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Src kinases and Flt3: phosphorylation, interference with receptor maturation and mechanism of association

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Erklärung

Diese Dissertation wurde im Sinne von §13 Abs.3 bzw.4 der Promotionsordnung vom 29. Januar 1998 von PD Dr. Haralabos Zorbas betreut.

Ehrenwörtliche Versicherung

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Abstract

Flt3 is the most recently discovered member of the platelet-derived growth factor receptor (PDGFR) subfamily of receptor tyrosine kinases (RTKs). It is expressed on early hematopoietic progenitor cells and is involved in their growth. Activating mutations of Flt3, such as internal tandem duplications (ITD) in the juxtamembrane (JM) region of Flt3, are among the most common genetic alterations found in patients with acute myeloid leukemia (AML). For this reason Flt3 is an attractive target for specific tyrosine kinase inhibitors, and a number of such compounds are currently being tested in clinical trials. However, the mechanisms of Flt3-mediated signal transduction are poorly understood.

Src family tyrosine kinases (SFKs) are involved in the signaling processes of various RTKs, including those of the PDGFR subfamily . Most SFKs are expressed predominantly or exclusively in hematopoietic cells, and at least Hck and Lyn were found to be activated in AML patients. These observations indicate that SFKs could also be involved in Flt3 signaling. In this work the role of SFKs in signal transduction of the Flt3 receptor was investigated. A panel of Hck and Flt3 mutants was used in biochemical and biological assays to deduce the contribution of kinase activity, SH2 and SH3 binding domains and tyrosine phosphorylation status to Flt3-SFK interaction.

The work presented shows that SFKs interfere with the maturation of wild type and Flt3 ITD receptors in human embryonic kidney 293 (HEK-293) cells in a kinase-dependent manner. The SFK-mediated effect on receptor maturation is reflected by accumulation of the immature, intracellular form of Flt3 at the expense of the mature, plasma membrane-inserted form of the receptor. This effect of SFK kinase activity on receptor maturation is not limited to Flt3, because analogous results were also obtained for the Kit receptor, another member of the PDGFR subfamily. The demonstration of the role of SFKs in regulation of receptor maturation is novel and the exact mechanism underlying this effect requires further investigation.

Using the HEK-293 cells it was also shown that Hck is able to phosphorylate Flt3 on tyrosine residues and to associate with autophosphorylated Flt3 in an SH2 domain-dependent manner. Hck-mediated tyrosine phosphorylation occurs in the JM region of Flt3. Tyrosine residues 589 and 591 in the JM region, when phosphorylated, were also identified as the docking sites for Hck. Although Hck phosphorylation and binding sites on Flt3 overlap, Hck-mediated phosphorylation of Flt3 is not sufficient for Flt3-Hck association.

In contrast to the situation in HEK-293 cells, Hck neither interferes with Flt3 maturation nor phosphorylates Flt3 on tyrosine residues, and does not detectably associate with the Flt3 receptor in the hematopoietic murine cell line 32D clone 3 (32D cl.3). However, the level of the ectopic expression of Hck in 32D cl.3 cells was lower than in HEK-293 cells. This difference in Hck expression level probably accounts for both the lack of the Hck-mediated phosphorylation of Flt3 and the interference with its maturation in 32D cl.3 cells. The lower Hck expression level, however, does not account for the lack of detectable association of Hck with Flt3 in 32D cl.3 cells. Using biological assays in which activation of Flt3 can partially overcome the requirement for cytokine stimulation in 32D cl.3 survival and proliferation, it was shown that Hck is not involved in Flt3 signal transduction leading to Flt3-mediated cell survival and Flt3 ITD-dependent cell growth. Therefore, biological relevance of SFKs in Flt3 signaling remains unclear.

In summary, although the binding and phosphorylation of Flt3 by Hck can be shown, the biological relevance of Hck in Flt3 signaling remains to be formally demonstrated. This research led to the novel finding that, at least when over-expressed or hyperactivated, SFKs interfere with the maturation process of RTKs. Various studies have shown that the ligand-independent activation of RTKs as well as their premature phosphorylation can interfere with maturation of RTKs. This study reveals that, by virtue of their phosphorylating of Flt3, Kit and possibly other RTKs, SFKs can regulate maturation of these RTKs and consequently alter their transport to the plasma membrane. Further studies are required to investigate the biological relevance of this function of SFKs.

Chapter 1

Introduction

1.1 Protein phoshorylation in signal transduction

The ability of cells to perceive and act upon a signal is essential for the development and functioning of multicellular organisms. Deregulation of this process leads to abnormal activities of cells, which underly the development of various diseases, including cancer and diabetes. Such deregulations are often associated with mutations in genes encoding proteins that are involved in signal transduction - a process during which an extracellular signal is converted to an intracellular event, like onset of expression of specific genes, leading to cell growth, differentiation or apoptosis. One group of signal transducing proteins includes protein kinases and phosphatases. These enzymes regulate phosphorylation of proteins - a post-translational modification that represents a key switch in the process of signal transduction.

1.2 Receptor tyrosine kinases (RTKs)

1.2.1 Diversity of RTKs

There are more than 520 protein kinases and 130 protein phosphatases encoded by the human genome [20]. Both types of proteins can be divided into phospho-tyrosine or phospho-serine/threonine specific enzymes based on their substrate amino acids. Receptor tyrosine kinases (RTKs) are transmembrane proteins which transduce an extracellular stimulus into an intracellular signal, which is then further propagated intracellularly. RTKs regulate almost all fundamental cellular processes including metabolism, division, differentiation, migration and death. There are currently 58 known RTKs, which can be classified on the basis of their primary structure into 20 subfamilies (Figure 1.9). The structure of RTKs usually includes an intracellular kinase domain, transmembrane domain and several immunoglobulin-like domains, Cystein-rich domains, epidermal growth factor (EGF)-like domains, fibronectin type III domains or other domains in the extracellular part.

1.2.2 Regulation of RTKs

Regulation of RTK activity by dimerization

In normal resting cells the activity of RTKs is tightly controlled and only a small fraction of active RTKs is present. This accounts for a basal level of RTKs activity in non-stimulated cells. The active state of the receptor is characterized by an "open" conformation of an activation loop (A-loop), which in most cases requires phosphorylation of one or more of its tyrosine residues, usually achieved by trans-autophosphorylation. In the inactive state of most RTKs, the A-loop assumes a so called "closed" conformation, which prevents substrate binding. Stimulation by the ligand leads

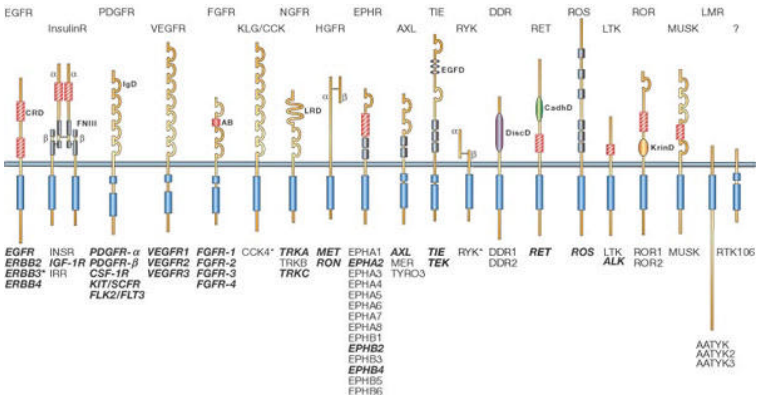

Figure 1.1: Family of human RTKs. The prototypic receptor for each family is indicated above the receptor, and the known members are listed below. Abbreviations of the prototypic receptors: EGFR, epidermal growth factor receptor; InsR, insulin receptor; PDGFR, platelet-derived growth factor receptor; VEGFR; vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; KLG/CCK, colon carcinoma kinase; NGFR, nerve growth factor receptor; HGFR, hepatocyte growth factor receptor, EphR, ephrin receptor; Axl, a Tyro3 PTK; TIE, tyrosine kinase receptor in endothelial cells; RYK, receptor related to tyrosine kinases; DDR, discoidin domain receptor; Ret, rearranged during transfection; ROS, RPTK expressed in some epithelial cell types; LTK, leukocyte tyrosine kinase; ROR, receptor orphan; MuSK, muscle-specific kinase; LMR, Lemur. Other abbreviations: AB, acidic box; CadhD, cadherin-like domain; CRD, cysteine-rich domain; DiscD, discoidin-like domain; EGFD, epidermal growth factor-like domain; FNIII, fibronectin type III-like domain; IgD, immunoglobulin-like domain; KrinD, kringle-like domain; LRD, leucine-rich domain. The symbols α and β denote distinct RTK subunits. RPTK members in bold and italic type are implicated in human malignancies. An asterisk indicates that the member is devoid of intrinsic kinase activity. (Adopted from [20].)

to dimerization of the receptor subunits, which increases the local concentration of the kinase domains. This in turn increases the probability of trans-autophosphorylation and increases the fraction of the active receptors (shown in Figure 1.2). Activated RTKs trans-autophosphorylate additional tyrosine residues in the cytoplasmic domains, which serve as binding sites for Src homology 2 (SH2)- and phospho-tyrosine binding (PTB) domain-containing signaling proteins [97]. The specificity of binding of SH2 domain-containing proteins is defined by 1-6 amino acids C-terminal of the phospho-tyrosine residue [205], while PTB domain-containing proteins bind to phospho-tyrosines in the context of 3-5 N-terminal amino acids [135]. Binding of these proteins to RTKs leads to the assembly of signaling complexes containing multiple signal transduction molecules. Over-expression of RTKs leads to their activation due to an increase in the concentration of dimers and is often associated with cancer development [20].

Regulation of RTKs by autoinhibition

In the absence of ligand stimulation RTKs are kept in the inactive state by various autoinhibitory mechanisms. Intracellular and extracellular regions of the RTKs can participate in the negative regulation of RTK activity. For

example, intramolecular interactions in the extracellular part of the EGFR prevent high affinity binding of the EGFR to its ligand [197]. Another example is fibroblast growth factor receptor (FGFR), which takes advantage of intramolecular interactions in the extracellular domain to keep the receptor in the inactive state. In FGFR the extracellular acid box domain binds to the D2 domain, which blocks D2 domains from binding heparin. As FGFR requires binding of both ligand and heparin for its full activation, this intramolecular interaction prevents the receptor activation [197]. In addition to extracellular domains, different regions in the cytoplasmic part of the receptors can have an autoinhibitory function (Figure 1.2). In the endothelium-specific receptor tyrosine kinase, Tie2, the C-terminal tail occludes the substrate binding site [201]. In other subfamilies of RTKs, the JM region participates in auto-inhibition of the kinase activity, though the mechanisms of autoinhibition vary among different receptor subfamilies. In Ephrin receptors, the JM region interacts with an αC helix in the N lobe of the kinase stabilizing its inactive conformation [14], [235]. Receptors of the PDGFR subfamily are also negatively regulated by the JM region. The mechanism of auto-inhibition for these receptors is described in detail in 1.3.4. In all cases described above, auto-inhibited receptors exist in an equilibrium with a small fraction of active receptors. Ligand binding to the active receptors stabilizes the receptors in their active conformation and thus shifts the equilibrium towards active receptors. Additionally, mutations in the autoinhibitory regions and their modifications by intracellular factors could relieve autoinhibitory constraints and increase the basal level of receptor activity.

Regulation of RTKs by intracellular kinases and phosphatases

As described above, activation of RTKs results in phosphorylation of multiple tyrosine residues in the intracellular part of the receptor. These phosphorylation events are required to achieve maximum catalytic activity, relieve autoinhibitory constraints and to create binding sites for signal transduction molecules. In addition to autophosphorylation, some of these tyrosine residues can also be phosphorylated by intracellular kinases. On the other hand, phoshatases can negatively regulate the activity of RTKs by de-phosphorylating these tyrosine residues. As a result, treatment of cells with tyrosine phosphatase inhibitors almost always leads to the activation of RTKs [197], [55]. It is therefore likely that the balance of kinases and phosphatases in the cell provides an additional level of control of RTKs activity.

