Transduced cell pools were selected with 2 μg/ml puromycin. 10^4 32D cells were infected three times with the pseudotyped particles in the presence of 8 μg/ml Polybrene. The transduced cell pool was selected with 2 μg/ml puromycin or 400 μg/ml G418 48 h post-transduction and subjected to analysis. In some cases cells were sorted for equal FLT3 levels before analysis. Independent rounds for the knockdown of DEP-1 were performed and analyzed five times independently. In each round of transduction, the corresponding control shRNA cell pools were generated and analyzed in parallel.

Physical Association of DEP-1 with Wild Type FLT3—HEK293 cells were co-transfected with plasmids encoding DEP-1 WT, DEP-1 C1239S, or DEP-1 D1205A along with a plasmid encoding human FLT3 in the molar ratio 1:1 using polyethyleneimine. 36 h after transfection, cells were starved for 8 h and stimulated with 100 ng/ml FL for 5 min. Cells were then lysed, and immunoprecipitation of DEP-1 or FLT3 was carried out as described above.

DEP-1-mediated Dephosphorylation of FLT3—THP-1 cells were starved in cytokine-free RPMI 1640 medium containing 0.5% FCS and stimulated with FL (100 ng/ml) for 2.5 min. Subsequently, cells were washed once with ice-cold PBS and lysed. For FLT3 immunoprecipitation, lysis was done in 0.5% Nonidet P-40, 50 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM EDTA. Immunoprecipitated FLT3 was incubated with the amounts of purified GST fusion proteins of wild type or catalytically inactive C1239S DEP-1 catalytic domain indicated in the figure in PTP assay buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 2 mM EDTA, 5 mM DTT) at 30 °C for 30 min. The samples were subjected to SDS-PAGE, followed by immunoblotting. Dephosphorylation of FLT3 was analyzed by assessing the ratio of phosphorylated FLT3 (by using phosphotyrosine-specific antibody pTyr-Y100) to total FLT3 protein (detected by anti-FLT3 antibody C-20).

Assessment of Cell Proliferation and Clonal Growth in Methylcellulose—To assess proliferation of the different 32D cell pools, cells were washed twice in IL-3-free medium, seeded in RPMI 1640 medium containing 10% heat-inactivated FCS, and sodium pyruvate (5 mg/ml) in cytokine-free medium, or supplemented with FL or IL-3. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (27), 20,000 cells per well were seeded in a 96-well plate and incubated in specified growth conditions. MTT was added in the final concentration of 0.5 μg/μl. The formazan crystals were solubilized, and absorbance was measured at 570 nm. The assay was performed in triplicate. For GFP-based assays, 2×10^5 cells were seeded in 96-well plates (white plates with clear bottom). At the time points indicated in the figure legends, proliferation of quadruplicate samples was determined measuring GFP fluorescence intensity with a TECAN reader (Crailsheim, Germany).

To analyze colony formation of 32D cell lines, 3×10^4 cells were plated in 1 ml of a culture mixture containing Iscove's modified Dulbecco's medium (Invitrogen), 1% methylcellulose, and 20% FCS per well of a 6-well plate. All samples were prepared in triplicate. The plates were incubated at 37 °C, 5% CO_2 for the indicated time periods before counting and photographing representative sections of the wells. Photographs were taken with a Zeiss AxioCam HRC digital camera attached to a Zeiss Axiovert 25 inverted microscope.

Flow Cytometry and Cell Sorting—To obtain cell populations with a similar FLT3 protein level, cell pools were sorted for a similar GFP level using an ARIA FACS cell sorter (BD Biosciences). For detection of expression of human DEP-1, retrovirally transduced 32D cells were labeled with primary anti-DEP-1 antibody and subsequently co-stained with Cy3-conjugated anti-mouse secondary antibody. Overexpression of DEP-1 was analyzed with a FACS Canto cytometer (BD Biosciences) using FlowJo software (BD Biosciences). For isolation of 32D cells stably overexpressing huDEP-1, an ARIA FACS cell sorter was used.

Signaling Analysis and FLT3 Immunoprecipitation—32D cells were washed twice with PBS and starved in serum- and cytokine-free RPMI 1640 medium for 4 h before stimulation at 37 °C with 50 ng/ml FL or 1 ng/ml IL-3 for the time periods indicated. THP-1 cell were starved in cytokine-free RPMI 1640 medium containing 0.5% FCS. Subsequently, cells were washed once with ice-cold PBS and lysed. For FLT3 immunoprecipitation, lysis was done in 0.5% Nonidet P-40, 50 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM EDTA. For detection of activated signaling proteins in cell lysates, RIPA buffer containing 1% Nonidet P-40, 0.25% deoxycholate, 50 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA was used. Lysis buffers were freshly supplemented with proteinase inhibitors and phosphatase inhibitors (2 μg/ml leupeptin, 1% aprotinin, 2 μg/ml pepstatin A, 1 mM PMSF, 1 mM Pefabloc, 1 mM sodium orthovanadate, 1 mM glycerol phosphate), and lysis was allowed on ice for 15 min before thorough vortexing and centrifugation. FLT3 immunoprecipitations were performed by incubating with anti-FLT3 S-18 antibodies at 4 °C overnight, followed by incubation with protein A- or protein G-Sepharose beads. Adjusted aliquots of the cell extracts were subjected SDS-PAGE, followed by transfer to PVDF membranes (Millipore, Bedford, MA), and probing with the indicated primary antibodies. After subsequent incubation with horseradish peroxidase-conjugated secondary antibodies, the blots were developed using Western Lightning chemiluminescence detection (PerkinElmer Life Sciences) and quantita-

tively evaluated using a CCD camera-based system (LAS3000, Fuji). For quantification of specific phosphorylation, blots for phosphorylated proteins were stripped and subsequently reprobed with pan-specific antibodies. Specific phosphorylation was calculated as the ratio of the signals for phosphorylated protein to the signal for total protein detected. Values of the nonstimulated samples of cells expressing the control shRNA cells were set to 1.0.

Radiation-induced Apoptosis—A total of 10^6 cells were starved from cytokines and serum for 4 h, placed in 6-well plates, and exposed to 5 gray γ-irradiation. Immediately after irradiation, cells were supplemented with FL (50 ng/ml), IL-3 (1 ng/ml), or no cytokine with 10% heat-inactivated FCS. Cell viability was analyzed using the annexin-V-PE apoptosis assay using flow cytometry. Cells staining negative for both annexin-V and 7-aminoactinomycin D were counted as viable cells.

Animal Experiments—Eight- to 10-week-old male C3H/HeJ mice, which are syngeneic to 32Dcl3 cells, were used to assess the *in vivo* development of leukemia-like disease. 32D muFLT3 shDEP-1 A2/A3 cells (2×10^6) were injected into the lateral tail vein. The experimental protocols were reviewed and approved by the local Committee on Animal Experimentation.

RESULTS

Screening of PTP Affecting FLT3 Signaling in Hematopoietic Cells—To analyze the role of individual PTP on the activity of FLT3, we used 32D cells stably expressing FLT3 wild type protein (28). This murine bone marrow-derived cell line is strictly growth factor-dependent and therefore suitable to study receptor-mediated signaling and physiologic activity. A panel of 20 classical PTP was selected according to their known expression in 32D cells or generally in hematopoietic cells (29) or because of a presumed role in oncogenesis or tumor suppression (supplemental Table S1)(17).

shRNA expression cassettes were introduced in 32D FLT3 cells using the MISSION® shRNA lentivirus transduction system. Nonvalidated target sets of 4–5 shRNAs targeting each a particular PTP were packed into lentiviral pseudoparticles and transduced into 32D FLT3 cells. Transduced cell pools were selected by antibiotic treatment starting 2 days post-transduction and were subsequently expanded. Care was taken to ensure relatively high transduction efficiencies (10–30%) and to carry out analyses with cell pools that were only propagated for short periods to minimize clonal drifts. The effect of PTP knockdown on FLT3-mediated signaling quality was characterized by analyzing FL-mediated phosphorylation of ERK as a functional readout. Cells were starved in cytokine-free medium and subsequently stimulated with FL. Whole cell lysates of PTP-shRNA-expressing cells pools were immunoblotted with phospho-ERK antibodies, and its activation was scored. Although many shRNAs had little effect, several PTP-shRNAs caused altered ERK activation. These were subjected to a second validation screen, repeating shRNA transduction and ERK activation measurements with independently derived cell pools. For some positively scoring PTP in the second round, knockdown was assessed by quantitative RT-PCR and immunoblotting (supplemental Table S1). Notably, two independent shRNAs targeting DEP-1 (further designated A2 and A3) reproducibly resulted in enhanced FLT3-mediated ERK phosphorylation (supplemental Fig. S1). To validate the knockdown, protein and mRNA levels of DEP-1 were analyzed. As indicated in Fig. 1*A*, DEP-1 protein was reduced to 7 or 38% using shRNA targets A2 or A3, respectively, as compared with cells expressing a nontargeting control shRNA (or nontransduced cells, supplemental Fig. S2*A*). DEP-1 mRNA expression was similarly reduced to 9 or 38%, respectively (Fig. 1*B*). Depletion of CD45 in 32D FLT3 cells also enhanced FL-stimulated phosphorylation of ERK1/2, indicating that this PTP may likewise interfere with FLT3 signaling (supplemental Table S1). A closer investigation of this finding revealed, however, that FLT3 surface expression was elevated in CD45-depleted cells by unknown mechanisms (data not shown). Therefore, the role of CD45 was not further investigated at this point. To analyze if DEP-1 depletion would lead to alterations in CD45 levels, the latter were checked in DEP-1-depleted cell pools. CD45 protein and mRNA levels were, however, unaffected by DEP-1 depletion (supplemental Fig. S2, *A* and *C*).

We considered the possibility that PTP depletion would affect signaling of FLT3-ITD, the transforming mutant version of FLT3. shRNA-mediated down-regulation of DEP-1 did not, however, alter the autophosphorylation of FLT3-ITD nor the magnitude of constitutive or FL-stimulated FLT3-ITD signaling (data not shown). It is possible that FLT3-ITD signaling can override the activity of DEP-1, and therefore DEP-1 depletion has no further consequences in FLT3-ITD-expressing cells.

Depletion of DEP-1 Stimulates FLT3 Signaling in Myeloid Cells—Subsequently, the effect of DEP-1 depletion on FLT3 signaling activity was characterized. Major signaling pathways downstream of the FLT3 wild type receptor are the PI3K/AKT and the RAS/ERK pathways. Therefore, phosphorylation of AKT and ERK was monitored. Enhanced phosphorylation of ERK in DEP-1-depleted cells lines with either shRNA A2 or A3 confirmed the screening results. Phosphorylation of ERK was enhanced reproducibly. In contrast, FL-mediated activation of AKT remained unchanged in response to DEP-1 knockdown as compared with control cells (Fig. 1*C*). Interestingly, the kinetics of signaling of control shRNA and DEP-1-depleted 32D FLT3 cell lines was not different (Fig. 1*C*). Additionally, we analyzed FL-mediated activation of STAT5. Although STAT5 is known to be not activated or only weakly activated by ligand-stimulated wild type receptor, oncogenic FLT3 variants mediate constitutive activation of STAT5 (9, 28, 30). Depletion of DEP-1 resulted in a slight but reproducibly elevated STAT5 phosphorylation in cells containing shRNA A2, which caused the more pronounced DEP-1 depletion (Fig. 1*D*). No significant alteration of STAT5 phosphorylation could, however, be observed in cells expressing DEP-1 shRNA A3 (data not shown).

Sacco *et al.* (31) have recently demonstrated that DEP-1 can directly regulate ERK activity by dephosphorylating pTyr in the pThr-202–Glu–pTyr-204 motif of the activation loop (numbering according to the human ERK1 protein). The pERK1/2 antibody employed for our screening and the experiments described above recognizes both phosphorylated Thr-202 and pTyr-204 with an unknown contribution of the two single sites to the combined signal intensity. Therefore, increased ERK1/2

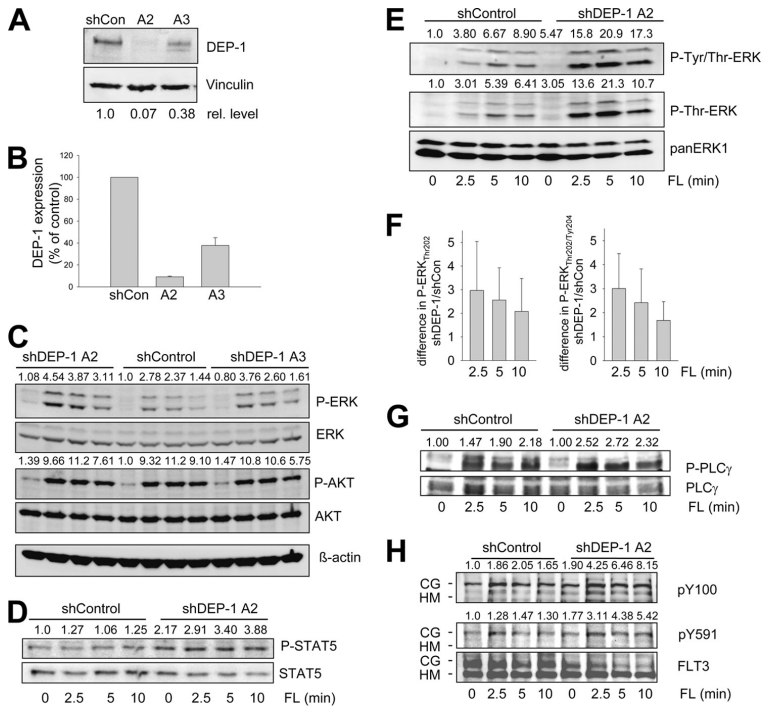

FIGURE 1. **Down-regulation of DEP-1 in 32D cells results in enhanced FLT3 signaling and enhanced phosphorylation of FLT3.** *A*, whole cell lysates of 32D cells expressing wild type muFLT3 and DEP-1-specific shRNA (targets A2 or A3) or a nontargeting control shRNA were subjected to SDS-PAGE, blotted to a PVDF membrane, and analyzed with antibodies recognizing DEP-1 and vinculin. Chemiluminescence signals were detected using a CCD camera-based chemiluminescence detection system and calculated relative to vinculin. Relative (*rel.*) levels of DEP-1 normalized to vinculin are indicated. *B*, quantitative RT-PCR for DEP-1 mRNA in the above cell lines. Mean ± S.D. of at least three independent experiments. *C–F*, indicated cell lines were starved for 4 h in serum- and cytokine-free medium and were stimulated with FL (50 ng/ml) for the indicated time periods. *C–H*, activation of ERK (*C* and *E*), AKT (*C*), STAT5 (*D*), or PLCγ (*G*) was analyzed using the indicated phospho-specific antibodies. Blots were reprobed for total signaling proteins. β-Actin was analyzed as loading control. *Numbers above* the phospho-specific blots represent quantification of the phosphor-specific signals, normalized to the corresponding signals with pan-specific antibodies, and relative to the signal in unstimulated cells harboring control shRNA, which was set to 1.0. The blots shown are representative for at least three experiments with consistent results. *F*, comparison of phosphorylation of ERK1/2 detected by conventional anti-pThr-202/pTyr-204 antibodies, and anti-pThr-202 antibodies. Ratio between ERK1/2 phosphorylation of shDEP-1 to shControl cells of three independent experiments is shown. *H*, FLT3 was immunoprecipitated and analyzed by immunoblotting with anti-FLT3, phosphotyrosine (*pY100*), or phospho-FLT3 (*pY591*) antibodies. *HM*, high mannose; *CG*, complex glycosylated FLT3. Quantification refers to complex glycosylated (surface) FLT3. Representative blots of at least three time repeated experiments are shown.

phosphorylation by DEP-1 knockdown in our experiments could potentially have been caused by reduced direct DEP-1 activity toward ERK1/2 pTyr-204. We reasoned that it should be possible to distinguish between direct dephosphorylation of ERK1/2 by DEP-1 or an event regulated by DEP-1 upstream of ERK1/2 by analyzing phosphorylation of ERK1/2 at Thr-202. Phosphothreonine 202 cannot serve as a direct substrate of DEP-1; therefore, the effects of DEP-1 knockdown on ERK1/2 pThr-202 could only be caused by upstream events. Indeed, depletion of DEP-1 caused stimulation of ERK1/2 Thr-202 phosphorylation to a comparable extent as the phosphorylation detected with the "conventional" antibody recognizing the doubly phosphorylated peptide (Fig. 1, *E* and *F*). We also analyzed the phosphorylation of PLCγ in response to FLT3 activation. This was likewise elevated in DEP-1-depleted 32D FLT3 cells compared with cells expressing the control shRNA (Fig. 1*G*).

Taken together, several signaling molecules downstream of FLT3 were found activated in DEP-1-depleted cells, including ERK1/2, PLCγ, and, weakly, STAT5. This observation and the finding of elevated ERK1/2 Thr-202 phosphorylation are consistent with a role of DEP-1 in attenuating an event proximal to receptor activation.

We therefore addressed the question whether FL-stimulated receptor phosphorylation was directly affected by DEP-1 depletion. Notably, FLT3 is detectable as a 130-kDa immature high mannose glycoprotein and as 150-kDa complex glycosylated protein (14). Only the latter form is accessible for FL at the cell surface, and consequently, only the 150-kDa band shows increased phosphorylation upon FL stimulation. Immunoblotting of immunoprecipitated FLT3 derived from DEP-1-depleted 32D cells with phosphotyrosine-specific antibodies revealed that total FLT3 phosphorylation was indeed somewhat elevated as compared with the FLT3 immunoprecipitated from control cells (Fig. 1*H*). Tyrosine residue 591 of FLT3 is known to be phosphorylated *in vivo* following ligand stimulation and has been identified as site contributing to Src binding and activation (19, 32). Phosphorylation of this specific tyrosine residue was also enhanced in cells where DEP-1 expression was

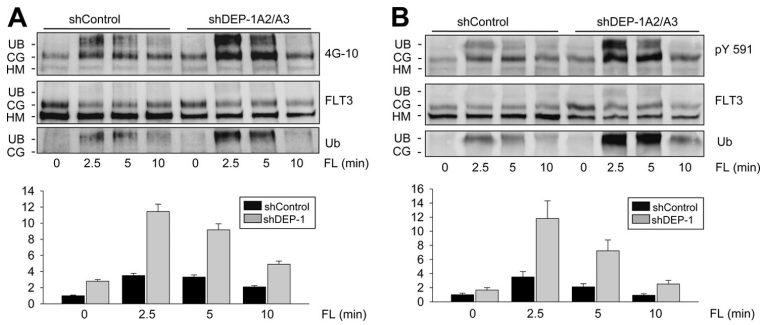

FIGURE 2. **Down-regulation of DEP-1 causes hyperphosphorylation of human FLT3.** 32D cells expressing human FLT3- and DEP-1-specific shRNAs A2 and A3 or a control shRNA were starved and stimulated with FL (50 ng/ml) for indicated time periods. FLT3 was immunoprecipitated and analyzed by immunoblotting with anti-FLT3 or phosphotyrosine (*4G-10*, *A*) or phospho-FLT3 (*pY591*, *B*) antibodies. *HM,* high mannose; *CG,* complex glycosylated FLT3; *UB*, ubiquitinated. *Graphs* demonstrate specific phosphorylation of total FLT3 receptor. Phosphospecific to total FLT3 receptor chemiluminescence signals are shown (sum of high mannose, complex glycosylated FLT3, and UB protein). Means ± S.D. are of at least three independent experiments.

down-regulated (Fig. 1*H*). The limited quality of muFLT3-specific antibodies, however, greatly limited analysis of FLT3 phosphorylation in these particular cells.

DEP-1 Affects Phosphorylation of FLT3 at Multiple Sites—To study DEP-1-mediated receptor dephosphorylation in more detail, we focused on cells expressing human FLT3. In this analysis, we took advantage of antibodies that are specific for individual FLT3 phosphorylation sites. These were previously used to comprehensively characterize the phosphorylation pattern of human FLT3 proteins in Ba/F3 cells (15). For this purpose, we generated 32D cells stably expressing the human FLT3 receptor following the previously described strategy of Grundler *et al.* (33). Human FLT3 encoding DNA was placed on a retroviral expression cassette upstream of a transcriptionally coupled GFP gene. 32D cells were transduced with corresponding pseudoviral particles, and cell populations with comparable GFP levels were obtained by sorting using flow cytometry. These cells were subsequently transduced with lentiviral particles encoding DEP-1-shRNA A2, A3, or nontargeting control shRNA. The obtained knockdown of DEP-1 was comparable with the one in 32D cells expressing the murine FLT3 receptor (supplemental Fig. S3). In addition, cells harboring both DEP-1-specific shRNAs were obtained by sequential transduction with DEP-1 A3 shRNA on a vector with puromycin resistance, followed by transduction of A2 shRNA on a vector allowing G418 selection. Transduction of cells with both shRNAs yielded a more pronounced down-regulation of DEP-1 (supplemental Fig. S3).