1.2.3 Activation of signaling cascades by RTKs

As described above, phosphorylation of RTKs on tyrosine residues, leads to an increase in the catalytic activity of RTKs and generates binding sites for

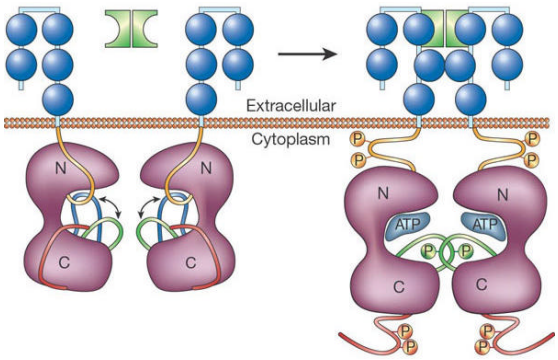

Figure 1.2: Activation of RTKs by dimerization and relief of autoinhibition. In the resting cells, majority of the receptors are present in the inactive state in which conformation of the A-loop (red) interfere with protein substrate binding and perhaps ATP binding. However, in a subset of receptors, conformation of the A-loop (green) is compatible with substrate binding. Ligand binding induces dimerization of receptor subunits and increases the probability of trans-autophosphorylation of the tyrosine residues in the A-loop, which stabilizes its active conformation. Autophosphorylation on additional tyrosine residues relieves autoinhibitory constrains and creates binding sites for downstream signaling proteins. (Adopted from [97].)

phospho-tyrosine-binding proteins. Binding of these proteins to an activated RTK mediates an assembly and recruitment of signaling complexes which participate in the signal transduction of an activated RTK [196].

A signaling programm initiated by RTKs involves a number of signaling pathways whose general principle of regulation and functions are highly conserved in evolution. The important rule is that these signaling pathways do not act separately in a linear fashion, but rather form a complex network which takes positive and negative inputs and employs various feedback regulatory mechanisms. RTKs induce activation of signal transduction molecules that trigger activation of the signaling pathways by three types of mechanisms: membrane translocation, conformational changes and phosphorylation on tyrosine residues.

Signaling pathways activated by RTKs are shown in Figure 1.3. Major signal transduction pathways activated by most RTKs include: Ras/Mitogen-activated protein kinase (MAPK) pathway, phospholipase C (PLC)-γ and Phosphatidylinositol 3-kinase (PI3-K) pathways and the signal transducers and activators of transcription (STAT) pathway. Principles of activation of these pathways are described below.

Ras/MAP Kinase signaling cascade

Activation of the small GTPase Ras in this pathway is achieved through recruitment of its guanine-nucleotide exchange factor Sos to the membrane, where Ras is located. Sos is recruited to the plasma membrane through its association with the SH2 domain-containing adaptor protein Grb2, which

binds to phosphorylated tyrosine residues on RTKs or other adaptor or docking proteins in the signaling complex. Once in its GTP-bound active state, Ras activates several effector molecules, including Raf, PI3-K and Cdc42. Raf activates MAPK kinase (MAPKK/MEK) by phosphorylating serine residues in the A-loop of the kinase. The activated dual specific Tyrosine, Serine/Threonine MAPKK then phosphorylates MAPK on threonine and tyrosine residues in the A-loop leading to its activation. MAPK phosphorylates a number of cytoplasmic and membrane bound targets. It also translocates to the nucleus where it phosphorylates and activates a number of transcription factors.

Pathways regulating Phosphoinositol metabolism

Activation of PLC-γ and PI3-K pathways involves activation of phophoinositol (PtdInst) metabolism and generation of second messengers. PLC-γ is recruited to the plasma membrane through binding of its SH2 domain to phosphorylated tyrosine residues on some RTKs. Activated PLC-γ cleaves its substrate PtdInst 4,5-biphosphate (PtdInst(4,5)P_2) to generate two types of second messengers: diacylglycerol (DG) and Inositol (Inst) 1,4,5-triphosphate (Inst(1,4,5)P_3 or IP_3). The latter binds to Ca^{2+} channel proteins on the surface of the endoplasmic reticulum (ER) and stimulates the release of Ca^{2+} from the ER into the cytoplasm. Calcium binds to Calmodulin, which activates a family of Ca^{2+}/Calmodulin-sensitive kinases (CaMK). In addition, together with DG, an elevated cytoplasmic Ca^{2+} level is required for activation of protein kinase C (PKC). So, through generation of second messengers, activation of PLC-γ leads to stimulation of various intracellular responses [98].

The PI3-K pathway is activated by binding of the SH2 domain of its regulatory subunit p85 to phosphorylated tyrosine residues in the activated RTKs or adaptor proteins in the signaling complex. Activated PI3-K phosphorylates PtdInst 4-phosphate (PtdInst(4)P) and PtdInst(4,5)P_2 to yield two kinds of membrane-bound second messengers: PtdInst(3,4)P_2 and PtdInst(3,4,5)P_3, respectively. These second messengers attract signaling proteins containing pleckstrin homology domains (PH) to the plasma membrane, including PDK-1 and Akt (also known as Protein kinase B (PKB)). PDK-1 phosphoryalates Akt on Thr 308 in the A-loop which is a prerequisite for activation of Akt. Akt phosphorylates a variety of target proteins including Forkhead transcription factors, Bad, S6-Kinase (S6-K) and glycogen synthase kinase-3 (GSK-3). Phosphorylation of Forkhead transcription factors (e.g FKHR1) by Akt results in their cytoplasmic retention and inactivation, which blocks the expression of their target genes involved in the control of the cell cycle, cell death and cell metabolism. Another substrate of Akt is pro-apoptotic Bcl-2-related protein, BAD. Phosphorylation of BAD by Akt

blocks its complex formation with with Bcl-2 and Bcl-x_L thereby preventing apoptotic cell death. PI3-K also induces activation of small GTPases Cdc42 and Rac1 via yet unknown mechanism [118], [230]. PI3-K activation (probably in a Rac1-dependent fashion) also stimulates generation of hydroxide peroxide (H_2O_2), which is catalyzed by NADPH synthase enzyme complex. H_2O_2 oxidizes active site cysteines in phosphatases, thereby inactivating them. Inactivation of the phosphatases leads to sustained phosphorylation of RTKs on tyrosine residues and their activation. Mutations in several proteins in PI3-K pathway have been associated with the development of cancers [225].

STAT-mediated signaling pathway

STAT transcription factors are activated by two types of receptors: those that lack intrinsic tyrosine kinase activity, but are constitutively associated with Janus kinases (JAKs) and by RTKs. Activation of STATs involves their phosphorylation on a single tyrosine residue in the C-terminus. Once phosphorylated, this tyrosine residue serves as a binding site for the SH2 domain of another STAT protein and induces formation of homo- or heterodimers. Phosphorylation of STATs upon RTK activation can be achieved directly or indirectly by intracellular tyrosine kinases. In the latter case, SFKs have been suggested to play a role [163], [226]. Dimerized STAT proteins translocate to the nucleus, where they bind to DNA and other transcription factors and modulate transcription of target genes [45] which results in growth related cellular responses.

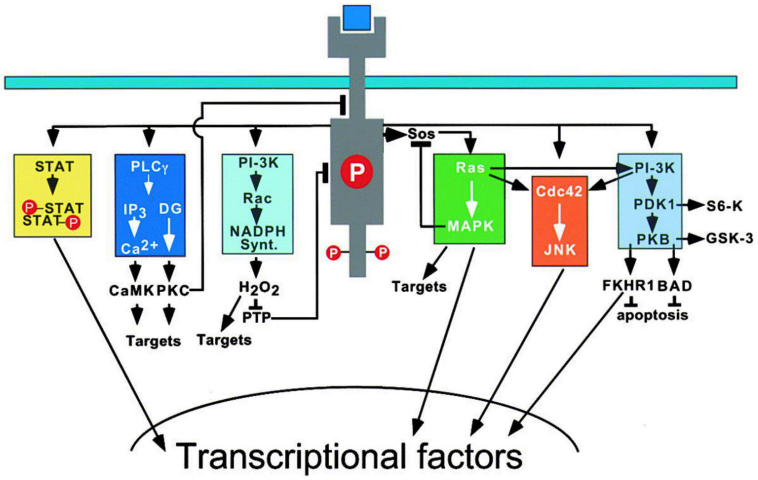

Figure 1.3: Signaling pathways activated by RTKs. Different signaling pathways are presented as distinct signaling cassettes (colored boxes). In several cases the signaling cassettes do not include all the known components of a given pathway. (Adopted from [196])

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1.2.4 Attenuation and termination of RTK activation

As described in 1.2.2, de-phosphorylation of RTKs by tyrosine phosphatases can negatively regulate the activity of RTKs. In addition, internalization and degradation of RTKs represents an important mechanism of down-regulation of RTK signaling.

Internalization and degradation of RTKs

Activated receptors are removed from the cell surface by endocytosis. Receptor-mediated endocytosis occurs via clathrin-coated pits and vesicles [176]. Clathrin polymerizes around the region of the membrane that contains a receptor targeted for internalization. This membrane region then invaginates to form a clathrin-coated pit and eventually pinches off in a form of a clathrin-coated vesicle (CCV). Protein complexes called assembly particles (AP) are located between clathrin and the membrane and determine whether membrane proteins would be included into the CCVs. In addition to clathrin and proteins in APs, many other proteins participate in endocytosis. For example, GTPase dynamin self-assembles into rings or spirals to wrap around the necks of the budding vesicles and is required for endocytosis [164]. Upon internalization, CCV shed clathrin and fuse with the internal vesicles to form early endosomes, which maturate into late endosomes. Upon internalization, RTKs are either recylced back to the plasma membrane or are internalized by the late endosomes into the internal vesicles. The latter process results in the formation of the multivesicular bodies (MVB), which eventually fuse with lysosomes where RTKs are degraded. RTKs can activate signal transduction pathways even after endocytosis, possibly through a different subset of signaling molecules and a termination of RTKs signaling takes place only upon incorporation of RTKs into MVBs and lysosomes [138].

1.2.5 Class III of RTKs

Genomics and structure

Class III of RTKs includes PDGFR α and β, Fms-like tyrosine kinase 3 receptor (Flt3), colony stimulation factor-1 receptor (CSF-1R) and the Kit receptor.

The chromosomal location and genomic structure of these receptors suggest their close evolutionary relationship and their origin from a common ancestor gene by cis and trans duplication. C-kit and PDFRα genes are located on chromosome 4q11-q13 [77], [70]. The PDGFRβ gene lies about 350 base pairs upstream of the major transcription site of c-fms gene, which encodes the CSF-1R, on chromosome 5q31-q33 [76], [178]. The Flt3 gene is located on chromosome 13q12 and is linked in head-to-tail fashion to Flt1,

a class V receptor tyrosine kinase [186]. In addition, fragments of the genes coding for intracellular parts of Kit, CSF-1R and Flt3 proteins, share the same number of exons, which are highly conserved in size, sequence and exon/intron boundary positions [2].

Members of class III of RTKs share a common structural organization which is discussed in detail for Flt3 in 1.3.1 and shown in Figure 1.4.