Initially, we analyzed total FLT3 tyrosine phosphorylation. FLT3 immunoprecipitated from FL-stimulated 32D cells expressing both shRNAs A2/A3 showed a more than 2-fold higher overall phosphorylation as compared with cells expressing the control shRNA (Fig. 2*A*). The kinetics of FL-mediated FLT3 phosphorylation was similar in both cell types as follows: 2.5 min after addition of the FL receptor, phosphorylation already reached a maximum, remained nearly stable until 5 min, and decreased to nearly control values 10 min after onset of stimulation. Noteworthy, a significant amount (up to 50%) of phosphorylated FLT3 molecules was detectable at a molecular mass above 200 kDa. Reprobing of the immunoblot with a specific antibody revealed ubiquitination of the receptor, whose change in intensity with time mirrored the kinetics of phosphorylation (Fig. 2*A*).

TABLE 1

Alteration of site-specific phosphorylation of human FLT3 in response to DEP-1 depletion

32D cells expressing human FLT3 and DEP-1-specific shRNAs A2 and A3 or a control shRNA were starved and stimulated with FL (50 ng/ml) for the indicated time periods. FLT3 was immunoprecipitated and analyzed by immunoblotting with site-specific phosphotyrosine FLT3 antibodies as indicated or phosphotyrosine antibody 4G-10. Total immunoprecipitated FLT3 was quantified using anti-FLT3 antibodies. Data represent the ratio of specific FLT3 phosphorylation to total receptor (sum of all of FLT3 forms, including ubiquitinated protein) of DEP-1-depleted cells divided by values obtained from control shRNA-expressing cells. The data are mean values of at least three independent experiments. Standard deviation was calculated using the ratios of three independent experiments. Because of the low phosphorylation of unstimulated samples, the calculation was error-prone and therefore excluded from the table.

	Specific phosphorylation (shDEP-1 to shControl)			
pY site	2.5	5	10	FL
				min
pY572	1.4 ± 0.3	1.4 ± 0.9	1.1 ± 0.2	
pY589	2.5 ± 0.2	2.2 ± 0.2	2.3 ± 0.1	
pY591	2.6 ± 0.9	2.6 ± 0.7	2.7 ± 0.4	
pY599	1.9 ± 0.7	2.4 ± 0.5	2.0 ± 0.5	
pY768	1.7 ± 0.6	1.6 ± 0.4	1.6 ± 0.1	
pY793	1.8 ± 0.4	1.9 ± 0.3	2.1 ± 0.3	
pY842	2.8 ± 0.5	2.3 ± 0.3	1.7 ± 0.6	
pY955	2.1 ± 0.9	2.2 ± 0.1	1.7 ± 0.3	
Total (4G10)	2.4 ± 0.7	2.4 ± 0.5	2.1 ± 0.6	

Next, FLT3 phosphorylation was studied in detail using site-specific antibodies. Antibodies recognizing the phosphorylation sites pTyr-572, pTyr-589, pTyr-591, pTyr-599, pTyr-768, pTyr-793, pTyr-842, and pTyr-955 have been described earlier and were all validated for specificity using appropriate tyrosine-mutant FLT3 versions (15, 19). Tyrosine phosphorylation at these positions was analyzed by immunoblotting of immunoprecipitated huFLT3. Multiple experiments were performed and quantified. An exemplary blot is shown for pTyr-591 in Fig. 2*B*. Example blots for the other pTyr sites used are shown in the supplemental Fig. S4, *A–G*. The stimulation of site-specific FLT3 tyrosine phosphorylation in the DEP-1-depleted cells compared with control cells is shown in Table 1. Values were normalized to pTyr of control cells.

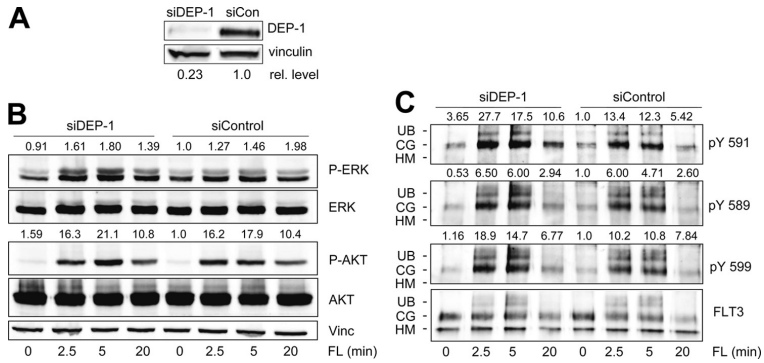

FIGURE 3. **Depletion of DEP-1 in THP-1 cells results in elevated FL-mediated signaling activity and FLT3 receptor phosphorylation.** Human THP-1 cells were transfected with a siRNA targeting DEP-1 (*siDEP-1*) or a nontargeting control siRNA (*siCon*). 48 h post-transfection, cells were starved and stimulated with FL (100 ng/ml) for the indicated time periods. Cells were lysed and subjected to SDS-PAGE and Western blotting. *A,* cell lysates were probed with antibodies recognizing DEP-1 and vinculin (*Vinc.*). Depletion of DEP-1 was quantified as ratio of DEP-1 to vinculin. Relative (*rel.*) levels of DEP-1, normalized to vinculin, are indicated. *B*, total cell lysates were probed with antibodies recognizing phosphorylated signaling proteins AKT or ERK. Blots were reprobed for total signaling proteins. *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. *HM,* high mannose; *CG,* complex glycosylated; *UB,* ubiquitinated FLT3. *Numbers above* the phosphotyrosine blots indicate specific phosphorylation of complex glycosylated FLT3. The blots are representative for at least four experiments.

In general, phosphorylation of all FLT3 tyrosine sites was elevated in the DEP-1 knockdown cells. This applied to the FL-stimulated receptor, but some enhanced specific phosphorylation was already visible in the unstimulated resting cells (Fig. 2, *A* and *B*; supplemental Fig. S4, *A–G*), suggesting that DEP-1 may interact with both unstimulated and ligand-stimulated FLT3. Kinetics of stimulation and decay of phosphorylated receptor remained unchanged upon DEP-1 depletion. The juxtamembrane tyrosine residues (Tyr-589, Tyr-591, and Tyr-599) showed a more pronounced elevation of phosphorylation in the DEP-1-depleted line. In addition, kinase domain-localized tyrosines Tyr-842 and Tyr-955 likewise showed a rather pronounced stimulation of FLT3 phosphorylation upon DEP-1 depletion. In contrast, phosphorylation of tyrosine residues Tyr-572, Tyr-768, and Tyr-793 was only moderately affected. To further link elevated FLT3 phosphorylation to the knockdown of DEP-1 expression, alteration of receptor phosphorylation was compared in cell lines expressing only shRNA A3, A2, or both. As shown in supplemental Fig. S5, enhancement of FLT3 phosphorylation occurred in all DEP-1-depleted cells, indicating that the effect is independent of the targeting shRNA sequence. Moreover, the stimulation of FLT3 phosphorylation was inversely correlated with the degree of DEP-1 down-regulation (supplemental Fig. S5), supporting the conclusion that DEP-1 depletion and FLT3 hyper-phosphorylation are directly linked.

To further characterize DEP-1 as a negative regulator of FLT3, we have chosen the human AML cell line THP-1 constitutively expressing wild type FLT3. For acute down-regulation of DEP-1, the cells were transiently transfected with a specific siRNA duplex. Using this technique, the most efficient DEP-1 knockdown was observed 48 h post-transfection. DEP-1 levels were reduced to 20–30% as compared with control cells (Fig. 3*A*). The effects of transient DEP-1 knockdown in this cell line were less pronounced than in 32D cells. However, analysis of downstream signals revealed that stimulation of FLT3 in DEP-1-depleted THP-1 cells weakly (about 1.5-fold) but reproducibly enhanced the activation of ERK as compared with the cells transfected with control siRNA, whereas activation of AKT remained unchanged (Fig. 3*B*). This was consistent with the findings in 32D cells. In addition, down-regulation of DEP-1 resulted in a slight elevation of phosphorylation of FLT3 autophosphorylation sites Tyr-589, Tyr-591, and Tyr-599 compared with control siRNA-transfected cells (Fig. 3*C*). 2.5 min after FL stimulation, phosphorylation of tyrosine Tyr-591 and Tyr-599 were 1.5–2-fold elevated in response to DEP-1 depletion.

Overexpression of DEP-1 Impairs FLT3 Receptor Phosphorylation and Activity—Because DEP-1 depletion led to FLT3 activation, overexpression of this phosphatase should result in inverse effects. To address this question, 32D cells expressing FLT3 were transduced with a retroviral expression cassette encoding DEP-1, and stable cell pools were selected and tested for elevated DEP-1 levels. As it turned out, initially detected DEP-1 overexpression was rather unstable and diminished rapidly. Therefore, a population of puromycin-resistant cells with significant synthesis of DEP-1 was enriched by sorting of immunofluorescence-labeled cells using a cell sorting system. These cells were briefly expanded and then analyzed immediately. As shown by immunoblotting of whole cell lysates and surface immunostaining with subsequent flow cytometry, the selected cells showed a significant overexpression of DEP-1 compared with control cells (Fig. 4, *A* and *B*). Assuming similar reactivity of the used antibody for (endogenous) murine and (exogenous) human DEP-1, calculations from immunoblotting data indicated an extent of overexpression of 8–10-fold in these sorted pools. 32D FLT3 cells overexpressing DEP-1 were characterized by reduced FL-mediated signaling activity (Fig. 4*C*). FL-mediated activation of ERK and AKT was drastically reduced in response to overexpression of DEP-1. This observation was different from the situation observed in DEP-1-depleted cells, where AKT remained unaffected. Moreover, gen-

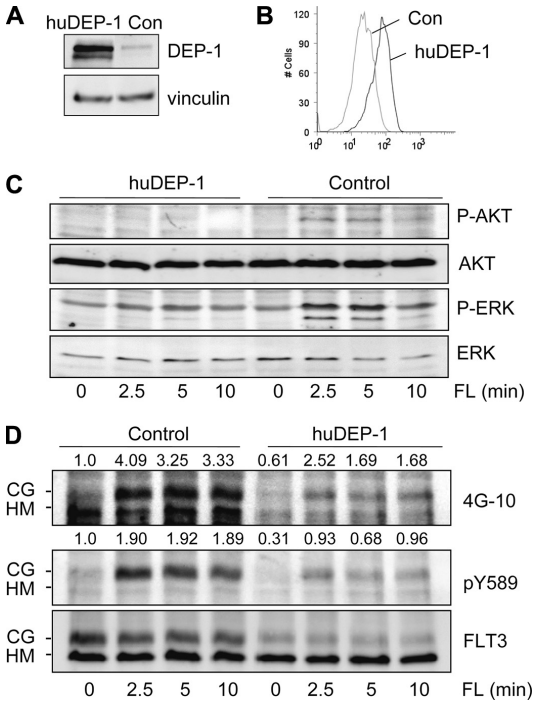

FIGURE 4. **Overexpression of DEP-1 impairs FLT3 signaling and phosphorylation.** 32D cells expressing huFLT3, stably transduced with an expression construct for human DEP-1 or the corresponding control vector, were analyzed for DEP-1 expression, FL-mediated signaling activity, and receptor phosphorylation. *A*, whole cell lysates were blotted with antibodies recognizing both endogenous murine and exogenous human DEP-1 and vinculin for control (*Con*). *B*, expression of transduced huDEP-1 was detected by labeling with a monoclonal antibody recognizing a surface epitope of human DEP-1 and subsequent labeling with anti-mouse-Cy3 antibody. Surface-localized DEP-1 was quantified by flow cytometry. *C* and *D*, indicated cell lines were starved for 4 h in serum- and cytokine-free medium and were stimulated with FL (50 ng/ml) for the indicated time periods. Whole cell lysates were blotted and activation of ERK or AKT was analyzed using phospho-specific antibodies. Blots were reprobed for total signaling proteins. *D*, phosphorylation of FLT3 was analyzed by immunoblotting with anti-phosphotyrosine antibody 4G-10 and anti-phospho FLT3 antibody pTyr-591, and blots were subsequently reprobed for total FLT3. *HM*, high mannose; *CG*, complex glycosylated FLT3. Representative blots of experiments repeated at least three time are demonstrated.

eral tyrosine phosphorylation (as shown using 4G-10 tyrosine-specific antibody), as well as specific phosphorylation of FLT3 (exemplified for the juxtamembrane tyrosine residue Tyr-589), was considerably reduced about 2-fold (Fig. 4*D*). Taken together, overexpression of DEP-1 in 32D cells resulted in impaired FLT3 phosphorylation and signaling activity.

DEP-1 Interacts Directly with FLT3—To elucidate the possibility that phosphorylated FLT3 serves as a direct substrate of DEP-1, we first analyzed the capacity of DEP-1 to dephosphorylate FLT3 *in vitro*. Phosphorylated FLT3 was immunoprecipitated from FL-stimulated THP-1 cells. Aliquots of immunoprecipitated FLT3 were then incubated with different amounts of recombinant GST-DEP-1 (catalytic domain) fusion proteins, and the dephosphorylation was subsequently assessed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 5*A*, catalytically active DEP-1 dephosphorylated FLT3 in a concentration-dependent manner. In contrast, incubation of FLT3 with catalytically inactive DEP-1 C1239S protein did not alter the phosphorylation status of FLT3.

In addition, dephosphorylation of FLT3 by DEP-1 was tested by co-expression experiments (Fig. 5*B*). FLT3 and DEP-1 were transiently expressed in HEK293 cells, and the cells were stimulated with FL, and FLT3 was immunoprecipitated and analyzed by immunoblotting. Overexpression of wild type DEP-1 strongly reduced FLT3 autophosphorylation, whereas the FLT3 phosphorylation status did not change upon co-expression of the inactive DEP-1 C1239S mutant. Interestingly, probing immunoprecipitated FLT3 for the presence of DEP-1 revealed an efficient association of the DEP-1 C1239S mutant protein but not of wild type DEP-1 with FLT3 (Fig. 5*B*).

Association of catalytically inactive PTP versions with their substrates is often comparatively stable, whereas the complex between active PTP and substrate rapidly decomposes because of efficient dephosphorylation. This phenomenon has been described as PTP "substrate trapping" (34). We also employed another DEP-1 substrate- trapping mutant DEP-1 D1205A, which has previously been used to find substrates (35). Again, a physical interaction of DEP-1 and FLT3 could be demonstrated by co-immunoprecipitation (Fig. 5*C, upper panel*). DEP-1 D1205A mutant protein efficiently co-immunoprecipitated with FLT3. FL-mediated stimulation of FLT3 phosphorylation even enhanced interaction of both proteins. Wild type DEP-1 was, however, not co-purified by FLT3, consistent with an effective dephosphorylation of FLT3 by DEP-1.

Reciprocal co-immunoprecipitation similarly revealed that both proteins interact directly. Although the wild type DEP-1 protein did not co-purify FLT3, DEP-1 D1205A efficiently co-immunoprecipitated FLT3. Again, co-purification of FLT3 was slightly elevated in response to ligand stimulation of FLT3 phosphorylation (Fig. 5*C*, *lower panel*). Interestingly, by probing immunoprecipitated DEP-1 with anti-phosphotyrosine antibodies, we could observe that the DEP-1 DA trapping mutant was phosphorylated by FLT3, indicating a mutual enzymatic interaction.

FLT3-ITD could be co-immunoprecipitated with DEP-1 C1239S protein with similar efficiency as wild type FLT3 (supplemental Fig. 6), excluding that the lack of an effect of DEP-1 knockdown on FLT3-ITD autophosphorylation (see above) was due to an inability of the mutant FLT3 for interaction with DEP-1.

Suppression of DEP-1 Stimulates FLT3-dependent Proliferation and Colony Formation of 32D Cells—To characterize the physiological consequences of altered FLT3 activity, first proliferation of 32D cell lines with shRNA-mediated DEP-1 knockdown was analyzed. Proliferation was measured using either MTT assays or measurement of GFP fluorescence, which is correlated with cell numbers. 32D muFLT3 cell lines remained cytokine-dependent, irrespective of DEP-1 depletion. No appreciable proliferation could be observed if cells were cultivated in the absence of cytokine. The FL-me-

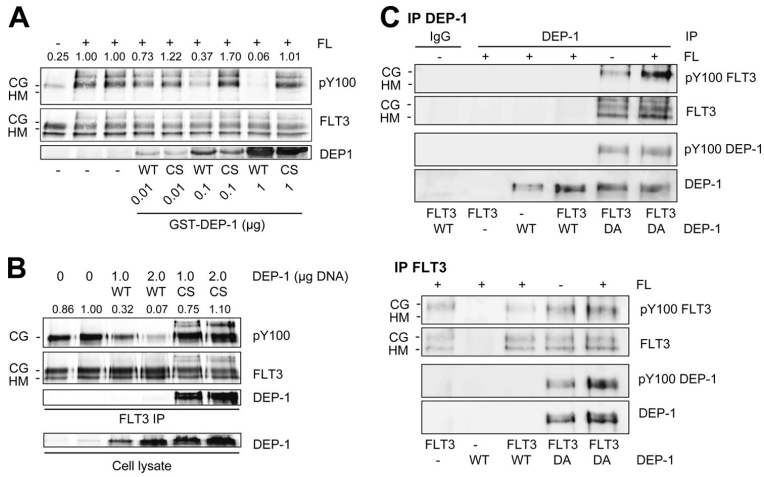

FIGURE 5. **DEP-1 directly interacts with and dephosphorylates FLT3.** *A*, THP-1 cell were starved in cytokine-free RPMI 1640 medium containing 0.5% FCS, and left unstimulated, or were stimulated with FL (100 ng/ml) for 30 min (*FL* +). Cells were subsequently lysed, and FLT3 was immunoprecipitated. Immunoprecipitated FLT3 obtained from stimulated cells was incubated with the indicated amounts of recombinant wild type GST-DEP-1 (catalytic domain) fusion protein (*WT*) or the corresponding catalytically inactive GST-DEP-1 C1239S protein (*CS*) in the indicated amounts (μg of protein) for 30 min. The samples were subsequently subjected to SDS-PAGE and immunoblotting. Blots were probed with anti-phosphotyrosine antibody (*pY100*) and reprobed with anti-FLT3 antibody, as indicated. Tyrosine phosphorylation of FLT3 was quantified as the ratio of phosphorylated FLT3 to total FLT3 (*numbers above pY100 blot*). This experiment was performed twice with consistent results. *B,* HEK293 cells were co-transfected with expression plasmids for human FLT3 and wild type (*WT*) DEP-1 or catalytically inactive DEP-1 C1239S (*CS*) in the indicated amounts (μg) using polyethyleneimine. 36 h after transfection, cells were starved for 8 h and then stimulated with 100 ng/ml FL for 5 min. The cells were subsequently lysed, and FLT3 was immunoprecipitated (*IP*). The blots were probed for phosphorylation of FLT3 by immunoblotting and reblotted for the amounts of FLT3. Quantification denotes the FLT3 phosphorylation signal normalized to the amounts of FLT3, relative to the control in absence of DEP-1. Co-purified DEP-1 protein in the immunoprecipitates was analyzed using anti-DEP-1 antibody. DEP-1 expression in the cell lysates was also analyzed (*lowest panel*). *HM,* high mannose; *CG,* complex glycosylated FLT3. A representative experiment of three with consistent results is depicted. *C,* HEK293 cells were co-transfected with plasmids encoding human FLT3 along with wild type DEP-1 (*WT*) or the DEP-1 D1205A trapping mutant (*DA*, as indicated) in the molar ratio 1:1. The cells were starved and stimulated with FL as in *A* and lysed, and either DEP-1 (*upper panel*) or FLT3 (*lower panel*) was immunoprecipitated. For a specificity control, immunoprecipitation was carried out with unspecific IgG. Immunoprecipitated and co-immunoprecipitated proteins were analyzed by immunoblotting with anti-FLT3, anti-DEP-1, or anti-phosphotyrosine (*pY100*) antibodies as indicated. *HM,* high mannose; *CG,* complex glycosylated FLT3. A representative experiment of three with consistent results is shown.

diated growth of cells expressing DEP-1 shRNA A2 was, however, stimulated compared with the shRNA control cells (Fig. 6, *A* and *B*). The effect was quite moderate when scored with the MTT assay after 3 days but more pronounced upon prolonged growth and detecting GFP fluorescence. No growth alteration could be observed for cells harboring the shRNA A3 (data not shown), indicating that the achieved suppression levels of DEP-1 in these cells were insufficient to translate to a growth response. Incubation of the cells in presence of IL3 resulted in strong growth stimulation. We also observed a very weakly elevated IL3-stimulated growth for the DEP-1-depleted cells as compared with the control cells (Fig. 6*B*). This finding may indicate that events downstream of IL3 receptor stimulation are perhaps also negatively controlled by DEP-1, a possibility that requires further investigation.

Because STAT5 acts as an important mediator of cellular transformation and we had observed a weakly enhanced activation of STAT5 upon DEP-1 depletion, we subjected the DEP-1-depleted cell lines to colony assays in the presence of FL in semisolid media. Although the control shRNA expressing cells did not grow in methylcellulose plates, the efficient knockdown of DEP-1 using shRNA A2 reproducibly translated into colony growth (Fig. 6*C*). In the presence of IL-3, abundant colony formation of all cell lines occurred, and colony numbers were not different in control cells or DEP-1-depleted cells. Somewhat increased size of colonies of DEP-1-depleted cells in the presence of IL-3 may correlate to the above described weak growth advantage. No colonies were formed in the absence of cytokine, indicating that colony formation of DEP-1-depleted cells in the presence of FL is specifically mediated by FLT3. Prompted by this capacity of DEP-1-depleted cells to form colonies in semisolid medium, we addressed the question as to whether the elevated FLT3 activity would possibly translate into driving tumor formation *in vivo*. Therefore, the ability of DEP-1-depleted 32D cells expressing wild type FLT3 to cause a leukemia-like disease in syngeneic C3H/HeJ mice was tested. As shown previously, in these experiments FLT3-ITD-expressing 32D cells caused development of a myeloproliferative disease within 2–3 weeks (18), but animals injected with DEP-1-depleted 32D FLT3 wild type cells did not develop an obvious disease up to 3 months after injection (data not shown).

It had been reported that constitutively active FLT3 induces resistance to radiation-induced apoptosis (9). Therefore, we finally also examined the effect of elevated FLT3 activity in DEP-1-depleted 32D FLT3 cells on DNA damage-induced apoptosis. As shown in supplemental Fig. S7, 32D FLT3 shDEP-1 cells remained highly sensitive to

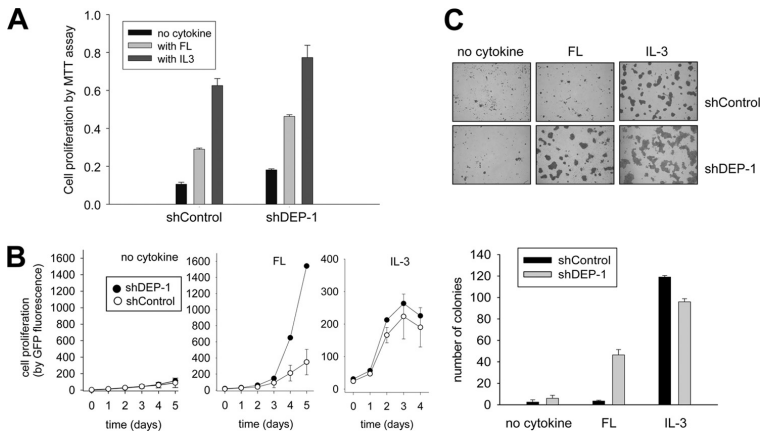

FIGURE 6. **Depletion of DEP-1 stimulates proliferation and clonal growth of 32D cell lines.** *A,* 32D cells expressing wild type muFLT3 and DEP-1 shRNA A2 or control shRNA were seeded in 96-well plates and cell growth in the absence of cytokines, in the presence of FL (20 ng/ml), or in the presence of IL-3 (2 ng/ml) and was measured after 2 days using the MTT method. *B,* cells were seeded in 96-well plates at a density of 0.8×10^5 per ml in RPMI 1640 medium supplemented with 10% fetal calf serum without cytokines, with FL (20 ng/ml), or IL-3 (2 ng/ml) as indicated. Cell growth was measured at the indicated time points by measuring cellular GFP fluorescence. Values are means of triplicates, and the shown data set is representative for three experiments with consistent results. *C,* clonal growth of 32D cells in methylcellulose. The above cell pools were subjected to colony formation assays in methylcellulose, in cytokine in the absence or presence of FL (20 ng/ml) or IL-3 (2.5 ng/ml). Representative sections were photographed after 12 days (FL samples) or 6 days (IL-3 samples) of culture, and colonies were quantified by counting representative fields of the wells.

γ-irradiation. Three days after exposure, less than 10% viable cells were detected.

DISCUSSION

By carrying out a screen of 20 different PTP, we identified DEP-1/PTPRJ as a phosphatase negatively affecting the signaling activity of FLT3. To perform the screen, we have chosen phosphorylation of ERK as a surrogate readout of altered receptor kinase activity. Depletion of DEP-1 reproducibly showed a robust stimulation of ERK phosphorylation for two independent shRNA cassettes, indicating that the observed effects were not caused by off-target effects. Furthermore, overexpression of DEP-1 in the same system led to a reduction of FLT3 signaling. We have previously established SHP-2 as a positive mediator of FLT3 signaling and FLT3-dependent cell proliferation in 32D cells (18). Several PTP can dephosphorylate FLT3 in an overexpression setting (14). DEP-1 is the first PTP for which a role in negative regulation of FLT3 signaling has now been demonstrated unequivocally with a loss-of-function approach.

DEP-1 could potentially affect FLT3 signaling by regulating receptor activity directly, by dephosphorylating downstream signaling molecules, or both. Elevated ERK activation upon FLT3 activation as seen in DEP-1-depleted cell lines could indicate a direct regulation of ERK phosphorylation by DEP-1, as has been described recently (31). Similar considerations could be made for the observed weak elevation of STAT5 phosphorylation in DEP-1-depleted cells. Showing elevated phosphorylation of ERK1/2 Thr-202 upon DEP-1 depletion, we could, however, rule out that increased ERK1/2 activation occurs solely through direct ERK dephosphorylation. Hyperphosphorylation at Thr-202 upon DEP-1 knockdown can only occur by an upstream event. Also, in addition to activation of ERK1/2 and weak STAT5 activation, we observed enhanced phosphorylation of PLCγ, another known signaling molecule downstream of FLT3. These findings are consistent with data sets demonstrating direct dephosphorylation of FLT3 by DEP-1. First, hyperphosphorylation of FLT3 occurred in DEP-1-depleted cells. Hyperphosphorylation was detectable at multiple phosphorylation sites, was observed with two different shRNA constructs and a distinct siRNA pool, was dependent from the extent of DEP-1 depletion, and was detectable in two different cell types. Second, recombinant DEP-1 dephosphorylated immunoprecipitated FLT3 *in vitro* and co-expressed DEP-1 dephosphorylated FLT3 in stably transfected 32D cells and transiently transfected HEK293 cells. Third, mutual co-immunoprecipitation of FLT3 and DEP-1 substrate trapping mutants further established FLT3 as a *bona fide* substrate of DEP-1.

DEP-1 has been described earlier as a PTP that can dephosphorylate receptor tyrosine kinases, including the EGF receptor (36, 37), PDGFβ receptor (38), VEGF receptor, and c-Met (39, 40). Our data demonstrate that DEP-1 can in addition dephosphorylate the receptor tyrosine kinase FLT3. As suggested previously (37), DEP-1 thus appears to be a relatively pan-specific PTP for receptor tyrosine kinases.

Interestingly, depletion of DEP-1 affected phosphorylation of particular tyrosine sites differently. Among the regulated sites, Tyr-572, which was identified to be phosphorylated in 32D but not in Ba/F3 cells (19), was least affected in response to DEP-1 down-regulation. Tyr-572 contributes to the autoinhibitory conformation of the juxtamembrane domain (41). Likewise Tyr-768, established as one of the binding sites for Grb2, showed only weak stimulation of phosphorylation in DEP-1-depleted cells. Phosphorylation of tyrosine residues Tyr-589 and Tyr-591, which are known as prominently contributing to signaling activity of wild type and oncogenic FLT3 protein,

including STAT5 activation downstream of FLT3-ITD (32, 42), were among the most strongly stimulated sites in DEP-1-depleted 32D cells. Hyperphosphorylation of these sites may cause the observed STAT5 activation upon DEP-1 knockdown. In addition, kinase domain-localized Tyr-842, and to a lesser extent Tyr-793, which are also involved in activity regulation, were also phosphorylated more strongly upon DEP-1 depletion (8, 43). Differential effects of DEP-1 on the different phosphorylation sites could reflect differential accessibility by steric reasons or be related to DEP-1 substrate specificity, as shown earlier for the PDGFβ receptor (44). Clearly, dephosphorylation of FLT3 by DEP-1 is another example of site selectivity of a PTP for a multiply phosphorylated cellular substrate. Whereas the DEP-1 knockdown affected mainly the extent of FL-stimulated phosphorylation of the mature, surface-located FLT3 and its ubiquinated counterpart, a weak elevation of basal phosphorylation could also be detected for some sites, for example Tyr-591 and Tyr-842. This observation indicates that DEP-1 may interact not only with a ligand-stimulated receptor but to some extent also with unstimulated FLT3, possibly to prevent basal phosphorylation.

Depletion of DEP-1 stimulated FL-dependent proliferation of 32D cells. This phenotype may relate to elevated ERK activation observed in these cells. Remarkably, DEP-1 depletion also caused 32D cells expressing wild type FLT3 to form colonies in semisolid medium in an FL-dependent manner. This may be explained by the slight but reproducible stimulation of STAT5 activation. Further pathways may, however, be involved, which we are currently trying to identify. This includes pathways activated by IL-3, because somewhat elevated responses to IL-3 were also observed in the DEP-1-depleted cells, including a moderately elevated proliferation. Despite driving colony formation *in vitro*, DEP-1 depletion did not lead to the development of myeloproliferative disease when the 32D cells were injected into syngeneic mice. Thus, DEP-1 depletion contributes to but is not sufficient for leukemic cell transformation via FLT3 activation. It will be important to analyze if loss of DEP-1 functionality occurs in the context of AML.

Although the data presented here identify DEP-1 as a FLT3-regulating PTP, the effects of shRNA-mediated depletion of DEP-1 on signaling and biological readouts were relatively moderate. It appears likely that other PTP cooperate with DEP-1 in the dephosphorylation of FLT3 and/or inactivation of downstream signaling events. Using *Ptprj* knock-out mice and CD45/*Ptprj* double knock-out mice, Zhu *et al.* (45) recently uncovered a role of DEP-1 for B-cell and macrophage immunoreceptor signaling, which only became apparent in the background of the CD45 knock-out mice. These studies clearly indicated a remarkable redundancy of the two only distantly related transmembrane PTP for regulation of Src family kinases in B-cells and macrophages (45). Similarly, other PTPs may cooperate with DEP-1 in regulation of FLT3 signaling and have the capacity to compensate DEP-1 loss-of-function. Although knockdown of CD45 in 32D FLT3 cells increased phosphorylation of ERK1/2 and of FLT3 by yet unclear mechanisms (supplemental Table S1 and data not shown). A combinatorial depletion of the DEP-1 and CD45 did not result in synergistic effects on FLT3-mediated signaling (data not shown). The possibility of redundant activities of DEP-1 and other PTP on FLT3 need to be explored in future studies.

Acknowledgments—We thank Drs. Ari Elson and Akepati Vasudheva Reddy for help with PTP-GLEPP1 expression analysis and Drs. Mohi, Neel, Tenev, and Nolan for reagents. We also thank Dr. Hubert Serve for providing array data of hematopoietic 32D FLT3 cells and DNA constructs.

REFERENCES

1. Stirewalt, D. L., and Radich, J. P. (2003) *Nat. Rev. Cancer* **3,** 650–665
2. Schmidt-Arras, D., Schwäble, J., Böhmer, F. D., and Serve, H. (2004) *Curr. Pharm. Des.* **10,** 1867–1883
3. Gilliland, D. G., and Griffin, J. D. (2002) *Blood* **100,** 1532–1542
4. Thiede, C., Steudel, C., Mohr, B., Schaich, M., Schäkel, U., Platzbecker, U., Wermke, M., Bornhäuser, M., Ritter, M., Neubauer, A., Ehninger, G., and Illmer, T. (2002) *Blood* **99,** 4326–4335
5. Small, D. (2006) *Hematology Am. Soc. Hematol. Educ. Program,* 178–184
6. Nakao, M., Yokota, S., Iwai, T., Kaneko, H., Horiike, S., Kashima, K., Sonoda, Y., Fujimoto, T., and Misawa, S. (1996) *Leukemia* **10,** 1911–1918
7. Heidel, F., Solem, F. K., Breitenbuecher, F., Lipka, D. B., Kasper, S., Thiede, M. H., Brandts, C., Serve, H., Roesel, J., Giles, F., Feldman, E., Ehninger, G., Schiller, G. J., Nimer, S., Stone, R. M., Wang, Y., Kindler, T., Cohen, P. S., Huber, C., and Fischer, T. (2006) *Blood* **107,** 293–300
8. Kindler, T., Breitenbuecher, F., Kasper, S., Estey, E., Giles, F., Feldman, E., Ehninger, G., Schiller, G., Klimek, V., Nimer, S. D., Gratwohl, A., Choudhary, C. R., Mueller-Tidow, C., Serve, H., Gschaidmeier, H., Cohen, P. S., Huber, C., and Fischer, T. (2005) *Blood* **105,** 335–340
9. Mizuki, M., Fenski, R., Halfter, H., Matsumura, I., Schmidt, R., Müller, C., Grüning, W., Kratz-Albers, K., Serve, S., Steur, C., Büchner, T., Kienast, J., Kanakura, Y., Berdel, W. E., and Serve, H. (2000) *Blood* **96,** 3907–3914
10. Kelly, L. M., Yu, J. C., Boulton, C. L., Apatira, M., Li, J., Sullivan, C. M., Williams, I., Amaral, S. M., Curley, D. P., Duclos, N., Neuberg, D., Scarborough, R. M., Pandey, A., Hollenbach, S., Abe, K., Lokker, N. A., Gilliland, D. G., and Giese, N. A. (2002) *Cancer Cell* **1,** 421–432
11. Choudhary, C., Müller-Tidow, C., Berdel, W. E., and Serve, H. (2005) *Int. J. Hematol.* **82,** 93–99
12. Schmidt-Arras, D., Böhmer, S. A., Koch, S., Müller, J. P., Blei, L., Cornils, H., Bauer, R., Korasikha, S., Thiede, C., and Böhmer, F. D. (2009) *Blood* **113,** 3568–3576
13. Choudhary, C., Olsen, J. V., Brandts, C., Cox, J., Reddy, P. N., Böhmer, F. D., Gerke, V., Schmidt-Arras, D. E., Berdel, W. E., Müller-Tidow, C., Mann, M., and Serve, H. (2009) *Mol. Cell* **36,** 326–339
14. Schmidt-Arras, D. E., Böhmer, A., Markova, B., Choudhary, C., Serve, H., and Böhmer, F. D. (2005) *Mol. Cell. Biol.* **25,** 3690–3703
15. Razumovskaya, E., Masson, K., Khan, R., Bengtsson, S., and Rönnstrand, L. (2009) *Exp. Hematol.* **37,** 979–989
16. Ostman, A., and Böhmer, F. D. (2001) *Trends Cell Biol.* **11,** 258–266
17. Ostman, A., Hellberg, C., and Böhmer, F. D. (2006) *Nat. Rev. Cancer* **6,** 307–320
18. Müller, J. P., Schönherr, C., Markova, B., Bauer, R., Stocking, C., and Böhmer, F. D. (2008) *Leukemia* **22,** 1945–1948
19. Heiss, E., Masson, K., Sundberg, C., Pedersen, M., Sun, J., Bengtsson, S., and Rönnstrand, L. (2006) *Blood* **108,** 1542–1550
20. Heyworth, C. M., and Spooncer, E. (1992) in *Haemopoiesis: A Practical Approach* (Molineux, G., and Testa, N. G., eds) pp. 37–53, Oxford University Press, New York
21. Jandt, E., Denner, K., Kovalenko, M., Ostman, A., and Böhmer, F. D. (2003) *Oncogene* **22,** 4175–4185
22. Kitamura, T., Koshino, Y., Shibata, F., Oki, T., Nakajima, H., Nosaka, T., and Kumagai, H. (2003) *Exp. Hematol.* **31,** 1007–1014
23. Petermann, A., Haase, D., Wetzel, A., Balavenkatraman, K. K., Tenev, T., Gührs, K. H., Friedrich, S., Nakamura, M., Mawrin, C., and Böhmer, F. D. (December 27, 2010) *Brain Pathol.* 10.1111/j.1750-3639.2010.00464.x
24. Gross, S., Knebel, A., Tenev, T., Neininger, A., Gaestel, M., Herrlich, P., and Böhmer, F. D. (1999) *J. Biol. Chem.* **274,** 26378–26386

25. Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., and Naldini, L. (1998) *J. Virol.* **72,** 8463–8471
26. Beyer, W. R., Westphal, M., Ostertag, W., and von Laer, D. (2002) *J. Virol.* **76,** 1488–1495
27. Mosmann, T. (1983) *J. Immunol. Methods* **65,** 55–63
28. Grundler, R., Miething, C., Thiede, C., Peschel, C., and Duyster, J. (2005) *Blood* **105,** 4792–4799
29. Mizuki, M., Schwable, J., Steur, C., Choudhary, C., Agrawal, S., Sargin, B., Steffen, B., Matsumura, I., Kanakura, Y., Böhmer, F. D., Müller-Tidow, C., Berdel, W. E., and Serve, H. (2003) *Blood* **101,** 3164–3173
30. Choudhary, C., Schwäble, J., Brandts, C., Tickenbrock, L., Sargin, B., Kindler, T., Fischer, T., Berdel, W. E., Müller-Tidow, C., and Serve, H. (2005) *Blood* **106,** 265–273
31. Sacco, F., Tinti, M., Palma, A., Ferrari, E., Nardozza, A. P., Hooft van Huijsduijnen, R., Takahashi, T., Castagnoli, L., and Cesareni, G. (2009) *J. Biol. Chem.* **284,** 22048–22058
32. Rocnik, J. L., Okabe, R., Yu, J. C., Lee, B. H., Giese, N., Schenkein, D. P., and Gilliland, D. G. (2006) *Blood* **108,** 1339–1345
33. Grundler, R., Thiede, C., Miething, C., Steudel, C., Peschel, C., and Duyster, J. (2003) *Blood* **102,** 646–651
34. Flint, A. J., Tiganis, T., Barford, D., and Tonks, N. K. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 1680–1685
35. Palka, H. L., Park, M., and Tonks, N. K. (2003) *J. Biol. Chem.* **278,** 5728–5735
36. Berset, T. A., Hoier, E. F., and Hajnal, A. (2005) *Genes Dev.* **19,** 1328–1340
37. Tarcic, G., Boguslavsky, S. K., Wakim, J., Kiuchi, T., Liu, A., Reinitz, F., Nathanson, D., Takahashi, T., Mischel, P. S., Ng, T., and Yarden, Y. (2009) *Curr. Biol.* **19,** 1788–1798
38. Kappert, K., Paulsson, J., Sparwel, J., Leppänen, O., Hellberg, C., Ostman, A., and Micke, P. (2007) *FASEB J.* **21,** 523–534
39. Chabot, C., Spring, K., Gratton, J. P., Elchebly, M., and Royal, I. (2009) *Mol. Cell. Biol.* **29,** 241–253
40. Grazia Lampugnani, M., Zanetti, A., Corada, M., Takahashi, T., Balconi, G., Breviario, F., Orsenigo, F., Cattelino, A., Kemler, R., Daniel, T. O., and Dejana, E. (2003) *J. Cell Biol.* **161,** 793–804
41. Fröhling, S., Scholl, C., Levine, R. L., Loriaux, M., Boggon, T. J., Bernard, O. A., Berger, R., Döhner, H., Döhner, K., Ebert, B. L., Teckie, S., Golub, T. R., Jiang, J., Schittenhelm, M. M., Lee, B. H., Griffin, J. D., Stone, R. M., Heinrich, M. C., Deininger, M. W., Druker, B. J., and Gilliland, D. G. (2007) *Cancer Cell* **12,** 501–513
42. Vempati, S., Reindl, C., Wolf, U., Kern, R., Petropoulos, K., Naidu, V. M., Buske, C., Hiddemann, W., Kohl, T. M., and Spiekermann, K. (2008) *Clin. Cancer Res.* **14,** 4437–4445
43. Griffith, J., Black, J., Faerman, C., Swenson, L., Wynn, M., Lu, F., Lippke, J., and Saxena, K. (2004) *Mol. Cell* **13,** 169–178
44. Persson, C., Sävenhed, C., Bourdeau, A., Tremblay, M. L., Markova, B., Böhmer, F. D., Haj, F. G., Neel, B. G., Elson, A., Heldin, C. H., Rönnstrand, L., Ostman, A., and Hellberg, C. (2004) *Mol. Cell. Biol.* **24,** 2190–2201
45. Zhu, J. W., Brdicka, T., Katsumoto, T. R., Lin, J., and Weiss, A. (2008) *Immunity* **28,** 183–196

Paper III

Ann Hematol
DOI 10.1007/s00277-011-1311-3

ORIGINAL ARTICLE

3,4-Diarylmaleimides—a novel class of kinase inhibitors—effectively induce apoptosis in FLT3-ITD-dependent cells

Florian H. Heidel · Thomas S. Mack · Elena Razumovskaya · Marie-Christine Blum · Daniel B. Lipka · Anne Ballaschk · Jan-Peter Kramb · Stanislav Plutizki · Lars Rönnstrand · Gerd Dannhardt · Thomas Fischer

Received: 2 February 2011 / Accepted: 16 August 2011

Abstract FLT3 kinase has become an attractive drug target in AML with up to 30% of cases harboring internal-tandem-duplication (ITD) mutations. For these, conferring a worse prognosis and decreased overall survival, several FLT3 tyrosine kinase inhibitors (TKIs) are currently being tested in clinical trials. However, when using these drugs as monotherapy, the problem of short duration of remissions and high incidence of TKI resistance has emerged. Here, we investigated two members of a novel class of tyrosine kinase inhibitors, 3,4-diarylmaleimides, for their efficacy on mutated FLT3 kinase. These compounds inhibit FLT3 kinase in an ATP-competitive manner and effectively inhibit phosphorylation of downstream targets. 3,4-Diarylmaleimides (DHF125 and 150) induce apoptosis in FLT3-ITD-dependent cells lines and patient blasts at low micromolar concentrations. They are retained in the cytoplasm of exposed cells for more than 24 h and synergize with chemotherapy and midostaurin. Both 3,4-diarylmaleimides show inhbition of FLT3-ITD-related kinase autophosphorylation at distinct tyrosine residues when compared to midostaurin. In conclusion, this novel group of compounds shows differential inhibition patterns with regard to FLT3 kinase and displays a promising profile for further clinical development. Currently, experiments evaluating toxicity in murine models and unraveling the exact binding mechanism are under way to facilitate a potential clinical application.