Functions of RTKs of class III in normal and malignant hematopoiesis

RTKs of class III in normal hematopoiesis RTKs of class III are involved in normal hematopoiesis and are associated with the pathogenesis of hematological disorders [173]. CSF-1R is the receptor for macrophage colony stimulating factor (M-CSF), which is essential for growth and differentiation of the monocyte-macrophage-osteoclast lineage [200]. Flt3 and Kit have partially overlapping, but distinct functions in the development of early hematopoietic progenitor cells [127] and Kit has an additional function in the development of germ cells and melanocytes, as well as of interstitial cells of Cajal [30]. The role of Flt3 in normal hematopoiesis is described in more detail in 1.3.6. PDGF also stimulates hematopoiesis, but unlike ligands of the CSF-1R, Kit and Flt3 which act directly on hematopoietic cells, PDGF activates stromal cells that express PDGFR [103]. Among these cells are fibroblasts, smooth muscle cells, osteoblasts and macrophages that produce a variety of growth factors which regulate the development of hematopoietic progenitors.

RTKs of class III in leukemogenesis Consistent with their role in normal hematopoiesis, deregulation of class III RTKs is associated with the development of various types of hematopoietic malignancies. Point mutations in the c-kit gene that lead to the activation of the tyrosine kinase activity of the receptor are found in patients with mast cell disease, AML and NK/T cell lymphomas [173]. PDGFR is often found as a translocation partner in a number of fusion proteins expressed in patients with AML and chronic myeloid leukemia (CML) [173]. In these fusion genes, PDGFR usually provides the kinase domain and the partner gene encodes a protein-protein interacting domain which leads to constitutive dimerization and activation of the PDGFR kinase activity. The role of mutations in the c-fms gene in leukemogenesis is not clear. Even though some early studies have identified point mutations in patients with AML and Myelodysplastic syndrome (MDS), further analysis was not able to confirm these findings [173]. Mutations in the Flt3 gene are the most common genetic lesions in patients with AML. The role of the Flt3 gene mutations in leukemogenesis is described in more detail in 1.3.7.

1.3 Flt3 and its ligand

1.3.1 Structure of the Flt3 receptor

The structure of the Flt3 receptor is characteristic of other RTKs of class III and is shown schematically in Figure 1.4. The extracellular part of the Flt3 receptor contains five immunoglobulin (Ig) domains, and is heavily glycosylated on asparagines (described in detail in 1.3.2). Ig domains are followed by a single transmembrane (TM) domain, JM region, kinase domain, interrupted by a kinase insert and C-terminal tail (C-tail).

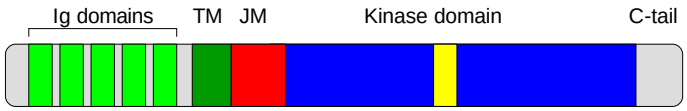


Figure 1.4: Domain structure of Flt3. Flt3 contains five Ig domains (light green), followed by a TM domain (dark green), JM region (red), kinase domain (blue) interrupted by a kinase insert (yellow) and C-tail (gray).

1.3.2 Maturation of Flt3 in the ER and Golgi complex

N-glycosylation of Flt3

Predicted molecular weight of Flt3 protein based on its primary sequence is about 110 kDa. However, Western blot analysis of Flt3 results in the two bands of 130-143 kDa and 155-160 kDa [131], [139]. Various studies showed that the difference in the calculated and actual molecular weight of Flt3 is due to its N-glycosylation. Treatment of immuno-precipitated Flt3 with tunicamycin, an agent that blocks the first step in the assembly of core oligosaccharide and therefore inhibits synthesis of all N-linked glycoproteins, results in a disappearance of both forms of the receptor and in the appearance of a new 110 kDa form [139]. Treatment of Flt3 immunoprecipitates with endo H, a glycosydase which cleaves only high mannose N-linked structures of glycoproteins, results in the reduction of the amount of lower, but not higher molecular weight form of Flt3 [139]. In contrast, no differences are observed when immuno-precipitates are treated with O-glycosidase [139]. Pulse chase experiments show that the higher molecular weight band arises from the lower molecular weight form [131]. In addition, labeling of cell surface proteins with ^{125}I detects only the higher molecular weight form on the cell surface [131]. Together, these studies suggest that the lower molecular weight form of Flt3 represents immature high mannose form of the receptor, which is probably confined to ER or ER-Golgi transition, while the higher molecular weight form of Flt3 represents mature cell

surface receptor which contains complex N-linked oligosaccharides.

Maturation of N-glycosylated proteins in the ER and Golgi complex

The maturation process of Flt3 has not been analyzed in detail. However, Flt3 most likely undergoes classical maturation steps in the ER and Golgi complex described in detail for other N-glycosylated proteins. The work described in this thesis shows that SFKs interfere with the maturation of Flt3. For this reason, detailed analysis of the maturation steps of a typical N-glycosylated protein is presented below.

The presence of the hydrophobic stretches of amino acids at the N-terminus (signal sequence) and in the middle of the polypeptide chain (transmembrane domain) ensures that the growing polypeptide is penetrating the ER membrane co-translationally. The protein spans the ER membrane in the same orientation it would have on the plasma membrane: the extracellular part is localized inside the ER lumen and the intracellular part is facing the cytosol [231]. At the same time N-glycosylation of the extracellular part of the growing polipeptide chain begins in the ER lumen. The first step in N-glycosylation of proteins is an assembly of the 14-saccharide "core" unit on a dolicholphosphate carrier by the addition of sugars one by one. The core oligosaccharide is then transferred *en bloc* to an asparagine residue that is part of Asn-X-Ser/Thr consensus sequence and is coupled to its side chain through an N-glycosidic bond. After coupling of the oligosaccharide to the polypeptide, all three glucose and one specific mannose residues are removed from the oligosaccharide by the action of glucosidases and mannosidases, which are resident enzymes of the ER. Then the glycoprotein moves to the Golgi complex, where further trimming and addition of sugars takes place. Terminal glycosylation in Golgi prepares the proteins for their specific functions. A typical maturation pathway of N-glycosylated proteins is shown on Figure 1.5.

In the ER, the most important function of N-glycosylation is the promotion of folding of newly synthesized proteins [87]. N-glycosylation-assisted folding of glycoproteins involves a unique chaperone system found in the ER, the so-called calnexin-calreticulin cycle. Calnexin (CNX) and calreticulin (CRT) are homologous ER lectins that bind specifically to monoglucosylated core oligosaccharides on glycoproteins. CNX or CRT bind to another folding factor thiol oxidoreductase ERp57. The complex of CNX or CRT with ERp57 modulates protein folding by promoting the formation of disulphide bonds [92].

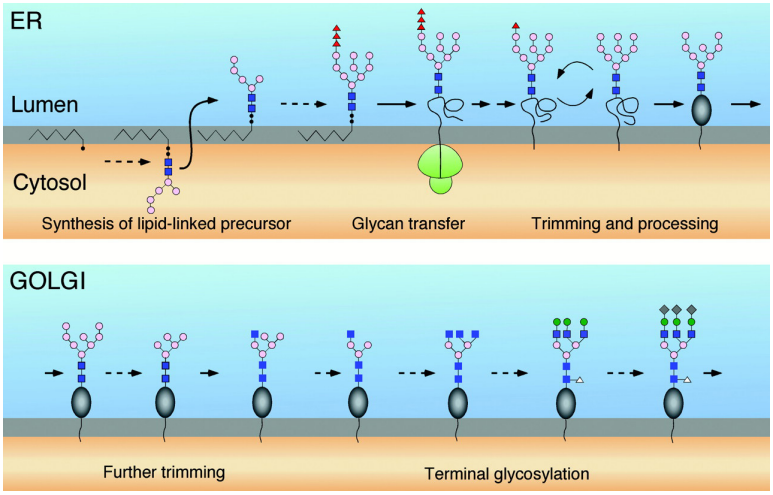


Figure 1.5: Typical steps of protein N-glycosylation in the ER and Golgi complex. Synthesis of the core olygosacharide takes place at the cytosolic face of the ER membrane by an addition of sugars to a dolichylphosphate carrier. A partly assembled oligosaccharide is flipped to the lumenal side of the membrane, where further sugars are added to generate a complete core oligosacharide. The core oligosacharide is then transfered to the asparagine residues of nascent, growing polypeptide chains. The three glucoses and terminal mannoses are trimmed away in the ER. The ER also contains a glucosyltransferase that can re-glucosylate glucose-free chains and thus establish, with glucosidase II, a deglucosylation-reglucosylation cycle. When the glycoprotein has folded (gray oval) and reached the Golgi complex, further trimming and terminal glycosylation occurs. Only one of many possible terminal glycosylation pathways is shown.(Adopted from [88].)

Role of N-glycosylation of class III RTKs

The role of N-glycosylation in the function of Flt3 and other class III RTKs is not clearly established. PDGFR is able to bind PDGF and is kinase active in the absence of the N-glycosylation, suggesting that N-glycosylation *per se* is not required for ligand-induced receptor activation [106]. Treatment of cells transformed by v-fms (viral oncogene derived from cellular c-fms gene) with the mannosidase inhibitor swaisonine, which results in the expression of abnormally glycosylated v-fms protein on the surface of the cells, does not interfere with their transformed phenotype [157]. In contrast, treatment of these cells with two glucosidase inhibitors which block glycosylation of v-fms protein at an early stage, prevents translocation of the oncogenic protein to the plasma membrane and reverts v-fms-transformed cells to the normal phenotype [157]. The latter results suggest that while proper glycosylation is not necessary for v-fms oncogenic potential, its glycosylation is required for membrane localization which is necessary for cellular transformation.

Presence of RTKs in the plasma membrane and their exposure to the ligands is most likely required for ligand-dependent activation of RTKs. On the other hand, hyperactivation due to the presence of mutations or overexpression of RTKs might result in the premature dimerization and/or activation

of RTKs in the secretory pathway and subsequent activation of the intracellular signaling processes. For example, premature auto-phosphorylation of Flt3 in Flt3 ITD impairs its maturation [198].

1.3.3 Expression of Flt3 in hematopoietic cells

Flt3 expression in normal human bone marrow cells was analyzed by various groups either by staining of cells with specific antibodies generated against the epitopes in the extracellular part of the receptor and subsequent fluorescence-activated cell sorting (FACS) analysis [185] or by Northern blot analysis of Flt3 mRNA [203]. Similar approaches were also applied to analyze Flt3 expression in murine bone marrow [82], [142], [50], [1].

Staining of human bone marrow cells with antibodies revealed that only a small fraction of bone marrow cells (about 2%) are Flt3 positive [185]. Most of these positive cells represent early progenitor or stem cells [185]. However, the most primitive human hematopoietic stem cells do not express Flt3 [185]. On the other hand, myeloid and B cell precursors in human bone marrow are Flt3 positive [185]. A study of murine Flt3 also showed that up-regulation of Flt3 expression is accompanied by a loss of self-renewal capacity of stem cells and that Flt3 positive cells have a prominent lymphoid reconstitution potential [1]. Expression of Flt3 on human T cell progenitors requires further analysis, but most of the studies in mice suggest that Flt3 is expressed on T cells and is required for their development [82], [1]. Flt3 expression in normal hematopoietic cells is summarized in Figure 1.6.

Analysis of Flt3 expression on human hematopoietic cell lines shows that most myeloid, monocytic, pro-B and pre-B cell lines are Flt3 positive, while erythroid, myeloma, NK and T cell lines are negative [185], [46], [27], [145].

1.3.4 Regulation of the kinase activity of Flt3 by the JM region

As described in 1.2.2, in the absence of ligand stimulation, RTKs are kept inactive by a number of autoinhibitory mechanisms.

Various genetic and biochemical studies suggest that the JM region of Flt3 could play an autoinhibitory role [156], [111], [110]. However, only the determination of the crystal structure of the autoinhibited form of Flt3 confirmed this hypothesis and revealed the mechanism of autoinhibition of the kinase activity by the juxtamembrane region [75].