Keywords AML · FLT3 · Tyrosine kinase inhibitor · Tyrosine phosphorylation

Electronic supplementary material The online version of this article (doi:10.1007/s00277-011-1311-3) contains supplementary material, which is available to authorized users.

F. H. Heidel (✉) · T. S. Mack · M.-C. Blum · D. B. Lipka · A. Ballaschk · T. Fischer
Department of Hematology and Oncology, Medical Center, Otto-von-Guericke University,
Leipzigerstr. 44,
39120 Magdeburg, Germany
e-mail: Florian.Heidel@med.ovgu.de

E. Razumovskaya · L. Rönnstrand
Experimental Clinical Chemistry, Wallenberg Laboratory, Department of Laboratory Medicine, Skåne University Hospital, Lund University,
20502 Malmö, Sweden

J.-P. Kramb · S. Plutizki · G. Dannhardt
Department of Pharmacy, Johannes-Gutenberg-University,
55131 Mainz, Germany

Introduction

Acute myeloid leukemia (AML) is an aggressive malignant disease characterized by abnormal proliferation of immature hematopoietic cells. Despite advances in chemotherapeutic treatment within the last decade, only 30–45% of patients below the age of 60 can be cured. The majority of patients, above the age of 60, have a dismal prognosis with only 5–15% long-term survival [1–5]. Several prognostic factors have been established within the last decade. While cytogenetic changes are established prognostic markers survival and response to chemotherapy, several molecular markers have been defined and analyzed for their prognostic influence [6]. Besides overexpression of the brain and acute leukemia gene [7], the v-ets erythroblastosis virus E26 oncogene [8, 9] and high meningioma 1 gene [10], mutation of nucleophosmin-1 (NPM1) [11], CAATT/

Published online: 01 September 2011

enhancer binding protein [12], mixed-lineage-leukemia gene, Wilms' tumor 1 gene [13] and FMS-related tyrosine kinase (FMS-like tyrosine kinase 3–internal tandem duplications (FLT3-ITD)) [14, 15] have shown prognostic influence.

The FLT3 gene is encoding a receptor tyrosine kinase that is part of the receptor tyrosine kinase III family (together with KIT, CSF-1R and PDGFRalpha and beta). FLT3 is expressed on the blasts of over 90% of AML cases. Mutations of FLT3 divide into length mutations (ITD) and point mutations. FLT3-ITD is a heterogenic group of genetic alterations found in up to 30% of AML cases [16–18]. These mutations confer a dismal prognosis with impaired progression free and overall survival [14, 15, 19, 20]. FLT3-ITDs lead to autophosphorylation and constitutive activation of the FLT3 receptor with consecutive phosphorylation of downstream signaling nodes such as STAT5, AKT, and ERK, presumably by disruption of the autoinhibitory region [21]. Transfection of murine hematopoietic cells with FLT3-ITD leads to growth factor independent proliferation and development of a myeloproliferative phenotype in a murine bone marrow transplant model [22].

Small molecule tyrosine kinase inhibitors are a newly established class of therapeutic drugs. In AML, several tyrosine kinase inhibitors are currently tested in advanced clinical trials. Using these drugs as monotherapy has revealed remarkable efficacy. However, at the same time the problem of short duration of remissions has emerged indicating rapid development of secondary resistance [23, 24]. In addition, up to 30% of patients may show primary resistance to currently available FLT3-TKIs. This imposes a strong need to further develop treatment strategies: besides immunotherapeutic approaches [25], development of novel "second-generation" inhibitors using different mechanisms of action to achieve increased efficacy are clearly warranted.

Here, we investigated two novel TKIs (3,4-diarylmaleimides, DHF125 and DHF150) previously described as angiogenesis inhibitors [26–28], to determine their mechanism of action and efficacy in FLT3-ITD-positive cell lines as well as in primary ITD-positive AML blasts.

Materials and methods

DNA constructs and generation of transfected cells

A human FLT3-ITD construct (amino acid position, N598; amino acid sequence, NEYFYVDFREYE), subcloned into the pAL expression vector under control of the 5′ long terminal repeat of the Moloney murine sarcoma virus and the plasmid pMAM/BSD were used as previously described [24]. The pAL-vector construct was stably transfected into the murine IL3-dependent myeloid cell line 32D by electroporation.

Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 20 mM Hepes (pH 7.3), 50-mM β-mercaptoethanol, and 2 mM L-glutamine.

Protein extract preparation, immunoprecipitation, and Western blotting

Cells at 2×10^6 were incubated in the presence of different inhibitor concentrations and in combination of different inhibitors for 1 h at 37°C. Preparation of cellular lysates was performed as described previously [24]. Protein lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose membrane (Amersham, Freiburg, Germany). The following antibodies were used: anti-phospho-FLT3 (pTyr591), anti-phospho-STAT5 (pTyr694/699), anti-phospho AKT (pSer473), anti-phospho ERK (pThr202/pTyr204) (all Cell Signaling Technology™, Frankfurt, Germany), antiphosphotyrosine (Biomol, Hamburg, Germany; and Santa Cruz, Heidelberg, Germany), anti-FLT3, anti-STAT5 (Santa Cruz, Heidelberg, Germany), anti-AKT (Cell Signaling Technology, Danvers, MA, USA), and anti-β-actin. Densitometric analysis was performed using the program Gel-Pro Analyzer®. For immunoprecipitation, 1×10^7 inhibitor/DMSO-treated MV4;11 cells were used for protein extraction; 100 μL of protein lysates were incubated with 20 μL of FLT3 antibody (sc-479, Santa Cruz, Heidelberg, Germany) for 24 h at 4°C. AG beads were added for 1 h at 4°C, followed by multiple washing steps. Finally, AG beads were covered with 30-μL β-mercaptoethanol-containing loading dye, heated for 5 min at 95°C followed by a quick spin-down. The supernatant was subjected to SDS-PAGE gel electrophoresis and Western blotting. Phospho-specific antibodies were produced and applied for Western blotting experiments as previously described [29].

Kinase assays

For determination of in vitro FLT3 kinase activity, we used HTScan™ FLT3 Kinase Assay Kit (Cell Signaling Technology, Inc., Danvers, MA, USA). Analysis was performed according to the manufacturer's protocol. For ATP competition experiments 10-fold of ATP concentration (200 μM instead of 20 μM) was used. Absorbance was measured using a standard ELISA reader at 450 nm.

A second kinase assay was performed on a commercial basis by Millipore UK Ltd. (Gemini Crescent; Dundee Technology Park, Dundee DD2 1SW, UK). Details of assay protocols can be found on Millipore's website at www.millipore.com/drugdiscovery/dd3/assayprotocols.

Apoptosis assays

Transfected murine 32D cells (1×10^5 cells/well) were incubated in 2 ml RPMI 1640 medium with different inhibitor concentrations for 48 h at 37°C. Primary human cells (2×10^5 cells/well) were incubated 72 h at 37°C. After incubation, cells were washed with ice-cold PBS, pelleted, and mixed with 300 μl of propidium-iodide-buffer (containing 50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton×100, Sigma) for 30 min at 4°C. Cell cycle analysis was performed as described previously [30] using FACS-Canto™ flow cytometers (BD Biosciences, Heidelberg, Germany). For AnnexinV/SytoxRed staining, treated 32D cells were washed with ice-cold PBS, resuspended in AnnexinV buffer and stained with AnnexinV-FITC and SytoxRed (Invitrogen) for 30 min on ice.

Isolation of primary AML blasts and cell culture

Bone marrow and peripheral blood samples with heparin as anticoagulant were obtained from AML patients (patient characteristics are indicated below) or donors with no evidence of malignant bone marrow infiltration after informed consent in a study approved by the local ethics committee. Mononuclear cells (MNC) were isolated immediately by means of Ficoll-Hypaque (Seromed, Berlin, Germany) density gradient centrifugation. For immunoblotting freshly isolated MNCs were either lysed directly or after incubation in RPMI 1640 medium supplemented with 20 mM Hepes (pH 7.3), 50-mM β-mercaptoethanol and 2 mM L-glutamine containing varying amounts of different tyrosine kinase inhibitors (midostaurin (protein kinase C (PKC)412), DHF125, and DHF150). For cell cycle analysis, MNCs were maintained in RPMI 1640 medium supplemented as above plus 10% FCS.

Patient characteristics

Patient characteristics (apoptosis assay)

Patient no.	Age/gender	FAB	WBC/uL	% of Blasts	Cytogenetics	FLT3 mutation*
Patient 1	79/F	M5	75,800	83	46,XX	ITD
Patient 2	78/M	M4	52,300	62	46,XY	ITD
Patient 3	88/F	M5	181,000	51	46,XX	ITD
Patient 4	39/F	M3	30,000	50	46,XX, *t*(15;17)	ITD

FLT3 mutation status was confirmed using standard diagnostic primer as follows: FLT3-ITD-fw 5-GCAATTTAGGTATGAAAGCCAGC-3 and FLT3-ITD-rev 5-CTTTCAGCATTTTGACGGCAACC-3.

Patient characteristics (cellular uptake assay)

Patient no.	Age/gender	FAB	WBC/uL	% of Blasts
Patient 1	88/F	M5	137,400	90
Patient 2	75/M	M1	80,500	66
Patient 3	76/F	M5	3,700	70
Patient 4	39/F	M3	30,000	50
Patient 5	78/M	M4	52,300	62
Patient 6	30/F	M4	151,400	70

Colony assays

Bone marrow MNCs were isolated as indicated above. Bone marrow samples were obtained from patients in complete remission after leukemia or lymphoma treatment and without evidence of any malignant bone marrow infiltration; 1×10^6 cells were plated in 1.1 ml Methocult™ (GF H4534) "complete" methylcellulose medium with recombinant cytokines (StemCell Technologies, Vancouver, Canada) and incubated for 10 days at 37°C. Colony formation was counted on day 10.

Inhibitors

3,4-Diarylmaleimides (academically developed at the Department of Pharmacy, Johannes-Gutenberg-University Mainz [26–28] and PKC412A (kindly provided by J. Roesel, Novartis Inc.) were dissolved and diluted in DMSO (Sigma, Munich, Germany). The chemotherapeutic drugs cytarabine and daunorubicin have been diluted in water. Equivalent doses of DMSO were added when used for combination treatment.

Detection of autofluorescence and cellular uptake

To determine uptake and persistence of 3,4-diarylmaleimides in AML blasts, primary cells were incubated at 37°C in a 5% CO_2-humified incubator with either compound in RPMI 1640 medium supplemented with 10% FCS. Uptake was determined by FACS analysis (FACSCanto, BD Biosciences, Heidelberg, Germany) by mean fluorescence intensity (DHF125, PE-Cy5-A mean; DHF150, PE-A mean). For determination of persistence of 3,4-diarylmaleimides in primary blasts, cells were incubated for 10 min with either compound, washed twice with PBS, and incubated at 37°C in RPMI 1640 medium supplemented with 10% FCS for 24 h. Autofluorescence was detected as indicated above.

Fluorescence of 3,4-diarylmaleimides was detected by confocal laser microscopy using a Zeiss Axiovert 100 M microscope attached to an LSM confocal unit. 3,4-Diary-

lmaleimides were added to murine 32D cells or human AML-blasts 5 min prior to microscope imaging. Images were edited using Zeiss LSM Image Examiner (Version 3.2.0.115).

Quantification of synergism and antagonism in drug combinations

For definition of synergy and/or antagonism in drug combinations we used the CompuSyn™ software (Chou, TC; ComboSyn, Inc. Paramus, NJ, USA) as previously described [30].

Statistical calculations

Statistical analysis was performed using GraphPad PrismTM v4.00. All experiments were performed in triplicates unless otherwise stated. For statistical comparison of colony formation upon diarylmaleimide treatment, a paired Student's *t* test was applied. Statistical significance was assumed for $p<0.05$.

Results

3,4-Diarylmaleimides inhibit FLT3 kinase in an ATP-dependent manner and reduce phosphorylation of its downstream signaling molecules

3,4-Diarylmaleimides are a novel class of inhibitors that have been initially described as potent inhibitors of angiogenesis in an in vivo chick embryo assay. Molecular modeling studies suggested binding of these compounds at the ATP-binding site of the model kinase CDK2. Screening for inhibitory activity in a panel of 12 selected protein kinases revealed a high affinity to VEGF-R but not to other kinases investigated (e.g., IGF-R, EGF-R, ABL, PDGFR, CDK2, CDK4, GSK3-b, and Aurora A&B), indicating a narrow spectrum of target structures [27, 28]. Herein, we aimed to investigate whether two structurally related 3,4-diarylmaleimides, DHF125 and DHF150 (Fig. 1a), inhibit FLT3 kinase and its downstream effectors. The majority of tyrosine kinase inhibitors are known to inhibit mutated FLT3 kinase by competing with ATP binding in the ATP-binding pocket. This reduces or abrogates phosphorylation of FLT3-ITD downstream targets, such as STAT5, AKT, and ERK.

To directly address inhibition of FLT3 kinase activity, we performed FLT3 kinase assays. DHF150 and DHF125 effectively inhibited FLT3 kinase with an IC_{50} of ~350 and ~450 nM, respectively. As suggested by the initial kinase screen, we aimed to confirm the ATP-competitive mechanism of 3,4-diarylmaleimides. As inhibitors binding in the ATP-pocket can be outcompeted by escalation of ATP [31], we applied a tenfold ATP concentration (Fig. 1b, left panel). These experiments revealed that both DM compounds could be antagonized by ATP escalation, indeed indicating an ATP-competitive mechanism of action. Midostaurin used as a positive control, also revealed ATP-dependent inhibition of FLT3 kinase (data not shown). This FLT3 kinase-specific mechanism of inhibition was confirmed using a commercially available FLT3 kinase assay performed by Millipore. In this assay, DHF125 showed inhibition of FLT3 kinase with an IC_{50} of 166 nM (Fig. 1b, right panel). The differences between the two kinase assays applied may be best explained by the different methodology: antibody-based array technology versus detection of kinase activity by radioactive labeled ATP, respectively.

In order to monitor inhibition of FLT3-ITD targets, we investigated the main downstream signaling nodes—STAT5, ERK, and AKT by Western blotting. Upon incubation of 32D-FLT3-ITD cells with either DM compound, a marked decrease in phosphorylation was detectable for STAT5 and ERK (Fig. 2c, left panel) while a slight reduction was seen for AKT phosphorylation. PKC412 served as a positive control for inhibition of these signaling nodes. These findings were confirmed using the human MV4;11 cell line (Fig. 2c, right panel). Incubation with either PKC412 or compound DHF125 led to reduced phosphorylation of STAT5 and ERK. In contrast, the human cell line HEL, harboring an activating JAK2V617F mutation, did not show any reduction in phosphorylation of STAT5 or ERK when compared to DMSO-treated control.

Thus, we were able to provide first evidence that both 3,4-diarylmaleimides inhibit FLT3 kinase and its downstream targets in an ATP-competitive manner.

3,4-Diarylmaleimides (DHF compounds) effectively induce apoptosis in FLT3-ITD-dependent cell lines

To determine the effects of 3,4-diarylmaleimides on mutated FLT3 tyrosine kinase in a cellular context, we transfected the murine IL3-dependent hematopoietic 32D cells with an FLT3 length mutation (FLT3-ITD)- or FLT3-wildtype (FLT3-WT)-cDNA construct. While FLT3-ITD-positive cells gain growth factor-independent proliferation, FLT3-WT cells generally remain IL3 dependent. 32D-FLT3-ITD-positive cells are known to undergo apoptosis upon incubation with FLT3 inhibitors like PKC412 in the absence of IL-3 [32]. However, when IL-3 is added, induction of apoptosis can be prevented bypassing FLT3-ITD dependency via growth factor receptor. Therefore, we incubated these cells with either DHF compound in the presence or absence of IL-3.

Induction of apoptosis was detectable at low micromolar concentrations (Fig. 2a), as detected by flow cytometry analysis using AnnexinV (as an early apoptosis marker) and SytoxRed (as a dead cell stain). Analyzing the subG1

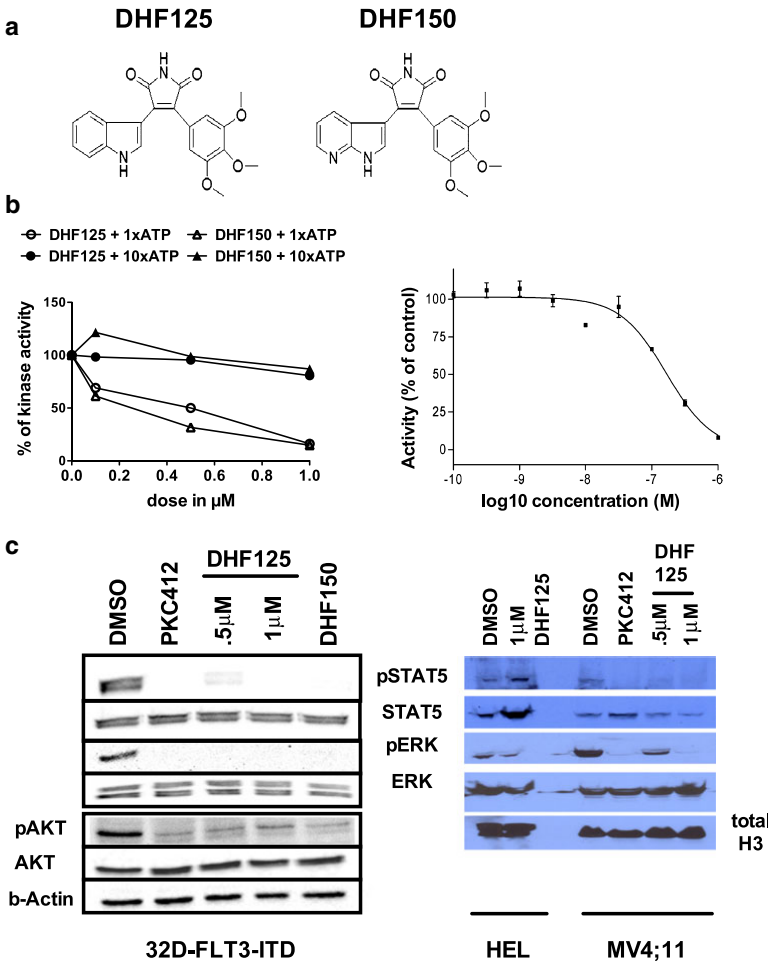

Fig. 1 3,4-Diarylmaleimides inhibit FLT3 kinase activity and phosphorylation of its downstream targets. **a** Chemical structures of both diarylmaleimide compounds investigated are displayed. **b** Using kinase assays, inhibition of mutant FLT3 kinase was evident at low micromolar concentrations. Using the HTScan assay, for DHF125 and DHF150, IC_{50} concentrations of ~350 and ~450 nM were detectable (*left panel*). In a second kinase assay performed by Millipore Inc., the IC_{50} for FLT3 kinase was 166 nM for compound DHF125 (*right panel*). **c** 32D-FLT3-ITD protein lysates show reduction of STAT5, ERK, and AKT phosphorylation upon incubation of cells (for 1 h) with PKC412 (100), compound DHF125 (0.5 and 1 µM) and compound DHF150 (1 µM), respectively

fraction in cell cycle analysis upon incubation with DHF125 and DHF150, we were able to define an IC_{50} of 375 and 850 nM, respectively (Fig. 2b, c). To confirm the FLT3 specificity of these effects, FLT3-ITD-positive cells were also cultured in the presence of IL-3 allowing the cells to bypass FLT3-ITD dependency by activation of alternative signaling pathways. As depicted in Fig. 2b, induction of apoptosis was reversible in both cases, confirming FLT3-ITD specificity of the effects observed. Induction of apoptosis was further confirmed by Western blotting experiments as indicated by caspase-3 cleavage (Fig. 2d).