The structure of the kinase domain of Flt3 in the inactive conformation is consistent with that of other Tyrosine and Serine/Threonine kinases (shown in Figure 1.7). It consists of N and C lobes with an ATP binding site

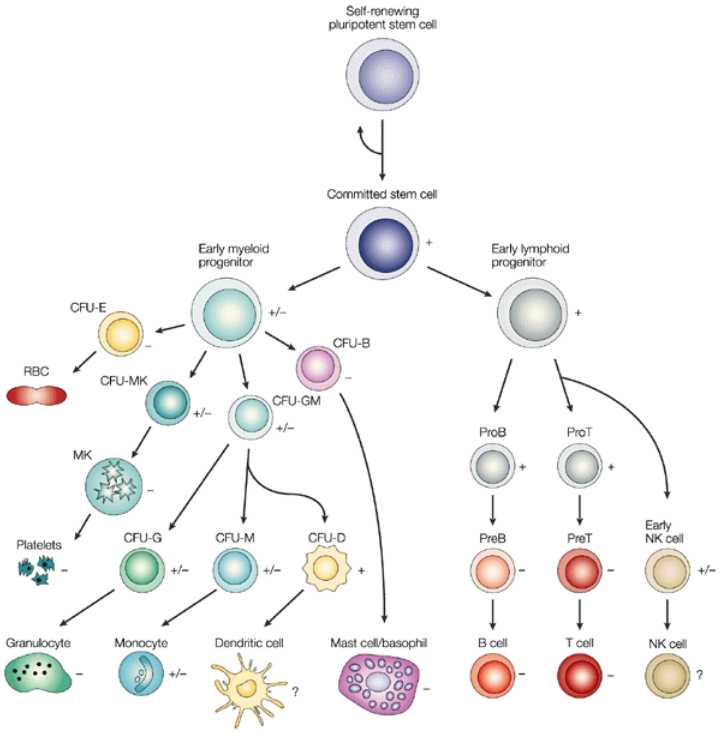
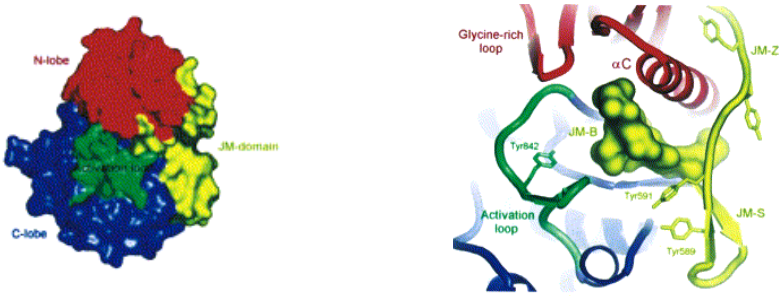


Figure 1.6: Expression of Flt3 in normal hematopoiesis Schematic view of maturation and differentiation of hematopoietic cells and Flt3 expression on these cells (+, +/- or -) is shown. Colony forming units for the erythroid (CFU-E), megakaryocytic (CFU-MK), granulocytic-monocytic (CFU-GM), basophilic (CFU-B), granulocytic (CFU-G), monocytic (CFU-M), and dendritic lineages are shown. NK cells are natural killer cells; RBC, red blood cells. (Adopted from [212].)

being situated in the cleft between the lobes and the activation loop folded between two lobes. The distinct feature of the Flt3 kinase domain structure is the position of the JM region in a way that it interacts with almost all key components of the kinase fold to inhibit an activation of the kinase. The JM region of the Flt3 receptor comprises amino acids 572-603 and can be divided into three topologically different regions from N- to C-terminus: juxtamembrane binding motif (JM-B), juxtamembrane switch motif (JM-S) and juxtamembrane zipper segment (JM-Z). 76% of the total area of the JM-B binds to the cleft between the N and C lobes where it forms interactions with key components of the kinase fold: glycine-rich P loop, activation loop and αC helix. In contrast to JM-B, JM-S is not buried in the structure of autoinhibited Flt3, but the side chains of Tyr 589 and Tyr 591 in this segment are positioned between the backbone of JM-S and the C lobe of the kinase. JM-Z folds alongside the N lobe and terminates in the JM hinge region.

The phosphorylation status of Tyr 589 and Tyr 591 plays a key role

[75]. Phosphorylation of one of these tyrosine residues is incompatible with the positioning of their side chains between the backbone of JM-S and the C-lobe and leads to dislocation of JM-B from its binding site within the catalytic domain. This allows the conformational changes in the kinase domain that are required for an activation of the kinase. In contrast, when Tyr 589 and Tyr 591 are de-phosphorylated and their side chains are positioned between JM-S backbone and C-lobe, JM-B fits snugly into its binding pocket, preventing the kinase domain from assuming an active conformation.



(a) (b)

Figure 1.7: Structure of the autoinhibited Flt3. Surface diagram of Flt3 (a) shows the structure of the kinase domain of Flt3 and its interaction with the juxtamembrane region of the receptor. The Flt3 kinase domain consists of the N lobe (red), the C-lobe (blue) and the activation loop (green), which is located in the cleft between two lobes. The JM region is shown in yellow. The ribbon diagram of Flt3 (b) highlights the interactions of the JM domain with the key components of the kinase domain: A-loop, αC helix, and Glycin-rich loop. (Adopted from [75].)

1.3.5 Structure and expression of Flt3 ligand (FL)

Structure of FL

There are several isoforms of Flt3 ligand (FL) [82], [130], [129], [128], [127]. The most abundant isoform of FL in humans is a transmembrane protein. Its N-terminal extracellular part contains four helical domains, a spacer region and a tether region. A single transmembrane domain is followed by a small C-terminal tail. Transmembrane FL is biologically active on the cell surface, however it can be cleaved by an unidentified protease to produce a soluble form of FL. The functional differences of the soluble ligand and transmembrane ligands are not known. Another isoform of FL arises from alternative splicing. For example, alternative splicing of the sixth exon generates a premature stop codon near the end of the extracellular part and therefore results in the soluble protein. This isoform is less abundant than the transmembrane isoform, but is also biologically active. In mice, one

more isoform of FL is known. It is produced by an alternative splicing of FL mRNA, which results in a frame shift. The resulted protein lacks spacer and tether regions and terminates in a stretch of hydrophobic amino acids, that anchor the protein to the membrane. This isoform, though the most abundant in mice, has not been found in humans.

FL contains two potential sites for N-glycosylation and is N-glycosylated [127]. Preliminary data suggest that it exists as a non-covalently linked homo-dimer [127]. The ligand dimer most likely binds to the dimeric form of the receptor, which leads to the activation of the latter. However, the exact mechanism of binding of FL to the Flt3 receptor is not known.

Expression profile of FL

Unlike the Flt3 receptor, whose expression is restricted to a small population of hematopoietic cells, FL is expressed in most tissues except brain [129]. Since FL is only known to stimulate the Flt3 receptor, and FL is the only known ligand for the Flt3 receptor, the pattern of receptor expression most likely restricts the biological outcome of receptor-ligand interaction.

1.3.6 Biological function of Flt3 in normal hematopoiesis

Role of Flt3 in growth of primitive hematopoietic progenitors

Early hematopoietic progenitor cells require activation by multiple cytokines to be recruited into the active cycling state [161]. Analysis of responsiveness of primary leukemia cells and primitive hematopoietic progenitor cell to FL have shown that FL acts synergistically with other cytokines to enhance growth of human and murine primitive hematopoietic progenitor/stem cells [131], [129], [69], [188], [143], [32]. In the absence of other cytokines, however, FL stimulation leads to sustained viability, but does increase proliferation of these cells [216], [224], [127].

Targeted disruption of Flt3 receptor and ligand in mice

The phenotype of Flt3 knock-out mice is in agreement with the suggested function of Flt3 in the development of primitive hematopoietic progenitors. Mice carrying a homozygous deletion of most of the gene encoding the Flt3 receptor are healthy animals with normal amounts of mature hematopoietic cells in peripheral blood [132]. However, the numbers of early B cell progenitors in these animals are reduced as compared to normal mice. In addition, stem cells isolated from Flt3 knock-out mice are deficient in their ability to repopulate lymphoid and myeloid compartments when introduced into lethally irradiated animals. Mice lacking FL also have reduced numbers of early B-lymphoid progenitors [144]. However in contrast to Flt3 knock-out

mice, these animals have a significant reduction of cells in peripheral blood, spleen and bone marrow. The numbers of NK cells and dendritic cells (DC) are also significantly reduced in FL-knock out mice. The reason for the differences between phenotypes of the Flt3 receptor and ligand knock-out animals is not clear. Mice strain variation and the depth of the analysis of animals have been suggested as possible explanations [127].

Role of FL in the immune system

Reduced numbers of NK and DC in mice lacking the FL gene suggests that FL has a specific function in the development of these cells. In agreement with this assumption, different groups demonstrated that FL induces expansion of functional mature DC [134], [133]. DC are the most efficient antigen presenting cells (APS) for T-cells. For this reason, FL-mediated expansion of DC was suggested for the use in the immunotherapy of cancer. Use of FL in mouse models of various cancers demonstrated a therapeutic potential of such an approach. FL treatment resulted in protection of mice from tumor development and regression of tumor growth [40], [38]. Clinical trials in humans employing immunization with cancer antigens and stimulation with FL also showed positive results [62]. However, in other studies no significant positive effect of FL could be shown and some treated patients developed autoimmune responses [52].

1.3.7 Role of Flt3 in hematopoietic malignancies

A great interest in understanding the biology of Flt3 is prompted by the finding that mutations in this gene as well as its over-expression are present in a large group of patients with AML.

Flt3 internal tandem duplications (ITD)

There are two types of mutations in the Flt3 gene that are associated with leukemia in humans and lead to constitutive activation of the kinase activity. The most common type of Flt3 mutation is an internal tandem duplication (ITD) [156], which is found in about 15-35% of patients with AML and 5-10% of patients with MDS but not in healthy donors [212]. ITD mutations result from duplications of a fragment of the JM region of the Flt3 receptor and its direct insertion in a head-to-tail fashion without a shift in the reading frame. The length of a duplicated fragment varies from 3 to 400 base pairs and in some cases insertions of a nucleotide sequence of unknown origin have also been detected [111]. One mechanism of kinase activation by an ITD-type mutation could be the dislocation of the JM-B segment of the JM region from its binding site, which relieves an auto-inhibitory constraint on

kinase activity [75]. This results in a constitutively elevated kinase activity of the receptor in the absence of ligand stimulation.

Flt3 tyrosine kinase domain mutations

The second type of Flt3 mutations found in AML patients includes missense mutations in the tyrosine kinase domain (TKD). TKD mutations occur in 5-10% of patients with AML, 2-5% of patients with MDS and in 1-3% of patients with acute lymphoblastic leukemia [212]. The most common mutation of this type is substitution of aspartic acid at the position 835 by tyrosine (D835Y), although other mutations of this codon have also been described [238]. Aspartic acid 835 is located in the activation loop of the Flt3 kinase domain and is highly conserved among RTKs. Substitutions of the corresponding aspartic acid in Kit (D816) were also found in AML and mast cell leukemia [12], [22].

Relevance of Flt3 mutations to leukemogenesis

Expression of Flt3 containing ITD or TKD mutations leads to transformation of hematopoietic cell lines characterized by factor-independent growth [147], [86], [61]. Moreover, Flt3 ITD induces a myeloproliferative disease in a murine bone marrow transplant model [108]. However, presence of Flt3 ITD alone is not sufficient to cause leukemia in mice, suggesting that additional mutations are required [108]. In support of this hypothesis, it was shown that Flt3 ITD cooperates with PML-RARα (a protein that results from an aberrant fusion of retinoic acid receptor α and promyelocytic leukemia protein) in the mouse model system to induce a short latency APL-like disease [107]. In patients, Flt3 ITD mutations are also often found in conjunction with other genetic abnormalities, such as translocations that include genes for transcription factors [71]. Based on these data, the "two-hit" model of leukemogenesis is favored by many researchers in the field [71]. According to this model, two classes of mutations cooperate in the development of AML: mutations of the first class (e.g Flt3 ITD or TKD, N- or K-Ras mutations) confer proliferative advantage to the cells, while mutations of the second class (e.g PML-RARα, AML/ETO, MLL fusions) impair differentiation and apoptosis. The presence of at least one mutation from each class is required for the development of AML.

1.3.8 Flt3-mediated signal transduction

Studies of Flt3-mediated signaling using a chimeric receptor

Before the cloning of FL, signal transduction studies of the Flt3 receptor were done using a chimeric receptor, which consists of the extracellular part of the CSF-1R and transmembrane and intracellular parts of murine Flt3

[139], [187], [13], [53]. The chimeric receptor was introduced into Rat-2, NIH-3T3 or Baf/3 cells and CSF-1. It was shown that stimulation with CSF-1 induces an increase in phosphorylation of the mature form of the receptor and enhanced the kinase activity of the receptor [187]. Upon stimulation with CSF-1, phosphorylation of a number of signal transducing molecules on tyrosine residues was induced. These molecules include proteins involved in the Ras signaling pathway: Ras GTPase activating protein (Ras GAP) and adaptor protein Shc and Vav, as well as proteins involved in phospholipid metabolism: PLCγ and PI3-K. It was also shown that SH2 domains derived from p85, Ras GAP, PLCγ and Grb-2, but not Vav or Nck can bind to the chimeric receptor [187], [53]. The PI3-K binding site on Flt3 was mapped to Tyr 958. However, mutation of the PI3-K binding site showed that it is not required for mitogenesis or internalization of the chimeric receptor [13]. A slight increase in phosphorylation of Src, but not Fyn was also detected upon CSF-1 stimulation. Additionally, both SFKs were also detected in immuno-precipitates of the chimeric receptor [53].

Studies of Flt3-mediated signaling using Flt3 ligand

After cloning of FL some, but not all, signaling data obtained with the chimeric receptor was confirmed using native Flt3 receptor. In Baf/3 cells stimulation of human Flt3 with FL induced auto-phosphorylation of the receptor, its association with Grb-2 and phosphorylation of Shc [242]. Activation of the Ras signaling pathway was also shown [242]. In addition, SH2 domain phosphatase-2 (SHP-2), but not SH2 domain phosphatase-1 (SHP-1), were found to be phosphorylated on tyrosine residues and inducibly associate with Grb-2 and Shc respectively upon FL stimulation [242]. In contrast to the data obtained with the chimeric receptor, the regulatory subunit of PI3-K, p85, did not associate with human Flt3 upon ligand stimulation, but instead formed a complex with SHP-2, SH2 domain-containing inositol polyphosphate 5-phosphatase (SHIP) and a tyrosine-phosphorylated 100 kDa protein upon stimulation of human Flt3 by FL in Baf/3 cells [242]. Stimulation of the native receptor with FL was also shown to induce phosphorylation of Gab1 and Gab2 adaptor proteins and their association with SHP-2, Grb2 and PI3-K [241]. Other signal transduction molecules involved in human Flt3-mediated signal transduction have also been identified. Ubiquitin ligase Cbl-b was shown to be phosphorylated on tyrosine residues upon FL stimulation and to form a complex with the p85 subunit of PI3K in THP-1 monocytic and JEA2 pro-B cell lines [115]. A novel zinc-finger protein, Fiz-1, was shown to associate with the catalytic domain of Flt3 in HEK-293 and Baf/3 cells upon Flt3 ligand stimulation [234]. Association of Flt3 with SFK and their phosphorylation upon FL stimulation of the native Flt3 receptor have not been reported so far.

Diversity of Flt3 signaling in various cell types

Activation of signaling pathways by Flt3 appears to be cell type specific [53], [242]. For example, in NIH-3T3 cells stimulation of the chimeric receptor does not lead to phosphorylation of p85 and leads to only a slight increase in phosphorylation of Shc, while phosphorylation of both proteins is strongly induced by Flt3 in Baf/3 cells [53]. Similarly, stimulation of human Flt3 leads to phosphorylation of SHP-2 and its association with p85 in Baf/3, but not in THP-1 cells [242]. These findings indicate that recruitment and activation of various signaling pathways by Flt3 depends on the expression of signaling molecules in a cell. Therefore, the biological outcome of Flt3 stimulation could also depend on the cell type in which the receptor is expressed.

Different signaling properties of Flt3 wt and Flt3 ITD

Initial studies showed that stimulation of the chimeric receptor provides an oncogenic signal and induces the transformation of Rat-2 cells and NIH-3T3 fibroblasts as well as abrogates IL-3 growth requirement of Baf/3 cells [139], [53]. However, later studies with human Flt3 receptor demonstrated that stimulation of Flt3 with FL does not lead to an oncogenic signaling and cellular transformation, but only sustains cell viability [147], [146]. In contrast, Flt3 ITD does transform cells and its expression blocks myeloid differentiation of 32D cl.3 cells into granulocytes [147], [244]. Some studies suggest that Flt3 ITD induces qualitatively different type of signaling than Flt3 wt. In support of this hypothesis, it was shown that while Flt3 wt induces the expression of some genes, Flt3 ITD represses or has no effect on transcription of the same genes. For example, Flt3 wt induces expression of myeloid transcription factors C/EBPα and PU.1, while Flt3 ITD strongly represses C/EBPα expression leaving PU.1 expression unchanged [148], [243]. As myeloid differentiation requires the induction of C/EBPα and PU.1 expression, repression of these processes might explain Flt3 ITD-associated differentiation block. Additionally, anti-apoptotic pathways induced by Flt3 ITD appear to be more divergent than those induced by Flt3 wt [146]. While dephosphorylation of BAD was shown to be sufficient to induce apoptosis in Flt3 wt expressing cells, additional down-regulation of Bcl-x_L was needed to induce apoptosis in cells expressing Flt3 ITD [146]. The mechanism underlying different signaling properties of Flt3 wt and Flt3 ITD is not known. It might include the length and strength of signaling induced by wild type and mutated receptors, as well as different intracellular localization of these receptors [198].

1.4 Src family of tyrosine kinases

1.4.1 Structure of Src family kinases (SFKs)

Domain structure of SFKs

A gene coding for Src (c-Src) was discovered as a cellular counterpart of a transforming gene in a genome of Rous sarcoma virus (RSV) v-Src [208], [209], [199]. This finding ultimately led to a number of break-through discoveries including that of an origin of viral oncogenes from normal cellular genes called proto-oncogenes [217] and protein phosphorylation on tyrosine residues [54] among many other findings. In contrast to the c-Src protein, v-Src lacks several amino acids at its C-terminus and contains several point mutations, which contribute to its transformation potential.

Src is a prototype of a family of nine non-receptor tyrosine kinases, which now includes Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes and Yrk. These proteins share a common domain structure (shown in Figure 1.8) and the mechanism of regulation (described in 1.4.3). Each member contains an N-terminal sequence, sometimes referred to as Src homology 4 (SH4) region, followed by a unique domain, Src homology 3 (SH3) domain, Src homology 2 (SH2) domain, a catalytic domain and a short cytoplasmic tail. This region contains a signal for myristylation (glycine at the position 2) and palmitoylation (cystein at the position 3). All SFKs are myristylated and most of them are also palmitoylated (except for Src, $p61^{Hck}$ and Blk). Myristylation is required for membrane binding of SFKs. Nonmyristylated mutants do not bind to the membranes and non-myristylated v-Src lacks a transforming potential, but is capable of inducing cellular proliferation [34]. The sequence of the unique domain is the least conserved among SFKs. The exact function of this domain is not known. However, in some SFKs the unique domain was shown to participate in specific protein-protein interactions [222], [72]. For example, Lck specifically interacts with co-receptors of T cell receptor (TCR), namely CD4 and CD8, by a di-cystein motif found in the unique domain [222]. Additionally, Src but not other SFKs, are phosphorylated on serine and threonine residues in the unique domain by Cdc2/cyclin complexes at the M phase of the cell cycle [152], [37]. The SH2 domain is a protein-protein binding domain that recognizes a phosphorylated tyrosine residues within a specific consensus sequence [205]. It is involved in the regulation of the kinase activity of SFKs by associating with the phosphorylated C-terminal tyrosine residue. It also associates with other phospho-tyrosine-containing proteins. The SH3 domain is another protein-protein interaction domain that binds to proline rich (XP-x-XP) motifs [36]. The SH3 domain is involved in auto-regulation of SFK activity via its interactions with the SH2-kinase linker region and in association with other signaling proteins.

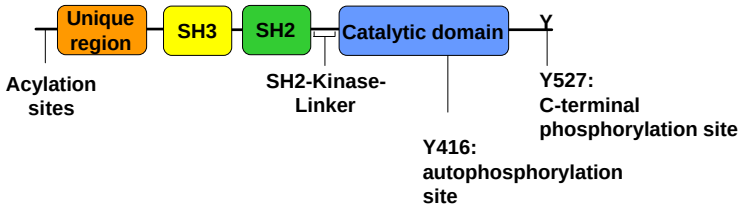


Figure 1.8: Domain structure of SFKs. SFKs share a common domain structure, which includes N-terminal stretch of amino acids, unique domain, SH3, SH2, kinase domain and C-terminal tail. Auto-phosphorylation site in the A-loop, Tyr 416 (amino acids numbering for Src), and C-terminal negative regulatory tyrosine, Tyr 527, are shown.

Isoforms of SFKs

Some SFKs are present in more than one isoform. Two isoforms of Fyn show mutually exclusive patterns of expression: Fyn^T is expressed exclusively in thymocytes and splenocytes, while another isoform of Fyn is mainly expressed in the brain [43]. Fyn isoforms arise from an alternative splicing, which results in the differences of the kinase domain. Specific functions of these two isoforms of Fyn are not known.

Hck also exists in two isoforms: $p59^{Hck}$ and $p61^{Hck}$. These isoforms result from the use of an alternative translation start on a single Hck mRNA. Translation of $p61^{Hck}$ starts at CTG, 21 codons upstream of an ATG translation start codon of $p59^{Hck}$ isoform [121]. Hck isoforms differ in their subcellular localization: the smaller $p59^{Hck}$ isoform localized primarily to the plasma membrane and the larger $p61^{Hck}$ isoform is found in the membrane and in the cytosol [121]. The difference in the intracellular localization of Hck isoforms is associated with the different post-translational modification of these two isoforms. While both isoforms are myristylated, only $p59^{Hck}$ is palmitoylated [177]. Palmitoylation was shown not only to increase the affinity for the membrane binding, but also to target Hck to caveolae [177] or lysosomes [35].

1.4.2 Expression of SFKs in various tissues

In vertebrates SFKs are expressed in various tissues and at least one SFK is expressed in each cell of the human body. Some SFKs are expressed ubiquitously (Src, Fyn, Yes and Yrk), while the expression of others is more restricted, usually to the cells of hematopoietic origin. Patterns of expression of different SFKs in various tissues are outlined in table 1.1.

1.4.3 Regulation of catalytic activity of SFKs

A kinase fold of SFKs is similar to all other known tyrosine and serine/threonine protein kinases. It consists of a smaller N lobe and larger C lobe. ATP binds

SFK	Tissue of expression
Blk	B cells
Fgr	Myeloid cells, B cells
Fyn	ubiquitous
Hck	Myeloid
Lck	T cells, NK cells, brain
Lyn	B cell, myeloid cell, NK cells, brain
Src	ubiquitous
Yes	ubiquitous
Yrk	ubiquitous

Table 1.1: Expression of SFKs in various tissues. Predominant tissues of expression for individual SFKs are shown. (Adopted from [167].)

in the cleft between two lobes and a peptide substrate binds in the extended conformation across the front end of the nucleotide binding pocket. The N lobe consists of five-stranded β sheet and an α helix, called αC, while the C lobe is mostly helical [99]. Conformational changes in αC play a key role in the regulatory mechanisms of SFK activity [202], [236]. In the active state of the kinase conserved Glu 310 in αC helix forms an ionic interaction with the side chain of the conserved Lys 295 in the N lobe. This interaction coordinates α and β phosphates of ATP. In addition, the A-loop, located in the center of the catalytic domain, is phosphorylated in the activated kinase and provides a platform for substrate binding. When not phosphorylated on Tyr 416, the A-loop collapses into the active site blocking the binding of ATP and the substrate [195].