As 32D cells transfected with an FLT3-WT construct are cultured in growth factor containing medium, any induction of cell death would potentially display toxic off-target effects. To elucidate the "maximal tolerable" or toxic dose for 32D transfectants, we incubated 32D-FLT3-WT cells with increasing concentrations of either compound until cell death occurred. 32D-FLT3-WT cells showed no significant increase in apoptosis up to 10 µM of either DHF compound (Fig. 2e; displayed is data for DHF125). However, higher doses of up to 50 µM revealed increasing rates of cell death in terms of cellular toxicity. This provides first evidence for a possible therapeutic range of this novel class of inhibitors.

To evaluate a potential broader spectrum of kinase inhibition, we analyzed the effects of DHF compounds on JAK2-kinase that had not been included in previous screens. Ba/F3 cells harboring the V617F mutation leading to constitutive activation of JAK2-kinase were incubated with increasing concentrations of either inhibitor. No significant induction of apoptosis could be demonstrated

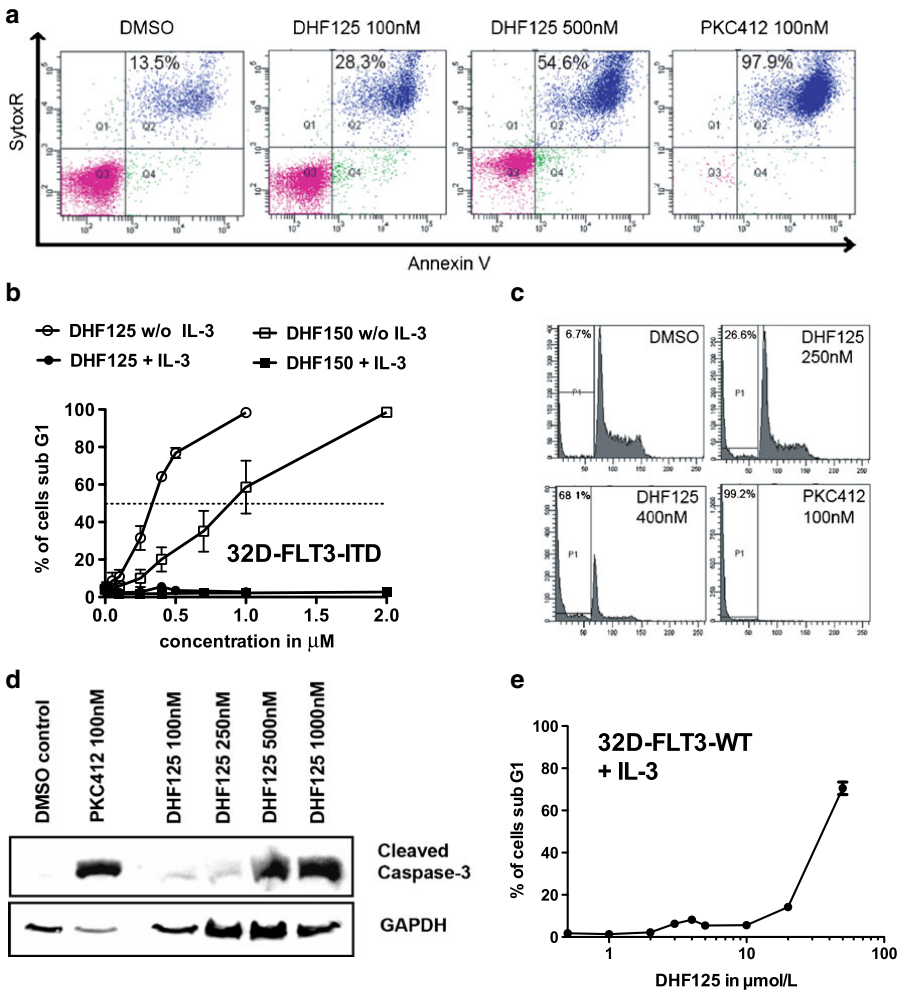

Fig. 2 3,4-Diarylmaleimides induce apoptosis in FLT3-ITD-dependent cell lines. Percentage of apoptotic cells was measured after a 48-h incubation period with either compound by flow cytometry using AnnexinV/SytoxRed staining (**a**), PI-based cell cycle analysis (**b**, **c**) and caspase-3 cleavage (**d**) in Western blotting. Calculated IC_{50} concentrations for DHF125 and DHF150 using 32D-FLT3-ITD cells were 375 and 850 nM, respectively (**b**). Addition of growth factor (*IL-3*) leads to rescue of cells upon incubation with either compound. **e** 32D-FLT3-WT cells grown in IL-3 containing medium show no toxic effects up to 10 μM of inhibitor concentration, as displayed for DHF125

using doses up to 5 μM while apoptosis could be induced using JAK inhibitor (data not shown).

This suggests that 3,4-diarylmaleimides effectively induce apoptosis in FLT3-ITD-dependent murine cell lines at low micromolar concentrations. These effects are specific for mutated FLT3 kinase and unspecific effects do not occur until doses above 50-fold the IC_{50}.

3,4-Diarylmaleimides show synergy with chemotherapy and PKC412

Applied as monotherapy, various tyrosine kinase inhibitors have shown promising responses. However, in most cases, these responses were short lived and insufficient for disease control. Currently, advanced clinical trials evaluate tyrosine

kinase inhibitors in combination with chemotherapy. As anthracyclines and cytarabine are standard drugs for the treatment of AML, we investigated the efficacy of a combination therapy with DHF inhibitors. Synergy was calculated using Chou–Talalay plots (CI Index).

Combination of the anthracycline daunorubicin with either DHF compound showed clear induction of apoptosis in 32D-FLT3-ITD cells (Fig. 3a). Increasing doses of either chemotherapeutic drug were combined with fixed inhibitor concentrations in a non-constant ratio design [30]. For daunorubicin, calculating the effective concentrations applied according to the Chou–Talalay model revealed synergy at higher concentrations, with slightly antagonistic/additive effects at lowest concentrations (Fig. S1 in the Electronic supplementary material). In contrast, simultaneous exposure of cells to cytarabine plus DHF inhibitors revealed synergistic effects at all concentrations applied (CI indices were calculated below 1 for all combinations).

Recent reports suggest that FLT3-TKI can display distinct mechanisms of action [33, 34]. This offers a potential for combination therapy. Thus, we combined both DHF compounds with midostaurin to investigate potential synergy. Interestingly, co-incubation of PKC412 with DHF125 or DHF150, using a constant ratio design, revealed synergy (CI index, <1) at all concentrations applied (Fig. 3b). PKC412 doses used in this assay varied from 0.5 to 7 nM. In clinical phase 2 trials, PKC412 trough levels of up to 40 nM have been shown to be reached and have shown limited clinical efficacy with tolerable toxicity [24, 35, 36]. Thus, our results offer a rationale to clinically investigate TKI combination treatment in order to reach increased efficacy. Moreover, this suggests a distinct mechanism of action for DHF compounds, as identical binding would rather lead to competition of both compounds and potentially to antagonistic effects.

3,4-Diarylmaleimides induce apoptosis in primary AML blasts harboring FLT3-ITD mutations in vitro while leaving colony formation of normal stem and progenitor cells unaffected

In contrast to cell culture models, primary blasts may display a variety of genetic and epigenetic modifications, leading to increased resistance to apoptosis or proliferation.

To determine the extent of apoptosis induction in primary blasts upon inhibition of mutated FLT3 kinase, we incubated primary AML cells for 72 h with increasing concentrations of either DHF-inhibitor. Induction of apoptosis was detected by measuring the subG1-fraction after propidium iodine staining. When taken in culture primary patient blasts revealed induction of apoptosis up to 30%. Therefore, we investigated cell death exceeding baseline apoptosis. Incubation with DHF125 led to increase of apoptosis by 10–17% at 1–2 μM with no further effect upon dose escalation up to 5 μM. In contrast, using

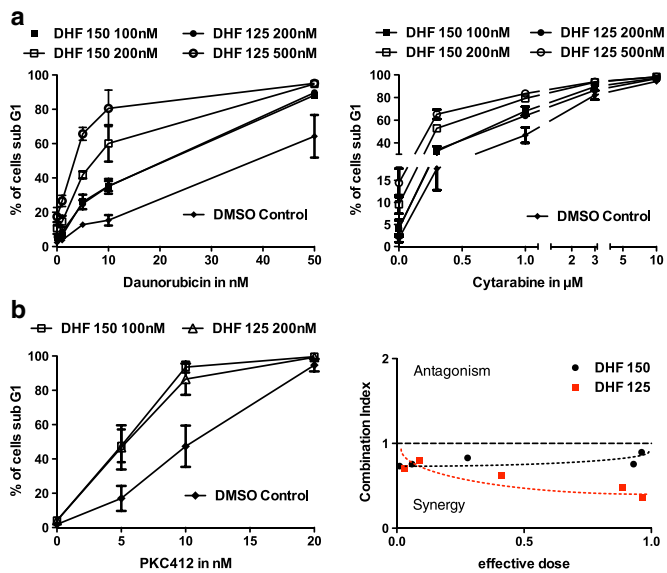

Fig. 3 3,4-Diarylmaleimide compounds synergize with chemotherapy and midostaurin in vitro. Combined treatment of 32D-FLT3-ITD cells with diarylmaleimides and daunorubicin (**a**, *left panel*) or cytarabine (**a**, *right panel*) for 48 h resulted in markedly increased amounts of apoptotic cells. Co-incubation of cells with DHF125 or DHF150 and midostaurin (PKC412) (**b**, *left panel*) demonstrated increased efficacy with regard to apoptosis induction. Synergy could be calculated at all effective doses applied (**b**, *right panel*)

DHF150, the rate of apoptotic cells increased by 12–15%. Upon dose escalation (5 μM) 17–30% of primary blasts became apoptotic after 72 h of incubation (Fig. 4a). Although conducted in an approach limited by spontaneous apoptosis, we were able to detect induction of cell death in primary patient blasts upon incubation with 3,4-diarylmaleimides.

To approach a possible therapeutic dose range, we investigated bone marrow cells of healthy donors or patients in stable remission after chemotherapy treatment. Toxicity was evaluated as impairment of colony formation. Usually, stem and progenitor cells in healthy human bone marrow have the ability to form colonies when plated in methylcellulose while further differentiated cells lack that ability. Therefore, choosing a colony formation assay seemed to be a relevant approach to investigate potential hematopoietic toxicity that would arise from impaired stem or progenitor compartments.

Addition of diarylmaleimide compounds showed no significant reduction in colony formation up to doses of 5 μM (p=0.39 for DHF125—5 μM compared to DMSO control; p=0.33 for DHF150—5 μM compared to DMSO control) (Fig. 4b). However, dose escalation of up to 10 μM revealed markedly impaired colony formation as readout for hematopoietic toxicity. This indicates that normal human stem and progenitor cells may not be affected by exposure to 3,4-diarylmaleimides concentrations of up to 5 μM of either compound (Fig. 4b). As we had investigated efficacy of a combination therapy using DM compounds with midostaurin (Fig. 3), we aimed to assess the impact of a combination therapy on colony formation of normal bone marrow. All doses investigated did not reveal any significant reduction in colony formation when compared to DMSO-treated controls (Fig. 4c)

These data are consistent with our results observed in murine cell lines (as described above). As FLT3-ITD-specific induction of apoptosis in murine cell lines and primary patient blasts occur at low micromolar concentration, these results can be interpreted as a first indicator for a potential therapeutic window.

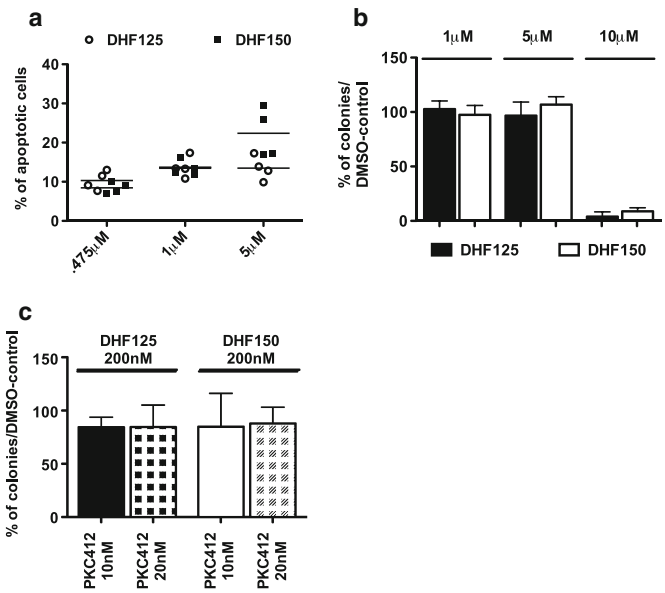

Fig. 4 3,4-Diarylmaleimide compounds induce apoptosis in FLT3-ITD harboring AML blasts at micromolar concentrations while leaving normal progenitor cells largely unaffected. diarylmaleimide compounds show induction of apoptosis in primary AML blasts. Primary cells were incubated for 72 h with different inhibitor concentrations. Apoptosis was determined as the amount of cells subG1 exceeding the baseline apoptosis rate of AML cells in culture (baseline apoptosis is graphed as 0%, every patient sample (*single dot*) is graphed as the mean of a triplicate). DHF125 led to increase of basal apoptosis by 10–17% while DHF150 elevated the rate of apoptotic cells by 17–30% (**a**). Using bone marrow cells from healthy donors, colony formation was analyzed upon increasing doses of diarylmaleimide inhibitors—in methylcellulose supplemented with cytokines—for 10 days. Impairment of colony formation was not detectable up to a dose of 5 μM of either compound. Incubation of these cells with 10 μM of either inhibitor led to almost complete loss of colony formation, suggesting toxicity on healthy hematopoietic stem and progenitor cells (**b**). Combination therapy of DM compounds with midostaurin (*PKC412*) did not reveal significant toxicity on colony formation at all doses applied (**c**)

3,4-Diarylmaleimides show rapid uptake and prolonged intracellular persistence in transfected cell lines and primary AML blasts

Exposure of malignant cells to tyrosine kinase inhibitors is one of the crucial issues in TKI treatment. Recent publications provide first evidence that prolonged exposure of kinases to the corresponding inhibitors is a crucial prerequisite for induction of cell death. Therefore, we aimed to investigate the retention of 3,4-diarylmaleimides in both, murine 32D cells and primary patient blasts. As 3,4-diarylmaleimides show autofluorescence that can be used as a correlate for inhibitor binding, we performed confocal fluorescence microscopy and flow cytometry analysis.

Fluorescence of exposed cells (as analyzed in the PE or PECy5 channel by flow cytometry) could be detected in primary patient blasts (Fig. 5a) and murine 32D cells upon incubation for less than 1 min. When co-incubated with an APC-anti-CD33 antibody, most of the blasts showed accumulation of either inhibitor (Fig. 5a). To determine the kinetics of drug uptake and persistence, we incubated

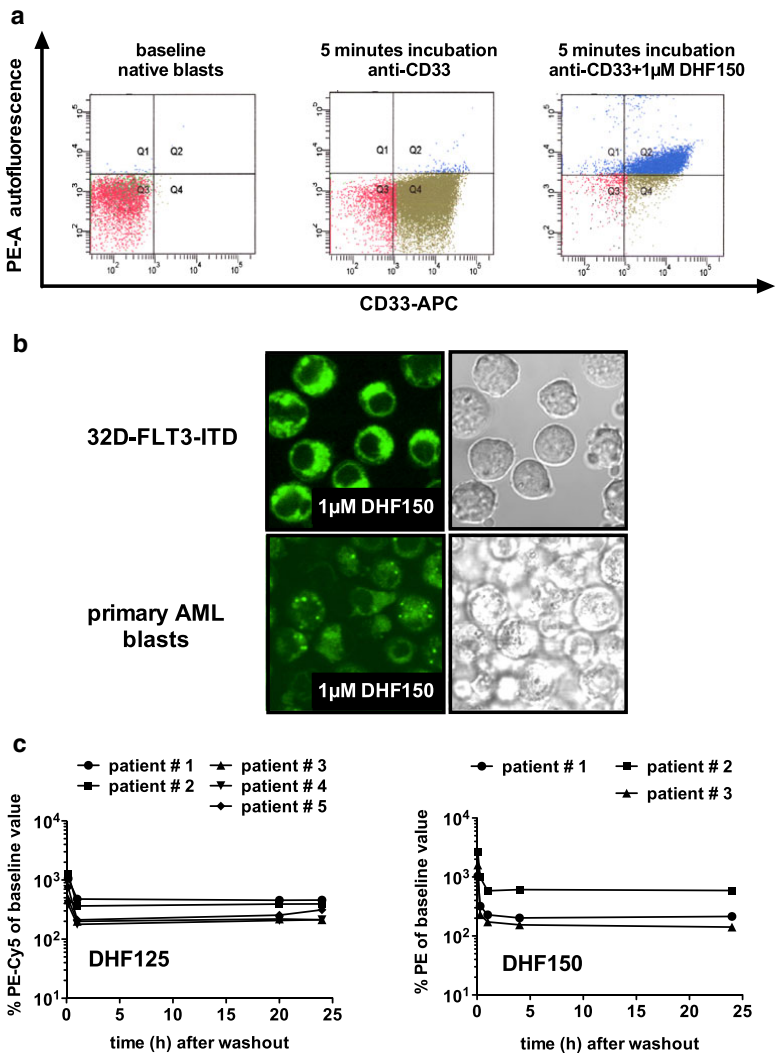

Fig. 5 3,4-Diarylmaleimides are incorporated and retained in primary patient blasts. Primary blasts of FLT3-ITD-positive AML samples were incubated with anti-CD33, APC-conjugated antibodies (according to previous positivity of patient samples in clinical flow cytometry analysis) and 1 μM of either DM inhibitor (**a**). Increase in fluorescence intensity was detected in the majority of patient blasts within 5 min of incubation as evaluated by flow cytometry. Confocal laser microscopy revealed cytoplasmatic accumulation in 32D-FLT3-ITD cells as well as in primary patients AML blasts (**b**). Cells treated with DHF125 or DHF150 (**c**) reached a peak in fluorescence intensity after 5–10 min of incubation. After washout, fluorescence level remained stable within a range of 200–600% of baseline fluorescence for either compound for up to 24 h

blasts with either DHF compound for 20 min. Increase in fluorescence was analyzed as described above. After 20 min, cells were washed twice with PBS and analyzed for fluorescence while cultured in RPMI 1640 medium at different intervals for 24 h. As increase in fluorescence could be due to inhibitor binding to the outer membrane of cells, we performed confocal fluorescence microscopy to determine whether 3,4-diarylmaleimides were accumulated in the cytoplasm. In both, murine 32D-FLT3-ITD cells as well as primary patient blasts, accumulation of 3,4-diarylmaleimide compounds could be detected in the cytoplasm within 5 min after exposure (Fig. 5b). Thus, confocal fluorescence microscopy confirmed that detection of fluorescence using flow cytometry was associated with rapid cytoplasmatic uptake and retention of either compound investigated.

Besides analysis of plasma levels in drug development, pharmacokinetics on a cellular level moves also into focus. As cells can retain inhibitors or even accumulate them in the cytoplasm, drug exposure can be prolonged and more effective. Therefore we aimed to address that specific question by incubating primary patient blasts for 20 min with 1 μM of either inhibitor, followed by washout and analysis of fluorescence levels compared to unstained controls.

For all patients investigated, a rapid uptake within 5–10 min with increase of mean fluorescence intensity of up to 2,600% of baseline values could be demonstrated. Of note, there was a large variability in uptake and accumulation of inhibitors as detected by increase of fluorescence between the different patient samples investigated. After washout, fluorescence levels decreased, but remained elevated up to 200–500% of baseline control within 24 h (Fig. 5c). This was detectable for both compounds with a comparable patient variability. However, none of the samples investigated showed a drop below 100% of baseline values.

In summary, these data show prolonged availability of both diarylmaleimides in the cytoplasm of leukemic cells for a sustained period of up to 24 h. This could be a first indicator for a promising clinical profile, as 3,4-diarylmaleimide may be retained or even accumulated in malignant blasts and thereby ensure a prolonged exposure of mutated FLT3 kinase.

3,4-Diarylmaleimides show a distinct profile for inhibition of FLT3-ITD-induced autophosphorylation

Activity of FLT3 tyrosine kinase inhibitors has been frequently measured using reduction of ITD-induced autophosphorylation as readout in Western blotting experiments. However, up to date few data exist on detailed analysis of the phosphorylation status of individual tyrosine residues upon inhibitor treatment. For example, it is not known, whether specific inhibition of autophosphorylation at distinct residues is required for inhibition of downstream targets and induction of apoptosis. The majority of publications investigating established FLT3 kinase inhibitors used total tyrosine phosphorylation (using the 4G10 antibody) as a correlate for kinase inhibition [32, 37]. In addition, analysis of Y591 phosphorylation has also been used. Recently, phosphorylation of tyrosine residues Y589, Y591, Y599, Y726, Y768, and Y955 has been shown to be involved in constitutive activation of FLT3 kinase induced by presence of ITD length mutations [29].