Inactive conformation of SFKs is stabilized by a set of intramolecular interactions. SH2 domain associates with phosphorylated Tyr 527 in the C-terminal tail of the kinase [202], [236]. This association directs the SH3 domain to interact with a linker connecting the SH2 and kinase domains. SH3 and the linker bind to the N lobe of the kinase domain and stabilize αC in the inactive conformation.

Dephosphorylation of the tyrosine residue in the C-terminal tail or binding of the proteins to the SH2 or SH3 domains disrupt intramolecular interactions and allow the αC to assume an active conformation. Since αC and the A-loop are structurally linked, the disruption of the intramolecular interactions also increases phosphorylation of the A-loop [99]. Phosphorylation of the A-loop on Tyr 416 is required for the full activation of SFKs. Upon phosphorylation, the A-loop assumes a conformation which no longer inhibits substrate and ATP binding. In addition, phosphorylation of the A-loop results in a reduced affinity of the SH2 and SH3 domains to their intramolecular ligands and promotes exogenous substrate binding [170], [73].

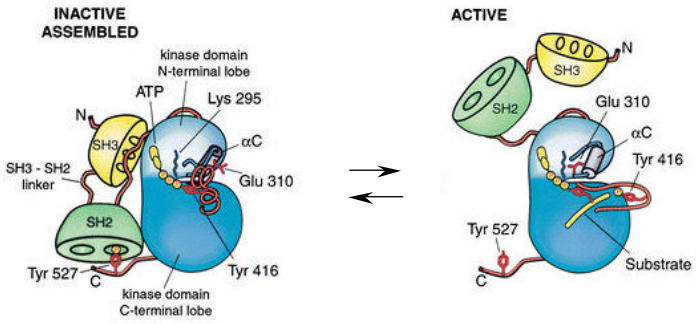


Figure 1.9: Regulation of the catalytic activity of SFKs. Intramolecular interactions between the SH2 domain and phosphorylated Tyr 527 (amino acids numbering for Src) at the C-terminus and between the SH3 domain and SH2-kinase domain linker stabilize an inactive conformation of SFKs (a). Full activation of SFKs requires the disengagement of the intramolecular interactions and phosphorylation of the A-loop on Tyr 416 (b).

1.4.4 Cellular mechanisms of SFK regulation

Regulation of the SFK activity by molecular displacement

Protein phosphatases in the regulation of SFK activity. Various protein tyrosine phosphatases have been involved in positive or negative regulation of the SFK activity by de-phosphorylating inhibitory C-terminal tyrosine residue or tyrosine residue in the activation loop of the kinase, respectively. Src activity was shown to be reduced in cells derived from SHP-1 deficient mice and was restored by over-expression of SHP-1 [204]. Moreover, Src activity was shown to be decreased upon over-expression of a dominant negative form of SHP-1 [204]. These studies suggest that SHP-1 is involved in positive regulation of Src activity. However, these findings could not be confirmed by others [66]. Furthermore, some studies suggest that SHP-1 negatively regulates the activity of SFKs. For example, SHP-1 was shown to directly de-phosphorylate Lck on tyrosine residues located in the A-loop and reduce its kinase activity [41]. In addition to direct de-phosphorylation of SFKs, SHP-1 was also shown to antagonize the action of Src by de-phosphorylating the substrates of Src [66]. Activity of another protein tyrosine phoshatase, PTP1B, was shown to be increased in human breast cancer cell lines with an increased Src activity, suggestsing the positive influence of PTP1B on the activation of Src [18]. The ability of PTP1B to de-phosphorylate and activate Src was confirmed *in vivo* and *in vitro* [18]. Other protein tyrosine phosphatases can also de-phosphorylate C-terminal tyrosine residue and activate Src. PTP-α de-phosphorylates and activates Src in fibroblasts and induces cellular transformation [245]. The hematopoietic cell phosphatase CD45 de-phosphorylates Lck and is considered a major regulator of Lck and Fyn in T cells [89]. These examples show that the level of activity of protein tyrosine phosphatases in cells can regulate the level

of SFK activity. However, further studies are required to understand the influence of tyrosine phosphatases on SFK activity.

Tyrosine kinases in the regulation of SFK activity. The activation of SFKs by protein tyrosine phosphatases can be antagonized by the action of kinases that phosphorylate negative regulatory tyrosine in the C-terminal tail of SFKs. The C-terminal Src kinase (Csk) is known to promote inactivation of SFKs in this manner [155]. A homolog of Csk, the Csk-homologous kinase (Chk), which is expressed primarily in hematopoietic cells and in the brain, was also suggested to possess a similar activity. However, in contrast to Csk, the function of Chk in the regulation of the catalytic activity of SFK is not clear. While kinase activity of SFKs is greatly enhanced in Csk knock-out mice [155], studies with Chk knock-out mice show that Chk does not affect the activity of Src, Hck and Fgr in the bone marrow cells [80]. On the other hand, some studies show that Chk can mediate inactivation of Src by kinase-dependent and by less understood kinase-independent mechanisms [42]. Restricted function of Chk in comparison with Csk was suggested to be at least in part due to the inability of the former to be recruited to certain cellular locations [48].

Regulation of the kinase activity of SFKs by kinases and phosphatases provides an additional mechanism for self-control of SFK activity. For example, Src was suggested to phosphorylate Cbp/PAG, a protein which binds and recruits Csk to the plasma membrane [17]. This leads to an activation of the latter and to the subsequent inactivation of SFKs. In a similar manner, SFKs can induce an activation of protein tyrosine phosphatases. Lck, for example, phosphorylates SHP-1, which in turn might modulate the catalytic activity of Lck [122].

Binding of the SH2 and SH3 domain ligands. Various proteins compete with intramolecular ligands for binding to the SH2 and SH3 domains of SFKs and thereby induce their activation. So far, no proteins which interact with phosphorylated C-terminal tyrosine or SH2-kinase linker have been identified. Examples of SH2 domain-binding proteins include RTKs [6], [114] and focal adhesion kinase (FAK) [193], [221]. SH3 domain-interacting proteins include Nef [149] and Sin [5], among others. An engagement of the SH2 and SH3 domains activates SFKs in response to RTK activation and integrin signaling [6], [114], [193], [221] [33]. SH2 and SH3 domain binding proteins not only activate SFKs, but can also serve as the substrates for SFKs and mediate biological responses of the activated SFKs.

Altered mechanisms of SFK expression and protein stability

Another mechanism involved in regulation of SFKs independently of molecular displacement is the control of SFK protein levels. SFKs were shown to be degraded by the ubiquitin-mediated pathway [85], [160], [79]. An active form of Src is more sensitive to degradation by the proteosomal machinery and is therefore less stable than an inactive form [85]. It was suggested that E3 ubiquitin ligase that binds specifically to the activated Src regulates its degradation. c-Cbl was later identified as an E3 ubiquitin ligase that binds specifically to the active Src and promotes its destruction via the proteosomal pathway [109], [240], [191]. Consistent with this, it was shown that c-Cbl suppresses Hck-mediated cellular transformation [94]. Therefore, once activated, SFKs induce attenuation of the signal by recruiting proteins responsible for their own degradation.

Nitric oxide-mediated activation of SFKs

Recent studies identified another mechanism that might be involved in the activation of SFKs. It was shown that Nitric oxide (NO) released from NO-generating agents activates Src [4]. The mechanism underlying the activation of Src by NO involves phosphorylation of the tyrosine residues in the activation loop of the kinase as well as formation of disulphide bonds (S-S). The latter finding is supported by data showing that treatment of NO-activated Src with reducing agents such as 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) abolished NO-induced catalytic activity of Src. NO may mediate S-S bond formation between different Src molecules and increase the probability of trans-phosphorylation. Alternatively, NO-induced S-S bond formation may mediate conformational changes of Src which would ultimately lead to an increase in its catalytic activity.

Regulation of SFKs by intracellular localization

SFKs associate with various cellular membranes, including cytoplasmic, perinuclear and endosomal membranes. Consistent with their membrane localization, SFKs are involved in biological functions associated with different cellular membranes [17]. At the plasma membrane, SFKs participate in the signal transduction from various cell surface receptors and regulate cell adhesion and migration. At endosomes and Golgi membranes, SFKs most likely regulate endosomal membrane transport [105], Golgi function [10] and secretion of proteins [166], [174]. In addition to membrane localization, a fraction of Src has been shown to localize to the nucleus and nucleolus depending on the phase of the cell cycle and the confluency of cells [47].

Membrane association of SFKs is dependent on its myristylation at the N-terminal glycine residue. However, myristylation provides only a general

membrane affinity and additional signals are responsible for targeting of SFKs to different cellular membranes [175]. For instance, six basic residues at the N-terminus and other regions of SFKs can participate in membrane targeting of SFKs. It was shown that a polypeptide derived from these regions of Src targets a cytoplasmic protein, piruvate kinase, to various cellular membranes [104]. It is possible that phosphorylation of SFKs within these sites could regulate its intracellular location [104]. Palmytoylation was also suggested to target SFKs to different membranes [177]. As palmytoylation is a reversible process, it might represent an additional mechanism involved in the control of the intracellular localization of SFKs. Another possible mechanism of the regulation of SFKs' localization is suggested by studies showing that upon activation Src is translocated from the perinuclear region of the cell to the cell periphery. This translocation depends on the SH3-mediated association of SFKs with the cytoskeleton through Rho GTPases and other cytoskeleton-associated proteins [64]. Therefore, deregulation of these proteins might alter the subcellular localization of SFKs. The specific functions of SFKs at various intracellular locations are poorly understood and would require identification of specific substrates of SFKs at these particular locations.

1.4.5 Functions of SFKs in hematopoietic cells

Hematopoiesis is a process in which a small population of slowly cycling self-renewing hematopoietic stem cells first undergoes commitment to generate common lymphoid or myeloid progenitors, which then give rise to the whole range of increasingly differentiated blood cells with specific functions. Normal hematopoiesis is tightly regulated by external stimuli, such as secreted and membrane-associated growth factors, extracellular matrix and cell-cell contacts.

Several lines of evidence point to the role of SFKs in hematopoiesis. These include expression profiles of SFK in hematopoietic cells, defects in hematopoiesis observed in animals with disrupted SFK-encoding genes, the suppression of growth of hematopoietic cells upon inactivation of SFKs and the cooperation of SFKs with cell surface receptors involved in hematopoiesis. In addition, SFKs are involved in specific functions of mature hematopoietic cells. These points are described in more detail below.

Expression of SFKs in hematopoietic cells

The expression pattern of SFKs suggests their function in hematopoietic system. Hck, Fgr, Lck and Blk are expressed exclusively and Fyn and Lyn predominantly in hematopoietic cells. Src, Yes and Yrk are expressed in a wide variety of tissues including cells of hematopoietic origin.

SFK	T cell	B Cell	NK Cell	Mast cell	Monocyte	Granulocyte	Platelet	Erythrocyte
Blk	-	+	-	-	-	-	-	?
Fgr	-	-*	-	-	+	+	-	+
Fyn	+	+	+	-	+	?	+	?
Hck	-	+	-	+	+	+	+	+
Lck	+	+[a]	+	-	-	-	-	?
Lyn	-*	+	+	+	+	?	+	+
Src	-*	-	+	+	+	?	+	?
Yes	+	-	+	+	?	?	+	?
Yrk	-	-	?	?	+	-	-	-

Table 1.2: Expression pattern of SFKs in hematopoietic cells. * indicates that a SFK is present in transformed of immortalized cells, but not found routinely in normal cells of that type. [a] indicates that SFK expression in the particular cell type is found in some studies, but not in others. (Adopted from [23] and modified using data for Yrk expression [141] and on SFK expression in erythrocytes [49].)

In hematopoietic cells, with the exception of Blk, whose expression is limited to B cells, SFKs are usually expressed in more than one cell type (outlined in Table 1.2) [23]. Likewise, each hematopoietic cell usually expresses more than one SFK. This expression pattern raises a question of whether each SFK has a unique function in a cell or a function of one SFK can be substituted by another family member. Another important observation is that cell lines derived from a particular type of hematopoietic cells sometimes express SFKs which are not detected in normal cells of that type. This observation suggests that immortalization or transformation process might induce a change in the expression of SFKs [23].

In the myeloid compartment, expression of SFKs is associated with the later stages of differentiation. For example, expression of Hck and Fgr increases as cells undergo myeloid differentiation. However, low levels of mRNA encoding these SFKs are also found in immature myeloid cells [24]. Consistent with their expression in more differentiated cells, mRNA encoding Hck and Fgr is detected in leukemic cells in which differentiation to granulocyte or monocytic lineages has taken place [233].

Hematological defects associated with genetic disruption of SFKs

Knock-out animals in which genes encoding SFKs are disrupted by homologous recombination, have specific defects in either development or function of hematopoietic cells. These findings provide direct evidence for the involvement of SFKs in hematopoiesis.

T cell abnormalities in mice deficient for SFKs. T cells express mainly Lck and Fyn. Disruption of the genes encoding these SFKs interferes with normal thymocyte development and functions. Disruption of the Lck gene results in the suppression of thymocyte development, which manifests in thymus atrophy, reduction in number of immature double-positive (CD4+/CD8+)

thymocytes and disappearance of mature single positive thymocytes [151]. Lck activity is also required for normal TCR signaling. T cell line Jurkat, which is defective in the expression of functional Lck is not responsive to TCR stimulation. However, re-introduction of Lck into these cells restores TCR specific cellular response [213]. Mice that lack the T-cell-specific isoform of Fyn show nearly normal thymocyte development, but their thymocytes are defective in TCR signaling [7], which is restored in mature T cells [210]. The mechanism underlying the function of Lck and Fyn in T cell development has been analyzed by various groups [165]. It was shown that Lck associates with CD4 and CD8, which brings it into the proximity of the TCR complex. A stabilization of TCR by its interaction with the MHC-peptide complex and by interaction of CD4 or CD8 with the non-variable region of MHC results in clustering and activation of Lck. Activated Lck phophorylates tyrosine residues within immunoreceptor tyrosine-based activation motifs (ITAMs) within the TCR signaling complex, which recruits the Syk family kinase ZAP-70 to the complex via SH2 domain-dependent interaction. Once in the complex, ZAP-70 is phosphorylated and activated by Lck, which results in the activation of the T cell specific kinase cascade. Activation of Fyn follows the activation of Lck and results in the phosphorylation of another subset of the intracellular proteins. Fyn can also partially substitute the function of Lck in peripheral T cells.

B cell abnormalities in mice deficient for SFKs. Blk is a SFK expressed exclusively in B cells. However, disruption of the gene encoding Blk does not alter B cell development, *in vitro* activation of B cells or humoral immune responses of B cells to T-cell-dependent and independent antigens [219]. On the other hand, animals that lack the Lyn gene have reduced numbers of peripheral B cells with a greater proportion of immature cells [39], develop autoantibodies and glomerulonephritis [91], [159]. B cells derived from $Lyn^{-/-}$ animals are also defective in B cell receptor (BCR) signaling. B cells from these animals have lower threshold for BCR signaling, enhanced MAP kinase activation and an increased proliferative response to BCR engagement [39]. These findings suggest that Lyn negatively regulates BCR signaling. Negative regulation of BCR signaling by Lyn is associated with phosphorylation of negative regulatory co-receptors of BCR, such as FcγRIIB and CD22 [158]. The lack of phosphorylation of these receptors prevents their association with SHP-1 and SHIP and abrogates suppression of BCR-mediated responses. However, studies with Lyn knock-in mice that over-express hyper-active form of Lyn, show that Lyn also plays a positive role in BCR signaling. Lyn knock-in mice have increased levels of Syk and PLCγ2 phosphorylation and show increased calcium flux in response to BCR stimulation [90]. Similar to Lyn deficient mice, these animals also develop autoantibodies and autoimmune glomerulonephritis [90]. These results sug-

gest that constant over-activation of Lyn can ultimately overcome negative signals controlled by Lyn in B cells.

Lyn is not the only SFK that controls B cell development. Studies with mice lacking Lyn, Fyn and Blk show the importance of these SFKs in pre-B cell receptor (pre-BCR)-mediated NF-κB activation and B cell development [189]. Therefore, various SFKs control B cell development and a function of one SFK can be at least partially substituted by another family member in these cells.

Abnormalities of myeloid cells in mice deficient for SFKs. Expression of Hck, Fgr and Src is drastically increased during myeloid differentiation, suggesting that these proteins might be involved in the differentiation process. However, no profound defects in myelogenesis are associated with the disruption of these SFKs. On the other hand, the functions of specialized cells derived from common myeloid progenitor are affected by disruption of genes encoding Hck, Fgr and Src.

Hck and Fgr single knock-out animals have no defects in hematopoiesis [126]. However, macrophages derived from $Hck^{-/-}$ mice have a defect in phagocytosis [126]. Impairment of phagocytosis is not due to a defect in FcγR signaling, but most likely involves alterations of some other molecular events such as phagosome membrane trafficking [126]. Disruption of the genes encoding Hck and Fgr results in a defect in natural immunity, which manifests in higher susceptibility of double knock-out animals to infection with *Listeria monocytogenes*, a bacterium that can survive intracellularly in the inactive macrophages [126]. The mechanism underlying this defect is not understood. The production of cytokines and of NO by macrophages, generally associated with the clearance of *Listeria* is not affected [126]. It is possible that reduced motility and/or reduced integrin signaling observed in these macrophages might contribute to an increased susceptibility to *Listeria* infection [214]. Additionally, neutrophils derived from $Hck^{-}/-$, $Fgr^{-}/-$ mice are defective in adhesion-dependent production of reactive oxygen species and degranulation [124] and [150].

Mice lacking a gene encoding Src have a defect in bone destruction - osteopetrosis [206]. This defect is a result of the inability of osteoclasts to resorb bone [123], while differentiation of monocytes into osteoblasts and then into osteoclasts is not affected in Src knock-out animals. The severity of osteopetrosis in mice lacking genes encoding Src and Hck (Src$-/-$, Hck$-/-$) is higher than in single Src knock-out mice and expression of Hck in osteoclasts from Src$-/-$ mice is increased [125]. These finding suggest that Hck can at least partially substitute the function of Src in osteoclasts.

Several mechanisms have been suggested to explain Src-mediated osteoclast deficiency. For example, Src could participate in the adhesion of osteoclasts to extracellular matrix or adhesion-mediated signaling required for bone resorption [100]. Alternatively, Src-mediated function of osteoclasts could involve regulation of the secretion of the lysosomal enzymes [168].

As described above, $Lyn^{-/-}$ mice have a phenotype associated with the defects in B cells. In addition to affecting B cells, disruption of the Lyn gene also alters myeloid development. With age $Lyn^{-/-}$ mice develop a dramatic increase in the number of myeloid progenitors, as well as splenomegaly and disseminated monocyte/macrophage tumors [84]. The same study shows that mice expressing a hyperactivated mutant of Lyn ($Lyn^{up/up}$), containing a substitution of the C-terminal tyrosine with phenylalanine, are tumor-free [84]. With respect to mechanism underlying Lyn-dependent myeloid phenotype, it was shown that phosphorylation and activity of phosphatases SHP-1 and SHP-2 as well as phosphorylation of tyrosine residues within immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in inhibitory receptors SIRPα and PIR-B are decreased in $Lyn^{-/-}$ as compared with ($Lyn^{up/up}$) mice [84]. Therefore, a possible mechanism explaining the negative role of Lyn in myelopoiesis includes activation of certain phosphatases and ITIM-dependent inhibitory signaling [84].

In summary, gene knock-out studies suggest that SFKs are involved in various molecular events required for hematopoiesis and function of mature hematopoietic cells. While some SFKs are engaged in unique functions of these cells, often a function of one SFK can be at least partially substituted by another SFK.

1.4.6 Role of SFKs in leukemogenesis

The function of SFKs in normal hematopoiesis suggests the possibility that these proteins are also involved in leukemogenesis. A number of recent studies explored this possibility with respect to different types of leukemia using genetic knock-out approaches, cell line models and agents inhibiting SFKs.

The most studied types of leukemia with respect to the involvement of SFKs are those caused by the Bcr-Abl oncogene. Bcr-Abl is associated with the development of CML and B cell acute lymphoblastic leukemia (B-ALL). SFKs are activated in myeloid cell lines which over-express Bcr-Abl, as compared with Bcr-Abl-negative cell lines [44]. Additionally, SFKs are more active in B-lymphoid cells derived from mice in which B-ALL was induced by infection with recombinant retrovirus containing Bcr-Abl, than in B cells derived from mice infected with a control retrovirus [95]. Bcr-Abl also physically associates with SFKs via a complex mechanism [227], [207].

A number of studies provide evidence for a biological relevance of SFK activity in Bcr-Abl-induced leukemias. One study shows that dominant negative mutants of Hck suppress transformation of myeloid cells by Bcr-Abl [120]. In the other study, mice lacking Lyn, Hck and Fgr failed to develop B-ALL, but not CML upon transplantation of the bone marrow infected with the recombinant retrovirus containing Bcr-Abl. This data suggest that Lyn, Hck and Fgr mediate signaling pathways required for the establishment of Bcr-Abl-induced B-ALL [95]. The latter study also shows that a SFK inhibitor, which does not affect Bcr-Abl kinase activity, significantly prolongs survival of mice with Bcr-Abl-induced B-ALL [95]. Additional evidence for the involvement of SFKs in the development of CML is presented by a study, which shows that disruption of Lyn using small interfering RNA (siRNA) induces apoptosis in primary, and drug-resistant Bcr-Abl-positive leukemic cells [171]. These findings set the stage for the development of improved specific therapies for the treatment of Bcr-Abl-positive leukemias based on the inhibition of SFK activity. However, more detailed analysis is required to understand the precise molecular events underlying the contribution of SFKs to the development of Bcr-Abl-positive leukemias.

In addition to CML and B-ALL, SFKs were suggested to be involved in the development of AML. In one study, blasts from the majority of AML patients were shown to have increased kinase activities of Lyn and Hck [180]. However, the sample size in this study was too small to provide any statistical significance of the finding. Positive contribution of Lyn to AML development is also suggested by the findings that treatment of cells with Lyn anti-sense oligonucleotide suppresses the growth of myeloid cell line MO7e in response to granulocyte-macrophage colony stimulating factor (GM-CSF), and that SFK inhibitor PD166285 decreases the growth of myeloid leukemic cell lines and blasts [180]. However, besides SFKs, PD166285 also inhibits other proteins, which could be relevant to the growth of myeloid cells. Together, these finding provide preliminary evidence for the involvement of SFKs in the development of AML. However, further studies along this line are required.

Besides positive regulation of leukemogenesis, SFKs can also negatively regulate the development of hematopoietic malignancies. As described above, Lyn knock-out animals develop monocyte/macrophage tumors while $Lyn^{up/up}$ animals are tumor-free [84]. Therefore, consistent with positive and negative function in hematopoiesis, SFKs can either positively or negatively regulate the development of different types of leukemias.