Given the fact that 3,4-diarylmaleimides acted synergistically with midostaurin in apoptosis assays, we hypothesized the possibility of distinct inhibition patterns using 3,4-diarylmaleimide compounds when compared to midostaurin. Indeed, Western blotting experiments using an FLT3-Y591 detecting phospho-specific antibody confirmed inhibition of autophosphorylation at this particular residue by midostaurin but not by any of the 3,4-diarylmaleimide compounds (Fig. 6a). This was consistent in murine 32D-FLT3-ITD cells (left panel) as well as in the human AML cell line MV4;11 harboring an FLT3-ITD mutation (right panel).

In order to investigate ITD-relevant tyrosine residues, we performed additional FLT3-ITD immunoprecipitation experiments in 32D-FLT3-ITD cells. Interestingly, investigating seven additional tyrosine residues, we found residues affected (1) by midostaurin treatment but not by 3,4-diarylmaleimides, (2) affected by neither group of compounds or (3) affected by both, midostaurin and 3,4-diarylmaleimides (Fig. 6b). None of the tyrosine residues was affected by 3,4-diarylmaleimides but not by midostaurin treatment.

In detail, tyrosine residue Y589 showed reduced phosphorylation upon midostaurin but was not affected by 3,4-diarylmaleimide treatment. Apparently, residues Y842, Y726, Y768, Y599, and Y955 were not significantly affected by any tyrosine kinase inhibitor treatment applied potentially indicating non-informative tyrosine residues in terms of TKI treatment. Residue Y793 showed reduction of autophosphorylation upon incubation with both 3,4-diarylmaleimides and PKC412. Total phosphorylation of FLT3, as determined using the 4G10 phospho-tyrosine antibody revealed a clear inhibitory effect of midostaurin as well as a minor reduction using DHF inhibitors (Fig. 6b). This effect could be seen best in the Y793 panel as no residual activity would be detected after stripping off pY793 and re-probing with 4G10.

These results implicate several novel findings: when investigating distinct tyrosine residues for their involvement in inhibitory activity of FLT3 kinase inhibitors, several residues involved in autophosphorylation caused by the presence of ITD mutations were not affected by midos-

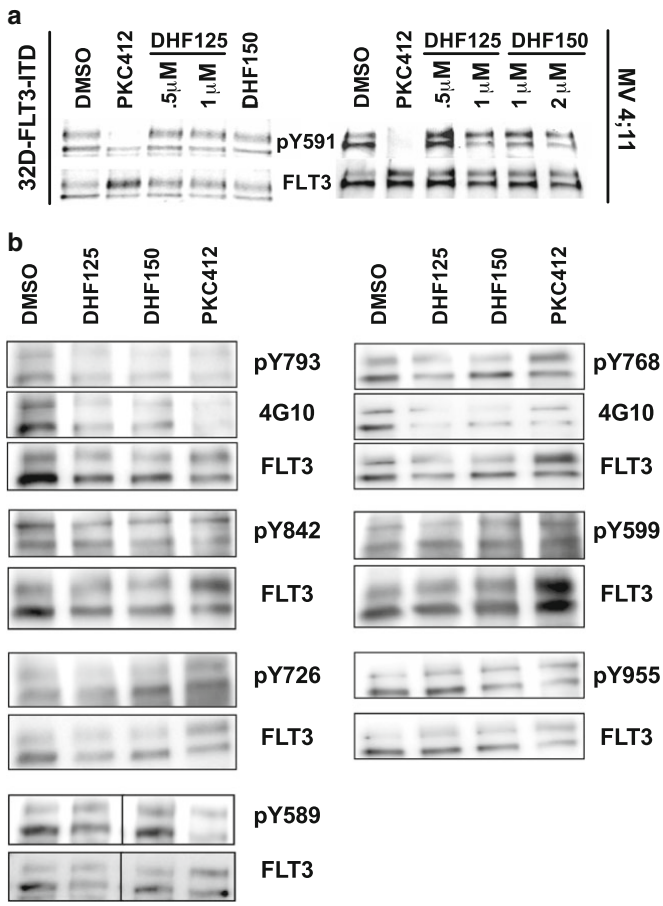

Fig. 6 3,4-Diarylmaleimides inhibit FLT-ITD-mediated autophosphorylation at distinct tyrosine residues compared to midostaurin. **a** Phosphorylation of FLT3 at Y591 (as detected by the P-Flt3-antibody applied) was abrogated by 100 nM of midostaurin (*PKC412*). In contrast, incubation with doses up to 1 μM of either DM compound did not affect autophosphorylation at Y591. **b** Immunoprecipitation experiments reveal distinct inhibition of phosphorylation at residue Y589, which is reduced by midostaurin but neither of the compounds investigated. For residues Y599, Y726, Y768, Y842, and Y955, neither midostaurin nor diarylmaleimides showed any effect on phosphorylation levels. Exclusively Y793 seems to be affected by both, diarylmaleimides and controls. Total FLT3 tyrosine phosphorylation (as detected by 4G10 antibody) showed clear reduction for midostaurin while diarylmaleimides merely caused a slight reduction

taurin. Thus it is tempting to speculate that these residues (namely Y842, Y726, Y768, Y599, and Y955) do not play a crucial role in midostaurin-mediated reduction of kinase activity. Moreover, 3,4-diarylmaleimides seem to carry out their inhibitory activity without affecting tyrosine residues Y589 and Y591, which are believed to play a pivotal role in FLT3-ITD-mediated transformation. Thus, in future studies, the phosphorylation status of these residues will be investigated using a broad spectrum of kinase inhibitors to gain a deeper insight into TKI-induced regulation of ITD-mediated autophosphorylation.

Discussion

AML is a malignant disease with high biological and genetic heterogeneity and survival rates of 30–40% in patients <60 years upon myelosuppressive chemotherapy [2–4]. However, the majority of patients affected by AML are above the age of 60, with a long-term disease-free survival of only 5–15% after myelosuppressive chemotherapy treatment. Besides cytogenetic risk-stratification, molecular markers—such as NPM1 or FLT3 mutations—have shown prognostic impact [14, 15, 19]. Therefore, inhibition of mutated FLT3 tyrosine kinase may improve response rates to chemotherapy and overall survival. Currently, advanced clinical studies are testing this hypothesis, using FLT3 tyrosine kinase inhibitors such as PKC412 or lestaurtinib (CEP-701) in combination with myelosuppressive chemotherapy. The majority of tyrosine kinase inhibitors currently investigated in clinical phase 2–3 trials for FLT3-ITD-positive AML originally have not been designed as "FLT3-inhibitors." Examples are PKC412 being developed as an inhibitor of PKC, BAY 43-9006 (Sorafenib) as

an inhibitor of Ras/Raf or CEP-701 (Lestaurtinib) as an inhibitor of Trkl. As a 'beneficial side effect', these compounds have shown to inhibit FLT3 kinase mostly at low micromolar or nanomolar concentrations. Currently, second-generation inhibitors, developed and designed for inhibition of FLT3 kinase are being tested in clinical and pre-clinical settings. [38] [33]. Here, we describe a novel class of inhibitors that was initially detected in a screening approach for angiogenesis inhibitors. These 3,4-diarylmaleimides are obviously potent inhibitors of mutant FLT3 kinase and display several characteristics that make them a promising group for further clinical development: (1) 3,4-diarylmaleimides are retained in the cytoplasm of exposed cells and suggest a prolonged intracellular exposure of mutant FLT3 kinase, (2) they synergize with chemotherapeutic drugs and notably with midostaurin which may be explained (3) by a distinct inhibition pattern with regard to the phosphorylation status of specific FLT3 tyrosine residues.

Exposure of malignant cells to tyrosine kinase inhibitors is one of the crucial issues in TKI treatment. Several inhibitors did not reach advanced clinical studies due to pharmacokinetic/-dynamic problems and inadequate exposure of target cells for TKI. In addition, uptake into malignant cells displays an important issue in the clinical use of tyrosine kinase inhibitors. Recently, irreversible commitment to apoptosis upon short-term high-dose exposure to TKIs has been suggested as a general mechanism [39].

Using a combination therapy of DM compounds with midostaurin, we could achieve synergy and decent activity against 32D-FLT3-ITD cells. The concentrations applied for PKC412 are achievable, even as trough levels (12.4–157 nM corrected for 99% protein binding) during long-term treatment [24]. Although no studies have been conducted using 3,4-diarylmaleimides in vivo so far, other FLT3 kinase inhibitors (such as Sorafenib) have been shown to reach peak plasma levels up to 10 μM (when given twice daily at 400 mg) [40], suggesting a potential therapeutic window.

More importantly, our experiments provide first evidence for prolonged retention of both diarylmaleimide inhibitors investigated in leukemic cells for a period of up to 24 h, resulting in apoptotic cell death. Thus, both compounds investigated seem to meet the requirements for sufficient intracellular target inhibition.

In general, monotherapy treatment of relapsed or refractory leukemia using tyrosine kinase inhibitors in early clinical trials showed short-lived responses. However, first results from trials using TKI in combination with chemotherapy revealed promising duration of remission rates and suggested further development in clinical phase 3 studies. These trials are currently under way and underline the importance of potential synergistic effects when applied simultaneously or sequentially. 3,4-Diarylmaleimides revealed synergistic effects with both, cytarabine and daunorubicin, which can be considered standard therapeutics for AML treatment. The concept of combining different kinase inhibitors could be a novel approach to overcome resistance in AML therapy.

Recently, several tyrosine residues of FLT3 have been investigated for their differential phosphorylation in FLT3-WT, FLT3-D835Y, and FLT3-ITD kinase [29]. While tyrosine residues Y589, Y591, Y599, Y726, Y768, and Y955 are phosphorylated sites in FLT3-ITD as well as in FLT3-D835Y mutants and FLT3-WT cells upon stimulation with its physiological ligand (Flt3 ligand), Y793 and Y842 seem to be exclusively phosphorylated in FLT3-D835Y mutants [29]. Moreover, functional analysis of tyrosine residues within an FLT3-ITD background has already been performed [41, 42]. Tyrosine to phenylalanine substitution of the FLT3 tyrosine residues Y589 and Y591 on an ITD background led to significant reduction of STAT5 phosphorylation while not affecting tyrosine kinase activity or whole FLT3 tyrosine phosphorylation. As tyrosines Y589 and Y591 were identified as two candidate STAT5 SH2 docking phosphorylation sites in the juxtamembrane (JM) domain, this finding raised the possibility that they might display occult STAT5 binding sites and that ITD mutations expose these sites by conformational disruption of the JM domain. Single amino acid exchanges from Y to F at known tyrosine phosphorylation sites (Y591F, Y726F, Y955F, and Y969F) and even some combined exchanges (such as Y726F/Y955F and Y955F/Y969F) did not lead to significant reduction of STAT5 phosphorylation [41]. Consistent with this finding, single Y to F mutations of Y589, Y591 or Y597 in the ITD background did not reduce FLT3-transforming potential of ITD mutations [42]. However, combined mutations of certain phosphorylation sites, showed an influence on phosphorylation of STAT5 or reduced transforming capacity of ITD mutation (Y589F/Y591F, Y589F/Y597F, Y589F/Y599F, Y591F/Y597F, and Y591F/Y599F).

In our hands, DM inhibitors clearly inhibited or even abrogated phosphorylation of FLT3 downstream targets (such as STAT5) and inhibited FLT3 kinase activity. However, inhibition of phosphorylation at residues affected by midostaurin (such as FLT3-Y591 and FLT3-Y598) was not evident in Western Blottting. This indicates that residual phosphorylation of single tyrosine residues may have no influence on transforming potential or phosphorylation status of important downstream targets such as STAT5. Our data suggest that even Y591 which is thought to be involved in STAT5 binding within an FLT3-ITD background and which has been shown to reduce phosphorylation of downstream targets when mutated to phenylalanine in

combination with cooperating phosphorylation sites may not be crucial for kinase inhibitor function.

Further studies investigating efficacy in cells resistant to currently available FLT3-TKIs and evaluation of toxicity in mouse models are clearly warranted and currently under way. Additional studies investigating differential inhibition patterns of FLT3 autophosphorylation at distinct tyrosine residues and experiments focusing on the exact binding mechanism of 3,4-diarylmaleimides are future aims but were beyond the scope of this report.

Acknowledgement We thank Fian Mirea and Ann-Kathrin Borrmann for technical assistance and Dr. D. Strand (Department of Gastroenterology, University Hospital Mainz) for support with confocal microscopy. This work was supported by grants from the Johannes-Gutenberg-University (MAIFOR, Mainz Research Funding no. 9728249, to F.H.H.) and the German Cancer Aid (DKH 108218 and DKH 108401 (TP6) to T.F.).

Disclosures F. Heidel, J.-P. Kramb, S. Plutitzki, G. Dannhardt, and T. Fischer filed a patent on the use of 3,4-diarylmaleimides in leukemia.

References

1. Godwin JE, Kopecky KJ, Head DR, Willman CL, Leith CP, Hynes HE, Balcerzak SP, Appelbaum FR (1998) A double-blind placebo-controlled trial of granulocyte colony-stimulating factor in elderly patients with previously untreated acute myeloid leukemia: a Southwest Oncology Group Study (9031). Blood 91(10):3607–3615
2. Goldstone AH, Burnett AK, Wheatley K, Smith AG, Hutchinson RM, Clark RE (2001) Attempts to improve treatment outcomes in acute myeloid leukemia (AML) in older patients: the results of the United Kingdom Medical Research Council AML11 trial. Blood 98(5):1302–1311. doi:10.1182/blood.V98.5.1302
3. Lowenberg B, Suciu S, Archimbaud E, Haak H, Stryckmans P, de Cataldo R, Dekker AW, Berneman ZN, Thyss A, van der Lelie J, Sonneveld P, Visani G, Fillet G, Hayat M, Hagemeijer A, Solbu G, Zittoun R (1998) Mitoxantrone versus daunorubicin in induction-consolidation chemotherapy–the value of low-dose cytarabine for maintenance of remission, and an assessment of prognostic factors in acute myeloid leukemia in the elderly: final report. European Organization for the Research and Treatment of Cancer and the Dutch-Belgian Hemato-Oncology Cooperative Hovon Group. J Clin Oncol 16(3):872–881
4. Mayer RJ, Davis RB, Schiffer CA, Berg DT, Powell BL, Schulman P, Omura GA, Moore JO, McIntyre OR, Frei E, The Cancer and Leukemia Group B (1994) Intensive postremission chemotherapy in adults with acute myeloid leukemia. N Engl J Med 331(14):896–903. doi:10.1056/nejm199410063311402
5. Stone RM, Berg DT, George SL, Dodge RK, Paciucci PA, Schulman P, Lee EJ, Moore JO, Powell BL, Schiffer CA, The Cancer and Leukemia Group B (1995) Granulocyte-macrophage colony-stimulating factor after initial chemotherapy for elderly patients with primary acute myelogenous leukemia. N Engl J Med 332(25):1671–1677. doi:10.1056/nejm199506223322503
6. Mrozek K, Heerema NA, Bloomfield CD (2004) Cytogenetics in acute leukemia. Blood Rev 18(2):115–136
7. Langer C, Radmacher MD, Ruppert AS, Whitman SP, Paschka P, Mrozek K, Baldus CD, Vukosavljevic T, Liu CG, Ross ME, Powell BL, de la Chapelle A, Kolitz JE, Larson RA, Marcucci G, Bloomfield CD (2008) High BAALC expression associates with other molecular prognostic markers, poor outcome, and a distinct gene-expression signature in cytogenetically normal patients younger than 60 years with acute myeloid leukemia: a Cancer and Leukemia Group B (CALGB) study. Blood 111(11):5371–5379
8. Marcucci G, Maharry K, Whitman SP, Vukosavljevic T, Paschka P, Langer C, Mrozek K, Baldus CD, Carroll AJ, Powell BL, Kolitz JE, Larson RA, Bloomfield CD (2007) High expression levels of the ETS-related gene, ERG, predict adverse outcome and improve molecular risk-based classification of cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B Study. J Clin Oncol 25(22):3337–3343
9. Mrozek K, Dohner H, Bloomfield CD (2007) Influence of new molecular prognostic markers in patients with karyotypically normal acute myeloid leukemia: recent advances. Curr Opin Hematol 14(2):106–114
10. Heuser M, Beutel G, Krauter J, Dohner K, von Neuhoff N, Schlegelberger B, Ganser A (2006) High meningioma 1 (MN1) expression as a predictor for poor outcome in acute myeloid leukemia with normal cytogenetics. Blood 108(12):3898–3905
11. Dohner K, Schlenk RF, Habdank M, Scholl C, Rucker FG, Corbacioglu A, Bullinger L, Frohling S, Dohner H (2005) Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood 106(12):3740–3746
12. Frohling S, Schlenk RF, Stolze I, Bihlmayr J, Benner A, Kreitmeier S, Tobis K, Dohner H, Dohner K (2004) CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. J Clin Oncol 22(4):624–633
13. Paschka P, Marcucci G, Ruppert AS, Whitman SP, Mrozek K, Maharry K, Langer C, Baldus CD, Zhao W, Powell BL, Baer MR, Carroll AJ, Caligiuri MA, Kolitz JE, Larson RA, Bloomfield CD (2008) Wilms tumor 1 gene mutations independently predict poor outcome in adults with cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B Study. J Clin Oncol 26(28):4595–4602
14. Frohling S, Schlenk RF, Breitruck J, Benner A, Kreitmeier S, Tobis K, Dohner H, Dohner K (2002) Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. Blood 100(13):4372–4380
15. Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U, Wermke M, Bornhauser M, Ritter M, Neubauer A, Ehninger G, Illmer T (2002) Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood 99(12):4326–4335
16. Breitenbuecher F, Schnittger S, Grundler R, Markova B, Carius B, Brecht A, Duyster J, Haferlach T, Huber C, Fischer T (2009) Identification of a novel type of ITD mutations located in nonjuxtamembrane domains of the FLT3 tyrosine kinase receptor. Blood 113(17):4074–4077
17. Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, Sonoda Y, Fujimoto T, Misawa S (1996) Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia 10(12):1911–1918
18. Yokota S, Kiyoi H, Nakao M, Iwai T, Misawa S, Okuda T, Sonoda Y, Abe T, Kahsima K, Matsuo Y, Naoe T (1997) Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. Leukemia 11(10):1605–1609
19. Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK, Linch DC (2008) The impact of FLT3 internal tandem duplication

mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood 111(5):2776–2784
20. Kayser S, Schlenk RF, Correa Londono M, Breitenbuecher F, Wittke K, Du J, Groner S, Spath D, Krauter J, Ganser A, Dohner H, Fischer T, Dohner K (2009) Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. Blood 114 (12):2386–2392
21. Griffith J, Black J, Faerman C, Swenson L, Wynn M, Lu F, Lippke J, Saxena K (2004) The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. Mol Cell 13(2):169–178
22. Kelly LM, Liu Q, Kutok JL, Williams IR, Boulton CL, Gilliland DG (2002) FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. Blood 99 (1):310–318
23. Cools J, Mentens N, Furet P, Fabbro D, Clark JJ, Griffin JD, Marynen P, Gilliland DG (2004) Prediction of resistance to small molecule FLT3 inhibitors: implications for molecularly targeted therapy of acute leukemia. Cancer Res 64(18):6385–6389
24. Heidel F, Solem FK, Breitenbuecher F, Lipka DB, Kasper S, Thiede MH, Brandts C, Serve H, Roesel J, Giles F, Feldman E, Ehninger G, Schiller GJ, Nimer S, Stone RM, Wang Y, Kindler T, Cohen PS, Huber C, Fischer T (2006) Clinical resistance to the kinase inhibitor PKC412 in acute myeloid leukemia by mutation of Asn-676 in the FLT3 tyrosine kinase domain. Blood 107 (1):293–300
25. Graf C, Heidel F, Tenzer S, Radsak MP, Solem FK, Britten CM, Huber C, Fischer T, Wolfel T (2007) A neoepitope generated by an FLT3 internal tandem duplication (FLT3-ITD) is recognized by leukemia-reactive autologous CD8+ T cells. Blood 109(7):2985–2988
26. Dannhardt G, Fischer T, Heidel F, Peifer C, Plutizki S, Kramb JP (2007) Use of 3-(4-indolyl)- or 3-(azaindolyl)-4-arylmaleimide derivatives in leukemia management. Patent no. WO 2009/071620 A1.
27. Peifer C, Krasowski A, Hammerle N, Kohlbacher O, Dannhardt G, Totzke F, Schachtele C, Laufer S (2006) Profile and molecular modeling of 3-(indole-3-yl)-4-(3,4,5-trimethoxyphenyl)-1 H-pyrrole-2,5-dione (1) as a highly selective VEGF-R2/3 inhibitor. J Med Chem 49(25):7549–7553
28. Peifer C, Stoiber T, Unger E, Totzke F, Schachtele C, Marme D, Brenk R, Klebe G, Schollmeyer D, Dannhardt G (2006) Design, synthesis, and biological evaluation of 3,4-diarylmaleimides as angiogenesis inhibitors. J Med Chem 49(4):1271–1281
29. Razumovskaya E, Masson K, Khan R, Bengtsson S, Ronnstrand L (2009) Oncogenic Flt3 receptors display different specificity and kinetics of autophosphorylation. Exp Hematol 37:979–989
30. Chou TC (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58(3):621–681
31. Lipka DB, Hoffmann LS, Heidel F, Markova B, Blum MC, Breitenbuecher F, Kasper S, Kindler T, Levine RL, Huber C, Fischer T (2008) LS104, a non-ATP-competitive small-molecule inhibitor of JAK2, is potently inducing apoptosis in JAK2V617F-positive cells. Mol Cancer Ther 7(5):1176–1184
32. Weisberg E, Boulton C, Kelly LM, Manley P, Fabbro D, Meyer T, Gilliland DG, Griffin JD (2002) Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. Cancer Cell 1(5):433–443
33. Heidel F, Lipka DB, Mirea FK, Mahboobi S, Grundler R, Kancha RK, Duyster J, Naumann M, Huber C, Bohmer FD, Fischer T (2009) Bis(1H-indol-2-yl)methanones are effective inhibitors of FLT3-ITD tyrosine kinase and partially overcome resistance to PKC412A in vitro. Br J Haematol 144(6):865–874
34. Kasper S, Breitenbuecher F, Hoehn Y, Heidel F, Lipka DB, Markova B, Huber C, Kindler T, Fischer T (2008) The kinase inhibitor LS104 induces apoptosis, enhances cytotoxic effects of chemotherapeutic drugs and is targeting the receptor tyrosine kinase FLT3 in acute myeloid leukemia. Leuk Res 32(11):1698–1708
35. Fischer T, Stone RM, Deangelo DJ, Galinsky I, Estey E, Lanza C, Fox E, Ehninger G, Feldman EJ, Schiller GJ, Klimek VM, Nimer SD, Gilliland DG, Dutreix C, Huntsman-Labed A, Virkus J, Giles FJ (2010) Phase IIB trial of oral Midostaurin (PKC412), the FMS-like tyrosine kinase 3 receptor (FLT3) and multi-targeted kinase inhibitor, in patients with acute myeloid leukemia and high-risk myelodysplastic syndrome with either wild-type or mutated FLT3. J Clin Oncol 28(28):4339–4345. doi:10.1200/JCO.2010.28.9678
36. Stone RM, DeAngelo DJ, Klimek V, Galinsky I, Estey E, Nimer SD, Grandin W, Lebwohl D, Wang Y, Cohen P, Fox EA, Neuberg D, Clark J, Gilliland DG, Griffin JD (2005) Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. Blood 105(1):54–60. doi:10.1182/blood-2004-03-0891
37. Levis M, Allebach J, Tse KF, Zheng R, Baldwin BR, Smith BD, Jones-Bolin S, Ruggeri B, Dionne C, Small D (2002) A FLT3-targeted tyrosine kinase inhibitor is cytotoxic to leukemia cells in vitro and in vivo. Blood 99(11):3885–3891
38. Zarrinkar PP, Gunawardane RN, Cramer MD, Gardner MF, Brigham D, Belli B, Karaman MW, Pratz KW, Pallares G, Chao Q, Sprankle KG, Patel HK, Levis M, Armstrong RC, James J, Bhagwat SS (2009) AC220 is a uniquely potent and selective inhibitor of FLT3 for the treatment of acute myeloid leukemia (AML). Blood 114(14):2984–2992
39. Shah NP, Kasap C, Weier C, Balbas M, Nicoll JM, Bleickardt E, Nicaise C, Sawyers CL (2008) Transient potent BCR-ABL inhibition is sufficient to commit chronic myeloid leukemia cells irreversibly to apoptosis. Cancer Cell 14(6):485–493
40. Huber S, Oelsner M, Decker T, Zum Buschenfelde CM, Wagner M, Lutzny G, Kuhnt T, Schmidt B, Oostendorp RA, Peschel C, Ringshausen I (2011) Sorafenib induces cell death in chronic lymphocytic leukemia by translational downregulation of Mcl-1. Leukemia 25(5):838–847. doi:10.1038/leu.2011.2
41. Rocnik JL, Okabe R, Yu JC, Lee BH, Giese N, Schenkein DP, Gilliland DG (2006) Roles of tyrosine 589 and 591 in STAT5 activation and transformation mediated by FLT3-ITD. Blood 108 (4):1339–1345
42. Vempati S, Reindl C, Wolf U, Kern R, Petropoulos K, Naidu VM, Buske C, Hiddemann W, Kohl TM, Spiekermann K (2008) Transformation by oncogenic mutants and ligand-dependent activation of FLT3 wild-type requires the tyrosine residues 589 and 591. Clin Cancer Res 14(14):4437–4445

Paper IV

Biochemical and Biophysical Research Communications xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD

Elena Razumovskaya, Jianmin Sun, Lars Rönnstrand *

Experimental Clinical Chemistry, Wallenberg Laboratory, Department of Laboratory Medicine, Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden

ARTICLE INFO

Article history:
Received 19 July 2011
Available online xxxx

Keywords:
FLT3
MEK5
ERK5
Apoptosis
Proliferation
Receptor tyrosine kinase
Acute myeloid leukemia

ABSTRACT

Fms-like tyrosine kinase-3 (FLT3) is a growth factor receptor normally expressed on hematopoietic progenitor cells. Approximately one third of all patients with AML carry an activating mutation in FLT3 that drives proliferation and survival of the leukemic cells. The most common activating mutation is the so-called internal tandem duplication (ITD), which involves an in-frame duplication of a segment of varying length in the region of the FLT3 gene that encodes the juxtamembrane domain. The pathways downstream of FLT3-ITD are partially known but further knowledge regarding the downstream signal transduction molecules is important in order to develop alternative strategies for pharmacological intervention.

In this paper we have studied the role of MEK/ERK5 in FLT3-ITD mediated transformation. We have found that both wild-type FLT3 and FLT3-ITD activate MEK5 leading to the activation of ERK5. By use of the selective inhibitor of MEK5, BIX02188, we have shown that activation of AKT downstream of FLT3 is partially dependent on ERK5. Furthermore, inhibition of MEK5/ERK5 induces apoptosis of both FLT3-ITD transfected Ba/F3 cells as well as the FLT3-ITD carrying leukemic cell lines MV4-11 and MOLM-13. These results suggest that MEK5/ERK5 is important for FLT3-ITD induced hematopoietic transformation and may thus represent an alternative therapeutic target in the treatment of FLT3-ITD positive leukemia.

1. Introduction

Fms-like tyrosine kinase-3 (FLT3, also called Flk-2) is a receptor tyrosine kinase (RTK) normally expressed on the cell surface of hematopoietic progenitor cells and which functions as inducer of proliferation, differentiation and survival of those cells [1,2]. Wild-type FLT3 is activated by binding of FLT3 ligand (FL) which causes dimerization and autophosphorylation of multiple tyrosine residues on the receptor and will in turn recruit signal transduction proteins and activate downstream signaling cascades [3].

The most common activating mutation of FLT3 is the so-called internal tandem duplication (ITD) located in the juxtamembrane domain of the receptor which leads to constitutive activation of the receptor even in the absence of ligand [4–6]. This mutation is the most frequent mutation in acute myeloid leukemia (AML) occurring in about 17–26% of patients with AML and is associated with a poor prognosis [7,8]. Several downstream signal transduction pathways have been shown to be of importance for transformation, including the ERK/MAP kinase pathway, the PI3-kinase/AKT pathway and STAT5A [9]. However, a more detailed analysis of downstream signaling targets is necessary in order to understand the process of leukemogenesis and also to provide us with diagnostic tools and potential targets for pharmacological intervention.

The mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated protein kinases (ERKs) are a family of serine/threonine kinases that transduce signals downstream of activated receptor tyrosine kinases and play an essential role in cell proliferation, differentiation, migration, survival, gene expression, cell cycle arrest and apoptosis, depending on the cell type. Several subfamilies are known including the ERK family of mitogen activated kinases, as well as the stress-activated kinases JNK and p38. An additional member of the MAP kinase family, extracellular signal-regulated protein kinase 5 (ERK5) was cloned in 1995 by Dixon and co-workers. The N-terminal kinase domain of ERK5 shares 50% homology with ERK1/2 [11,12] and is important for cytoplasmic targeting, interaction with the upstream MAPK kinase MEK5 and for oligomerization. The C-terminal domain is a unique for ERK5. It encodes two proline-rich regions (PR1 and PR2), as well as nuclear localization and nuclear export signals which are responsible for nuclear shuttling of ERK5 [13]. It has been shown that the C-terminal domain undergoes autophosphorylation at

* Corresponding author. Fax: +46 40 33 11 04.
E-mail address: Lars.Ronnstrand@med.lu.se (L. Rönnstrand).
URL: http://www.ronnstrandlab.com (L. Rönnstrand).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.07.089

Please cite this article in press as: E. Razumovskaya et al., Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD, Biochem. Biophys. Res. Commun. (2011), doi:10.1016/j.bbrc.2011.07.089

several sites upon its activation which lead to conformational change that exposes the docking sites and the nuclear localization signal [13]. The phosphorylation of the C-terminus plays role in regulation of gene expression [14–16]. It has been shown that ERK5 interacts directly with MEK5 but not with MEK1/2 [10]. ERK5 can be activated by oxidative stress, hyperosmolarity, serum [17], and several growth factors like nerve growth factor (NGF), epidermal growth factor (EGF) [18–21] and platelet-derived growth factor (PDGF) via dual phosphorylation of the TEY motif by MEK5 [22–25]. ERK5 has been associated with cell survival, proliferation and migration [12,26,27]. Important role of ERK5 has been shown in phosphorylation of BAD and AKT, suppression of caspase-3 cleavage and inhibition of nuclear apoptotic alterations, thus explaining the anti-apoptotic function of ERK5. [24,27,28].

In the present study, we show that activation of FLT3 leads to activation of ERK5. By inhibiting the activation of ERK5 by the selective MEK5 inhibitor BIX02188 [29] we also demonstrate a decreased phosphorylation of AKT in Ba/F3 cells expressing wild-type FLT3 as well as in cells expressing FLT3-ITD. Interestingly, cells expressing the oncogenic FLT3-ITD mutant were more sensitive to the inhibitor than cells expressing wild-type FLT3. Treatment of Ba/F3 cells expressing FLT3-ITD with the BIX02188 inhibitor induced apoptosis in a dose-dependent manner. These results were confirmed in human AML cell lines, MV4-11 and MOLM-13 which express FLT3-ITD endogenously.

2. Material and methods

2.1. Cell cultures

MV4-11 cells was obtained from ATCC, USA. Ba/F3 and MOLM-13 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were cultured in RPMI-1640 medium (Thermo Scientific HyClone, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria), 10 ng/mL of murine IL-3 (ProSpec Tany, Ness-Ziona, Israel), 100 units/mL penicillin and streptomycin (PAA Laboratories GmbH, Pasching, Austria). Ba/F3 cells expressing either wild-type or mutant FLT3 were generated as previously described [11]. All cell lines were cultured in a humidified incubator at 37 °C with 5% CO_2.

2.2. Reagents/Antibodies

BIX02188 was synthesized by Shanghai Yingxuan Chempharm Co., Ltd. (Shanghai, China). U-0126 was from Larodan Fine Chemials AB (Malmö, Sweden). Anti-phosphotyrosine antibody 4G10 was from Millipore (Billerica, MA), Anti-FLT3 antibody has been described [30], pERK5 (Thr218/Tyr220) from Cell Signaling Technology (Danvers, MA), ERK1/2 and ERK5 were detected by an antibody recognizing ERK1, ERK2 and ERK5. The antibody was raised against the synthetic peptide DHTGFLTEYVATRWC and affinity purified essentially as described [31]. AKT antibody from Santa Cruz Biotechnology (Santa Cruz, CA) and pAKT antibody was from Epitomics (Burlingame, CA).

2.3. Cell proliferation/survival assay (Trypan Blue)

Ba/F3, MV4-11 or MOLM-13 cells were washed three times with PBS prior to cell proliferation assays. Cell proliferation assay was performed by incubating cells in media in the presence or absence of IL-3 or with FL and in the presence or absence of different concentrations of BIX02188. DMSO was used as control for BIX02188. Cells were seeded at 70,000 cells/well in 24-well plates and incubated with the different media for 48 h. Viable cells were counted using Countess™ Automated Cell Counter and trypan blue dye (Invitrogen, CarlsBAD, CA).

2.4. Apoptosis assay

Apoptosis was measured by PE Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen, CA, USA), according to the manufacture's instructions. FACSort instrument (BD Biosciences, CA, USA) was used to perform flow cytometry. Results were analyzed by CellQuest software (Becton Dickinson, CA, USA).

2.5. Cell stimulation, lysis, immunoprecipitation, SDS–PAGE and Western blotting

Ba/F3 cells were starved for 4 h in RPMI-1640 medium (Thermo Scientific HyClone, Logan, UT) without HI-FBS and IL-3. Cells stimulation, immunoprecipitation and Western blotting of lysates were done as described elsewhere [32]. Immunodetection was performed by enhanced chemoluminescence using Immobilon Western chemoluminescent HRP substrate (Millipore, Billerica, MA) according to the manufacture's instructions. Bands were detected by CCD camera (LAS-3000, Fujifilm, Tokyo, Japan) and quantified using MultiGauge software (Fujifilm).

2.6. Data presentation and statistical analysis

All experiments were repeated at least 3 times and one representative experiment is presented. For quantifications the averages of three independent experiments and error bars showing standard deviations were used.

3. Results

3.1. Ligand stimulation of FLT3 induces phosphorylation of ERK5

In order to assess the involvement of ERK5 downstream of FLT3, we used Ba/F3 cells stably transfected with either wild-type FLT3 or FLT3-ITD. Following ligand stimulation, FLT3 was rapidly activated with peak phosphorylation occurring at around 5 min of stimulation followed by a decreased receptor phosphorylation (Fig. 1). ERK5 was fully phosphorylated already at 2 min of stimulation and remained high for at least 30 min. In cells expressing the constitutively active mutant FLT3-ITD, constitutive ERK5 phosphorylation was, but increased upon ligand stimulation of the receptor and remained high for at least 30 min.

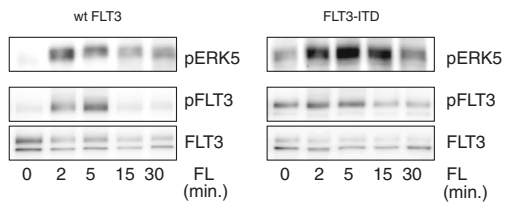

Fig. 1. Ligand stimulation of FLT3 receptor activates ERK5. Ba/F3 cells stably transfected with either wild-type FLT3 or FLT3-ITD, were starved for 4 h and stimulated with FL for the indicated periods of time, lysed and subjected to immunoprecipitation (IP) with antibodies against FLT3 and/or total cell lysates (TCL) were collected for ERK5 identification. The samples were analyzed by Western blotting and the membranes with IP samples were probed with antibodies against phospho-tyrosine (pFLT3) and then FLT3. The membranes with TCL samples were probed with pERK5 antibodies.

Please cite this article in press as: E. Razumovskaya et al., Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD, Biochem. Biophys. Res. Commun. (2011), doi:10.1016/j.bbrc.2011.07.089

3.2. The MEK5 selective inhibitor BIX02188 reduces phosphorylation of ERK5, ERK1/2 and AKT in FLT3 expressing Ba/F3 cells

In order to assess the impact of ERK5 activation on signaling downstream of FLT3 as well as its role in proliferation and survival signals, we employed the MEK5 selective inhibitor BIX02188 [29]. Since MEK5 is the kinase upstream of ERK5, inhibition of MEK5 leads to a decreased ERK5 phosphorylation. Ba/F3 cells expressing wild-type FLT3 were incubated with different concentrations of the MEK5 inhibitor, BIX02188. Phosphorylation of ERK5 was almost completely blocked by the inhibitor (Fig. 2). A marked reduction of ligand-induced AKT phosphorylation was also seen, despite the fact that AKT is reported not to be sensitive to BIX02188 [29]. Surprisingly, the phosphorylation of ERK1/2 was also partially reduced. Given the fact that neither MEK1/2 or ERK1/2 are inhibited by BIX02188 at the concentration used [29], this suggests that MEK5 could act upstream of ERK1/2. Cells expressing the oncogenic mutant FLT3-ITD responded similarly to BIX02188, although the inhibition of ERK5 was seen already at 6 μM of inhibitor. As we have previously shown, despite the fact that FLT3-ITD is constitutively active, we still see ligand-induced AKT and ERK phosphorylation which is partially inhibited by BIX02188.

3.3. Inhibition of MEK5 decreases proliferation and survival in FLT3-ITD expressing Ba/F3 cells

Ba/F3 cells expressing wild-type FLT3 or FLT3-ITD were incubated in the absence or presence of 12 μM BIX02188 for 48 h. Cells were stained with trypan blue and the number of viable cells was counted in an automated cell counter. Cultures of cells expressing wild-type FLT3 had almost the same number of cells after incubation in the absence (black bars) or presence (white bars) of BIX02188, respectively (Fig 3A). In contrast, cells expressing FLT3-ITD mutation had a decreased number of living cells in the presence of BIX02188 compared to medium without inhibitor (Fig. 3C). In order to analyze the apoptosis rate of wild-type FLT3 or FLT3-ITD expressing Ba/F3 cells we used the PE Annexin V Apoptosis Detection Kit. Cells were stained with PE Annexin V and 7-AAD and analyzed by flow cytometry according to the manufacturer's protocol. In the absence of ligand-stimulation, wild-type FLT3 expressing Ba/F3 cells showed no significant difference in the amount of apoptotic cells in the presence or absence of BIX02188 inhibitor (Fig. 3B). Cells stimulated with FL were however sensitive to treatment with BIX02188, but the most striking effect could be seen in FLT3-ITD expressing Ba/F3 cells (Fig. 3D). In order to rule out a general effect of BIX02188 on cell proliferation and survival, cells were grown in the presence of IL-3 which is known to support growth and survival of the Ba/F3 cells. Under those conditions, the BIX02188 inhibitor had no effect on proliferation or survival of the Ba/F3 cells. This indicates that the effect we are seeing is specific for FLT3 and not a general effect on the signal transduction machinery of the cells.

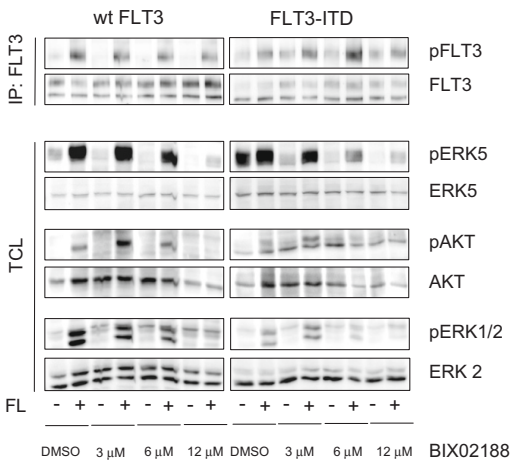

Fig. 2. BIX02188 inhibitor blocks the activation of MEK5/ERK5 and AKT in Ba/F3 cells expressing wild-type FLT3 or FLT3-ITD. Ba/F3 cells expressing wild-type FLT3 or FLT3-ITD were starved in medium containing different concentrations of BIX02188 for 4 h. Cells were then stimulated with FL for 0 or 5 min, and lysed. pERK5, pERK1/2 and pAKT were detected by immunoblotting of TCL. IP of FLT3 followed by Western blotting was used to detect the degree of phosphorylation of FLT3 and the total FLT3 expression Cells incubated in medium containing DMSO were used as controls.

3.4. Effect of BIX02188 on FLT3-ITD downstream signaling, proliferation and apoptosis in MV4-11 and MOLM-13 leukemic cells

In order to analyze the influence of BIX02188 in leukemic cell signaling, we incubated the leukemic cell lines MV4-11 and MOLM-13, which both express FLT3-ITD endogenously, with varying concentrations of the MEK5 inhibitor BIX02188 (Fig 4A). In both MOLM-13 and MV4-11 cells, ERK5 activation was inhibited by BIX02188, but MOLM-13 were slightly more sensitive to the inhibitor. This difference in sensitivity to BIX02188 between MOLM-13 and MV4-11 was also reflected the ability of the inhibitor to induce apoptosis and reduced proliferative rate between MOLM-13 and MV4-11 (Fig. 4B). It should be noted that also the pERK1/2 signal is decreased to some extent in the presence of BIX02188, despite the fact that ERK1/2 are not directly affected by the inhibitor at the concentrations used [29].

4. Discussion

In this study we demonstrate that ERK5 is activated downstream of both the wild-type FLT3 as well as in the oncogenic mutant FLT3-ITD commonly found in patients suffering from acute myeloid leukemia. By use of the selective MEK5 inhibitor BIX02188, we were able to inhibit the activation of ERK5, but we also noticed that there was a dose-dependent decrease in phosphorylation of both AKT and ERK1/2. Previous work has shown that the inhibitor is selective for MEK5 and that, at the concentration used in these experiments, AKT or ERK1/2 are not affected [29]. Thus, it is likely that AKT and ERK1/2 at least to some extent lie downstream of MEK5 in signaling. Similarly, in the closely related platelet-derived growth factor receptor β, ligand-induced AKT activation is decreased upon knockdown of ERK5 expression [33]. Also in the VEGF receptor system, ERK5 is involved in regulation of AKT phosphorylation which is further linked to anti-apoptotic signaling [34]. Since it is well known that AKT is involved in anti-apoptotic signaling via diverse signaling pathways, such as phosphorylation of BAD [35] or phosphorylation of FOXO3A [36], we investigated whether inhibition of ERK5 activation could influence proliferation and survival in both ligand-stimulated wild-type FLT3 and the constitutively active mutant FLT3-ITD. We could clearly demonstrate that inhibition of MEK5 led to a decreased proliferative rate and an even more pronounced increase of apoptotic death. The FLT3-ITD mutation leads to a constitutively active receptor tyrosine kinase and induces proliferative signals as well as survival signals. Therefore we wondered whether the effect of BIX02188 on Ba/F3 cells expressing FLT3-ITD could also be seen in FLT3-ITD expressing leukemic cells derived from patients with acute myeloid

Please cite this article in press as: E. Razumovskaya et al., Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD, Biochem. Biophys. Res. Commun. (2011), doi:10.1016/j.bbrc.2011.07.089

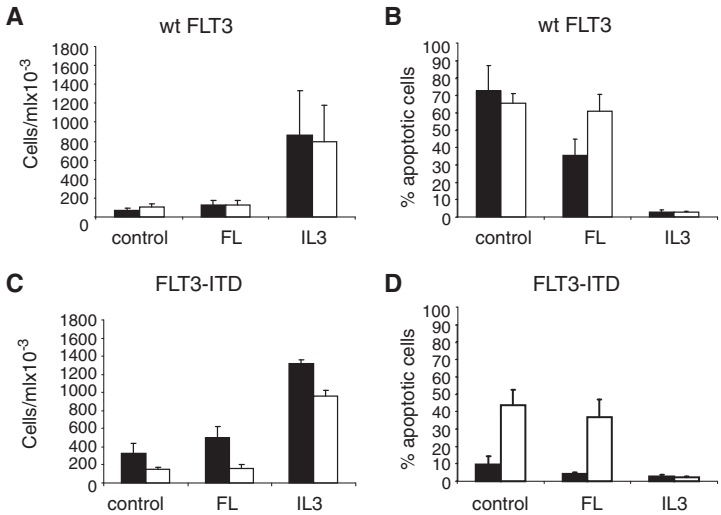

Fig. 3. Proliferation and survival of Ba/F3 expressing wild-type FLT3 or FLT3-ITD in the presence or absence of BIX02188 inhibitor. Cells were incubated in different media with or without 12 μM BIX02188 for 48 h. DMSO was used as control for BIX02188. Viable cells were counted with trypan blue dye exclusion (A and C). Cells were stained with PE Annexin V and 7-AAD and analyzed by flow cytometry. Cells were incubated either in the absence (white bars) or presence (black bars) of BIX02188 (B and D).

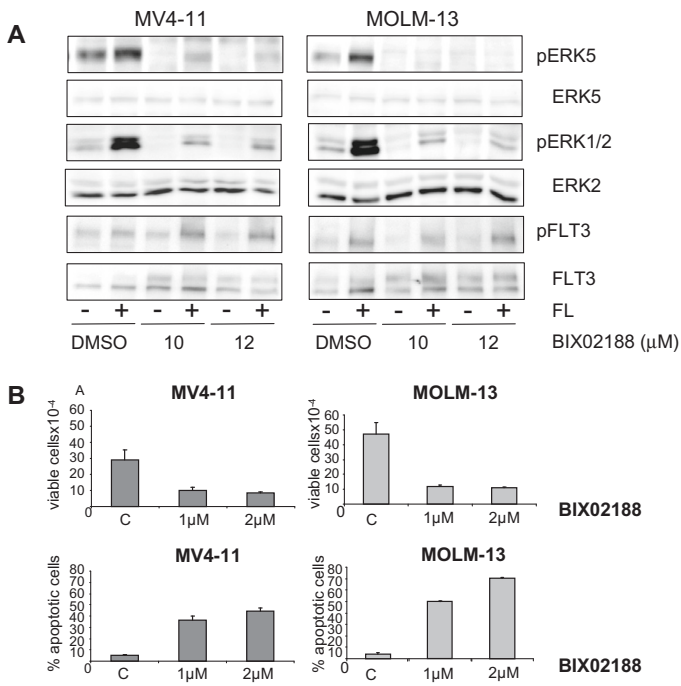

Fig. 4. Effect of BIX02188 on downstream signaling, proliferation and apoptosis in MV4-11 and MOLM-13 leukemic cell lines. (A) Cells were starved for 4 h and stimulated with ligand for 5 min, lysed and subjected to immunoprecipitation of FLT3 and TCL were collected for ERK5 identification. Samples were separated with SDS–polyacrylamide electrophoresis, electrotransferred to Immobilon filters and probed with antibodies against phospho-tyrosine (pFLT3) and then FLT3. The membranes with TCL samples were probed with pERK5 and then re-probed with pERK1/2, ERK5 and ERK2 antibodies. (B) Cells were incubated in media with different concentrations of BIX02188 for 48 h and then analyzed by trypan blue dye exclusion assay (proliferation) and stained with Annexin V-PE apoptosis kit and analyzed by flow cytometry (apoptosis).

Please cite this article in press as: E. Razumovskaya et al., Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD, Biochem. Biophys. Res. Commun. (2011), doi:10.1016/j.bbrc.2011.07.089

leukemia. For this purpose we utilized the MV4-11 and MOLM-13 cell lines. MV4-11 is homozygous with respect to the FLT3-ITD mutation [37], whereas the MOLM-13 cell expresses both wild-type FLT3 as well as FLT3-ITD [38]. It has been speculated that the presence or absence of wild-type FLT3 could influence the behavior of FLT3-ITD expressing AML cells. FLT3-ITD is known to be weakly expressed on the cell surface as a result of trapping within the endoplasmic reticulum [39]. The presence of a wild-type allele could simplify recruitment to the cell surface where it could encounter ligand. Despite the fact that the FLT3-ITD is a constitutively active mutant, its activity can be further increased by binding of its ligand [40]. In our experiments we could show that the MOLM-13 cells were more susceptible to induction of apoptosis by the BIX02188 inhibitor than the MV4-11 cells, which maybe could be explained to the presence of two copies of FLT3-ITD in the MV4-11 cells.

A role of ERK5 has been implicated in cancer through several lines of observation. ERK5 has been shown to be highly expressed in oral squamous cell carcinoma where it is associated with the later, metastatic stages [41]. Furthermore, knockdown of ERK5 in leukemic T cells decreased nuclear accumulation of the NF-kB p65 subunit and suppressed the induction of tumors in mice [42]. It was recently demonstrated that ERK5 is involved in regulating the activity of the tumor suppressor PML by direct phosphorylation [43].

In this study we have shown a role of ERK5 in FLT3-ITD mediated leukemogenesis. Given the inherent problems with the presently available pharmacological inhibitors of FLT3 (primary resistance [44] as well as acquired resistance to inhibitors [45]), ERK5 provides us with a potential target for pharmacological intervention in AML patients with FLT3-ITD mutations. Future studies are aiming at using the BIX02188 inhibitor in animal studies of the FLT3-ITD expressing leukemic cell lines.

Acknowledgments

We want to thank Corrado Cilio and Per-Anders Bertilsson for helping with flow cytometry analysis.

This work was supported by grants form the Swedish Cancer Society, the Swedish Children's Cancer Society, the Swedish Research Council, The Knut and Alice Wallenberg Foundation, Skåne University Hospital Foundation, Malmö University Hospital Cancer Foundation, the Biocare Network- Biomarkers in Cancer Medicine, and the Royal Physiographical Society, Lund.

References

[1] N. Maroc, R. Rottapel, O. Rosnet, S. Marchetto, C. Lavezzi, P. Mannoni, D. Birnbaum, P. Dubreuil, Biochemical characterization and analysis of the transforming potential of the FLT3/FLK2 receptor tyrosine kinase, Oncogene 8 (1993) 909–918.

[2] A.M. Turner, N.L. Lin, S. Issarachai, S.D. Lyman, V.C. Broudy, FLT3 receptor expression on the surface of normal and malignant human hematopoietic cells, Blood 88 (1996) 3383–3390.

[3] O. Rosnet, H.J. Bühring, O. deLapeyriere, N. Beslu, C. Lavagna, S. Marchetto, I. Rappold, H.G. Drexler, F. Birg, R. Rottapel, C. Hannum, P. Dubreuil, D. Birnbaum, Expression and signal transduction of the FLT3 tyrosine kinase receptor, Acta Haematol. 95 (1996) 218–223.

[4] H. Kiyoi, M. Towatari, S. Yokota, M. Hamaguchi, R. Ohno, H. Saito, T. Naoe, Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product, Leukemia 12 (1998) 1333–1337.

[5] H. Kiyoi, R. Ohno, R. Ueda, H. Saito, T. Naoe, Mechanism of constitutive activation of FLT3 with internal tandem duplication in the juxtamembrane domain, Oncogene 21 (2002) 2555–2563.

[6] J. Griffith, J. Black, C. Faerman, L. Swenson, M. Wynn, F. Lu, J. Lippke, K. Saxena, The structural basis for autoinhibition of FLT3 by the juxtamembrane domain, Mol. Cell 13 (2004) 169–178.

[7] M. Nakao, S. Yokota, T. Iwai, H. Kaneko, S. Horiike, K. Kashima, Y. Sonoda, T. Fujimoto, S. Misawa, Internal tandem duplication of the flt3 gene found in acute myeloid leukemia, Leukemia 10 (1996) 1911–1918.

[8] D.G. Gilliland, J.D. Griffin, The roles of FLT3 in hematopoiesis and leukemia, Blood 100 (2002) 1532–1542.

[9] B. Scheijen, J.D. Griffin, Tyrosine kinase oncogenes in normal hematopoiesis and hematological disease, Oncogene 21 (2002) 3314–3333.

[10] G. Zhou, Z.Q. Bao, J.E. Dixon, Components of a new human protein kinase signal transduction pathway, J. Biol. Chem. 270 (1995) 12665–12669.

[11] S. Nishimoto, E. Nishida, MAPK signalling: ERK5 versus ERK1/2, EMBO Rep. 7 (2006) 782–786.

[12] X. Wang, C. Tournier, Regulation of cellular functions by the ERK5 signalling pathway, Cell. Signal. 18 (2006) 753–760.

[13] M. Buschbeck, A. Ullrich, The unique C-terminal tail of the mitogen-activated protein kinase ERK5 regulates its activation and nuclear shuttling, J. Biol. Chem. 280 (2005) 2659–2667.

[14] Y. Kato, V.V. Kravchenko, R.I. Tapping, J. Han, R.J. Ulevitch, J.D. Lee, BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C, EMBO J. 16 (1997) 7054–7066.

[15] C. Yan, H. Luo, J.D. Lee, J. Abe, B.C. Berk, Molecular cloning of mouse ERK5/BMK1 splice variants and characterization of ERK5 functional domains, J. Biol. Chem. 276 (2001) 10870–10878.

[16] H. Morimoto, K. Kondoh, S. Nishimoto, K. Terasawa, E. Nishida, Activation of a C-terminal transcriptional activation domain of ERK5 by autophosphorylation, J. Biol. Chem. 282 (2007) 35449–35456.

[17] J. Abe, M. Kusuhara, R.J. Ulevitch, B.C. Berk, J.D. Lee, Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase, J. Biol. Chem. 271 (1996) 16586–16590.

[18] Y. Kato, R.I. Tapping, S. Huang, M.H. Watson, R.J. Ulevitch, J.D. Lee, Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor, Nature 395 (1998) 713–716.

[19] S. Kamakura, T. Moriguchi, E. Nishida, Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus, J. Biol. Chem. 274 (1999) 26563–26571.

[20] F.L. Watson, H.M. Heerssen, A. Bhattacharyya, L. Klesse, M.Z. Lin, R.A. Segal, Neurotrophins use the Erk5 pathway to mediate a retrograde survival response, Nat. Neurosci. 4 (2001) 981–988.

[21] Y. Obara, A. Yamauchi, S. Takehara, W. Nemoto, M. Takahashi, P.J. Stork, N. Nakahata, ERK5 activity is required for nerve growth factor-induced neurite outgrowth and stabilization of tyrosine hydroxylase in PC12 cells, J. Biol. Chem. 284 (2009) 23564–23573.

[22] Y. Izawa, M. Yoshizumi, K. Ishizawa, Y. Fujita, S. Kondo, S. Kagami, K. Kawazoe, K. Tsuchiya, S. Tomita, T. Tamaki, Big mitogen-activated protein kinase 1 (BMK1)/extracellular signal regulated kinase 5 (ERK5) is involved in platelet-derived growth factor (PDGF)-induced vascular smooth muscle cell migration, Hypertens. Res. 30 (2007) 1107–1117.

[23] E. Rovida, N. Navari, A. Caligiuri, P. Dello Sbarba, F. Marra, ERK5 differentially regulates PDGF-induced proliferation and migration of hepatic stellate cells, J. Hepatol. 48 (2008) 107–115.

[24] J. Lennartsson, F. Burovic, B. Witek, A. Jurek, C.H. Heldin, Erk 5 is necessary for sustained PDGF-induced Akt phosphorylation and inhibition of apoptosis, Cell Signal 22 (2010) 955–960.

[25] N. Mody, D.G. Campbell, N. Morrice, M. Peggie, P. Cohen, An analysis of the phosphorylation and activation of extracellular-signal-regulated protein kinase 5 (ERK5) by mitogen-activated protein kinase kinase 5 (MKK5) in vitro, Biochem. J. 372 (2003) 567–575.

[26] J.E. Cavanaugh, Role of extracellular signal regulated kinase 5 in neuronal survival, Eur. J. Biochem. 271 (2004) 2056–2059.

[27] K.G. Finegan, X. Wang, E.J. Lee, A.C. Robinson, C. Tournier, Regulation of neuronal survival by the extracellular signal-regulated protein kinase 5, Cell Death Differ. 16 (2009) 674–683.

[28] X. Wang, K.G. Finegan, A.C. Robinson, L. Knowles, R. Khosravi-Far, K.A. Hinchliffe, R.P. Boot-Handford, C. Tournier, Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress, Cell Death Differ. 13 (2006) 2099–2108.

[29] R.J. Tatake, M.M. O'Neill, C.A. Kennedy, A.L. Wayne, S. Jakes, D. Wu, S.Z. Kugler Jr., M.A. Kashem, P. Kaplita, R.J. Snow, Identification of pharmacological inhibitors of the MEK5/ERK5 pathway, Biochem. Biophys. Res. Commun. 377 (2008) 120–125.

[30] E. Heiss, K. Masson, C. Sundberg, M. Pedersen, J. Sun, S. Bengtsson, L. Rönnstrand, Identification of Y589 and Y599 in the juxtamembrane domain of Flt3 as ligand-induced autophosphorylation sites involved in binding of Src family kinases and the protein tyrosine phosphatase SHP2, Blood 108 (2006) 1542–1550.

[31] O. Voytyuk, J. Lennartsson, A. Mogi, G. Caruana, S. Courtneidge, L.K. Ashman, L. Rönnstrand, Src family kinases are involved in the differential signaling from two splice forms of c-Kit, J. Biol. Chem. 278 (2003) 9159–9166.

[32] M. Pedersen, L. Rönnstrand, J. Sun, The c-Kit/D816V mutation eliminates the differences in signal transduction and biological responses between two isoforms of c-Kit, Cell. Signal. 21 (2009) 413–418.

[33] J. Lennartsson, F. Burovic, B. Witek, A. Jurek, C.H. Heldin, Erk 5 is necessary for sustained PDGF-induced Akt phosphorylation and inhibition of apoptosis, Cell. Signal. 22 (2010) 955–960.

[34] O.L. Roberts, K. Holmes, J. Müller, D.A. Cross, M.J. Cross, ERK5 is required for VEGF-mediated survival and tubular morphogenesis of primary human microvascular endothelial cells, J. Cell Sci. 123 (2010) 3189–3200.

[35] P. Blume-Jensen, R. Janknecht, T. Hunter, The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136, Curr. Biol. 8 (1998) 779–782.

Please cite this article in press as: E. Razumovskaya et al., Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD, Biochem. Biophys. Res. Commun. (2011), doi:10.1016/j.bbrc.2011.07.089

[36] C. Möller, J. Alfredsson, M. Engström, H. Wootz, Z. Xiang, J. Lennartsson, J.I. Jönsson, G. Nilsson, Stem cell factor promotes mast cell survival via inactivation of FOXO3a-mediated transcriptional induction and MEK-regulated phosphorylation of the proapoptotic protein Bim, Blood 106 (2005) 1330–1336.
[37] M. Levis, J. Allebach, K.F. Tse, R. Zheng, B.R. Baldwin, B.D. Smith, S. Jones-Bolin, B. Ruggeri, C. Dionne, D. Small, A FLT3-targeted tyrosine kinase inhibitor is cytotoxic to leukemia cells in vitro and in vivo, Blood 99 (2002) 3885–3891.
[38] H. Quentmeier, J. Reinhardt, M. Zaborski, H.G. Drexler, FLT3 mutations in acute myeloid leukemia cell lines, Leukemia 17 (2003) 120–124.
[39] D. Schmidt-Arras, S.A. Böhmer, S. Koch, J.P. Müller, L. Blei, H. Cornils, R. Bauer, S. Korasikha, C. Thiede, F.D. Böhmer, Anchoring of FLT3 in the endoplasmic reticulum alters signaling quality, Blood 113 (2009) 3568–3576.
[40] R. Zheng, E. Bailey, B. Nguyen, X. Yang, O. Piloto, M. Levis, D. Small, Further activation of FLT3 mutants by FLT3 ligand, Oncogene (2011).
[41] C. Sticht, K. Freier, K. Knöpfle, C. Flechtenmacher, S. Pungs, C. Hofele, M. Hahn, S. Joos, P. Lichter, Activation of MAP kinase signaling through ERK5 but not ERK1 expression is associated with lymph node metastases in oral squamous cell carcinoma (OSCC), Neoplasia 10 (2008) 462–470.
[42] J. Garaude, S. Cherni, S. Kaminski, E. Delepine, C. Chable-Bessia, M. Benkirane, J. Borges, A. Pandiella, M.A. Iniguez, M. Fresno, R.A. Hipskind, M. Villalba, ERK5 activates NF-kappaB in leukemic T cells and is essential for their growth in vivo, J. Immunol. 177 (2006) 7607–7617.
[43] Q. Yang, X. Deng, B. Lu, M. Cameron, C. Fearns, M.P. Patricelli, J.R. Yates 3rd, N.S. Gray, J.D. Lee, Pharmacological inhibition of BMK1 suppresses tumor growth through promyelocytic leukemia protein, Cancer Cell 18 (2010) 258–267.
[44] R. Grundler, C. Thiede, C. Miething, C. Steudel, C. Peschel, J. Duyster, Sensitivity toward tyrosine kinase inhibitors varies between different activating mutations of the FLT3 receptor, Blood 102 (2003) 646–651.
[45] K. Bagrintseva, R. Schwab, T.M. Kohl, S. Schnittger, S. Eichenlaub, J.W. Ellwart, W. Hiddemann, K. Spiekermann, Mutations in the tyrosine kinase domain of FLT3 define a new molecular mechanism of acquired drug resistance to PTK inhibitors in FLT3-ITD-transformed hematopoietic cells, Blood 103 (2004) 2266–2275.

Please cite this article in press as: E. Razumovskaya et al., Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD, Biochem. Biophys. Res. Commun. (2011), doi:10.1016/j.bbrc.2011.07.089

Biochemical and Biophysical Research Communications 415 (2011) 214

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Corrigendum

Corrigendum to "Inhibition of MEK5 by BIX02188 induces apoptosis in cells expressing the oncogenic mutant FLT3-ITD" [Biochem. Biophys. Res. Commun. 412 (2011) 307–312]

Elena Razumovskaya, Jianmin Sun, Lars Rönnstrand *

Experimental Clinical Chemistry, Wallenberg Laboratory, Department of Laboratory Medicine, Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden

The author regrets an error in the published Fig. 3 legend, which appears correctly below:

Fig. 3. Proliferation and survival of Ba/F3 expressing wild-type FLT3 or FLT3-ITD in the presence or absence of BIX02188 inhibitor. Cells were incubated in different media with or without 12 μM BIX02188 for 48 h. DMSO was used as control for BIX02188. Viable cells were counted with trypan blue dye exclusion (A and C). Cells were stained with PE Annexin V and 7-AAD and analyzed by flow cytometry (B and D). Cells were incubated either in the presence (white bars) or absence (black bars) of BIX02188.

DOI of original article: 10.1016/j.bbrc.2011.07.089
* Corresponding author.
E-mail address: Lars.Ronnstrand@med.lu.se (L. Rönnstrand).

doi:10.1016/j.bbrc.2011.10.070