1.4.7 SFKs in signal transduction mediated by RTKs

Activation of SFKs downstream of RTKs

Activated RTKs recruit SFKs via their SH2 domains. This leads to a disruption of intramolecular interaction of SH2 domain with C-terminal tyrosine and induces activation of SFKs. However, full activation of SFKs in response to RTK activation requires additional factors such as recruitment of protein tyrosine phosphatases that activate SFKs and the translocation of SFKs to the plasma membrane.

Methods used to study the roles of SFKs downstream of RTKs

Four main approaches were used to study the involvement of SFK in RTK-mediated signaling. First approach includes microinjections or transfections of SFKs or cDNA encoding these proteins or their their domains or antibodies inactivating SFKs into the cells. Second approach involves the analysis of signal transduction induced by mutant receptors which do not associate with SFKs. Third approach takes advantage of cell lines derived from mice lacking SFKs, and involves the comparison of receptor-induced responses in these and normal cell lines. Finally, pharmacological inhibitors of SFKs are often used to determine specific functions of SFKs in RTK signal transduction. The role of SFKs in RTK-mediated cell growth or motility is usually analyzed using these approaches.

Examples of SFK-dependent signaling downstream of class III RTKs

SFKs are the key signaling molecules downstream of RTKs of different subfamilies. Examples of SFK-dependent signaling downstream of class III RTKs are described below.

Role of SFKs downstream of the PDGFR. Among class III RTKs, association with SFKs is the most extensively studied in case of the PDGFR. Phoshorylation and activation of SFKs in response to the stimulation of quiescent fibroblasts by PDGF was the first discovery suggesting the role of SFKs in RTK-mediated signaling [172]. Further studies showed that in addition to being activated by PDGF stimulation, Src, Yes and Fyn also associate with the PDGFR following its stimulation by the ligand [114]. Small fraction of Src molecules activated in response to PDGF stimulation correlates with low stoichiometry of association of Src with the PDGFR and suggests that activation accompanies complex formation [114]. SFKs bind to phosphorylated Tyr 579 and Tyr 581 in the JM region of the PDGFR via their SH2 domains [6], [154].

Following these findings, various groups have attempted to investigate whether SFKs are required for biological functions mediated by the PDGFR.

Microinjection studies have revealed that SFKs are required for PDGF-induced DNA synthesis and entry into the mitosis and that SH2 domain of SFKs is essential for this function [179]. On the other hand, studies with the mutant PDGFR that is not able to bind SFKs do not confirm these data. For example, the substitution of two critical tyrosines in the JM of the PDGFR (Tyr 579 and Tyr 581) with phenylalanines prevents the association of the PDGFR with SFKs, but has no effect on mitogenic signaling and transcription of Myc induced by PDGF stimulation [51]. On the other hand, PDGF-mediated chemotaxis of cells expressing the mutant PDGFR is either inhibited or unaffected, depending on the cell type used [183], [93]. Another study that argues against the involvement of SFKs in PDGFR-mediated signaling, shows that PDGF-induced phosphorylation of intracellular proteins, cell cycle progression as well as chemotaxis are not affected in fibroblasts derived from mice lacking Src, Yes and Fyn [112]. However, a cell line used in this study (SYF) was derived from the knock-out animals by transformation with the large T antigen of simian virus 40 (SV-40) [112], which might overcome the requirement of SFKs for PDGFR-mediated mitogenic response [29].

Role of SFKs downstream of the Kit receptor. Similarly to the stimulation of fibroblasts with PDGF, stimulation of human megakaryoblastic leukemia cells with the Kit receptor ligand, stem cell factor (SCF), leads to an increase in phosphorylation and kinase activity of Lyn [119]. Lyn associates specifically with phosporylated fusion protein consisting of the Glutathione S-transferase (GST) and the JM region of Kit [119]. Similar to the PDGFR, autophosphorylated tyrosine residues 568, and to a lesser extent 570, in the JM region of Kit are required for the activation of SFKs upon stimulation of Kit with SCF [116]. Several studies suggest that SFKs mediate biological responses of Kit which lead to cell growth and chemotaxis. For example, treatment of cells with Lyn anti-sense oligonucleotides results in a reduced proliferation of cells in response to SCF stimulation [119]. Moreover, transfection of dominant negative Lyn into normal murine mast cells and analysis of SCF-induced growth of mast and hematopoietic progenitor cells derived from Lyn knock-out mice, shows that Lyn is required for SCF-induced growth and chemotaxis of these cells [162]. On the other hand, activation of SFKs in porcine aortic endothelial cells (PAE) does not correlate with SCF-induced DNA synthesis [116].

Role of SFKs downstream of the CSF-1R. CSF-1R is expressed on monocytes and macrophages and supports their proliferation, differentiation and survival [194]. Stimulation of the CSF-1R over-expressed in the myeloid cell line M1 results in phosphorylation of Hck, Fyn and Yes [137]. In addition, following stimulation by the ligand, SFKs are recruited to phoshorylated Tyr

559 in the JM region of the CSF-1R, and mutation of this tyrosine to Phe results in a decreased response of cells to CSF-1 with respect to cell growth and differentiation [137]. In contrast to the data obtained using M1 cells, over-expression of the CSF-1R Y559F mutant in the murine myeloid 32D cells results in an increased proliferation in response to CSF-1 as compared with cells over-expressing wild type CSF-1R [181], which suggests that SFKs negatively regulate the CSF-1R (described in detail in 1.4.8).

Role of SFKs downstream of the Flt3 receptor. Of the several studies analyzing the role of SFKs in signaling of class III RTKs, only one investigated the involvement of SFKs in Flt3-mediated signaling [53]. This study shows that stimulation of the chimeric receptor, which consists of the extracellular part of the CSF-1R and the transmembrane and cytoplasmic parts of Flt3, with CSF-1 induces a slight increase in phosphorylation of Src, but not Fyn. Additionally, this study shows that both Src and Fyn co-immunoprecipitate with the chimeric receptor. There has been no confirmation of these observations with Flt3 or Flt3 ITD reported, and the role of SFKs in Flt3-mediated signal transduction requires further analysis.

SFK-mediated signaling downstream of RTKs leading to cell growth

Above examples, as well as studies with other RTKs, show that at least in certain cell types and certain cellular contexts SFKs promote mitogenesis induced by RTKs. Possible mechanisms of SFK-mediated signaling with respect to cell growth are described below and summarized in Figure 1.10.

A number of studies suggest that an increase in mRNA encoding a transcription factor Myc is required for SFK-mediated mitogenic response of RTKs. Over-expression of Myc rescues cells from cell cycle block caused by over-expression of dominant negative Src [11]. A direct role of Myc downstream of SFKs in PDGF-induced mitogenic response is demonstrated in a study with the specific SFK inhibitor SU6656, which does not inhibit the PDGFR [19]. Treatment of cells with this inhibitor prevents an increase in Myc mRNA levels and DNA synthesis following PDGF stimulation. However, re-introduction of Myc into these cells abrogates the block caused by SU6656 [19]. How exactly activation of SFKs leads to an increase in Myc mRNA level is not clear. Some preliminary reports suggest that SFK-mediated signaling increases the stability of Myc mRNA without inducing its transcription, and that multiple signaling molecules phosphorylated and/or activated by SFKs (e.g Abl, Shc, Stat3, Vav2 and Rac) play a role in this process [28].

Pro-apoptotic transcription factor p53 has also been involved in the mitogenic signaling of RTKs mediated by SFKs. Antibodies neutralizing SFKs

have no effect on mitogenesis induced by PDGF stimulation in cells expressing dominant negative form of p53 or derived from p53-null mice [29]. The role of SFKs in opposing the effects of p53 also explains the fact that cells over-expressing Large T antigen of SV-40, which inactivates p53, do not require SFKs for transduction of the mitogenic signal [29]. It is not known if SFK-mediated inactivation of p53 is coupled to SFK-dependent increase in Myc mRNA levels.

Besides Myc and p53, other pathways were suggested to be involved in the downstream signaling of SFKs leading to mitogenesis. For example, SFKs phosphorylate Shc and FAK, which could recruit adaptor protein Grb2 to the plasma membrane and thereby activate Ras-MAPK pathway, which is often associated with the mitogenic responses of RTKs. Another possible mechanism of SFK-mediated mitogenic signaling involves SFK-induced phosphorylation of PLCγ. PLCγ induces translocation of the Ras exchange factor RasGRP1 to the Golgi complex and subsequently activates Ras in the Golgi complex, but not on the plasma membrane [16], [28]. It has been suggested that Ras-mediated signaling from Golgi contributes to cell proliferation [81]. In addition, SFKs modulate the activity of Rho GTPases, which, besides their more extensively studied role in the organization of cytoskeleton, also regulate cell growth [223].

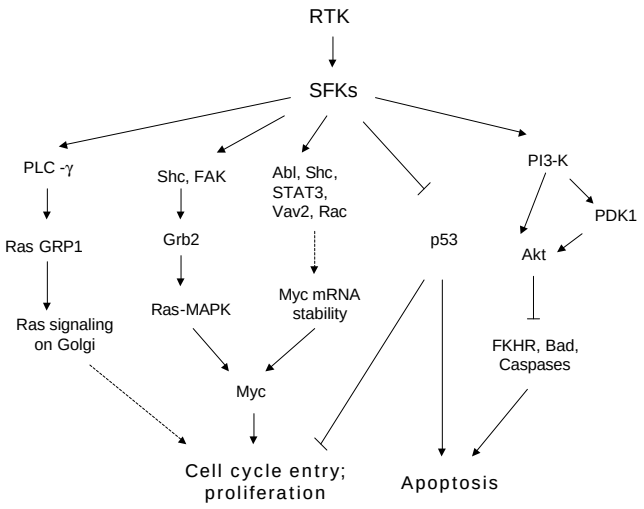


Figure 1.10: A model of SFK-mediated mitogenic responses downstream of RTKs. Possible mechanisms of SFK-mediate mitogenic and anti-apoptotic signals downstream of RTKs are outlined in a simplified manner. Myc and p53 are required for SFK-mediated mitogenesis upon PDGFR stimulation. An increase in Myc mRNA stability depends on various signaling molecules, including Abl, Shc, Stat3, Vav2 and Rac. In addition to Myc and p53, activation of Ras on the Golgi and plasma membranes, as well as activation of PI3-K signaling pathway, could promote SFK-mediated mitogenesis downstream of RTKs.

SFK-mediated signaling downstream of RTKs leading to cytoskeletal effects

The cytoskeletal effects induced by activated SFKs lead to a decrease in cell-cell and cell-matrix adhesion and an increase in cell motility [65], [239]. These SFK-mediated effects could promote metastatic and invasive growth of advanced tumors in which SFKs are activated [239]. However, only a few studies address the question of SFK-mediated signaling downstream of RTKs leading to cytoskeletal changes. The results of these studies suggest that at least under certain circumstances, SFKs are involved in the modulation of cellular migration induced by several RTKs.

An increase in the motility of a cell requires modification of cellular attachments to the extracellular matrix and disruption of the intracellular contacts. These processes are regulated by two types of subcellular structures: focal adhesions and adherent junctions, respectively. SFKs regulate both of these structures [239]. Although the exact functions of SFKs downstream of RTKs with respect to cellular motility are poorly understood, various molecular mechanisms underlying SFK-dependent cytoskeletal effects in cells expressing activated SFKs are known. These mechanisms could also be employed by SFKs in response to their activation by RTKs (outlined in Figure 1.11).

Src physically associates with and phosphorylates components of focal adhesions including FAK and p130 ^{Cas} [192], [169]. In addition to components of focal adhesions, Src can inhibit the functions of Rho which is involved in the regulation of the formation of stress fibers and turn-over of focal adhesions [223]. Src-mediated down-regulation of Rho is mediated by phosphorylation of Rho GTPase activating protein (Rho GAP) [182], which increases the intrinsic GTPase activity of Rho, converting it into inactive GDP-bound form. Another cytoskeletal target of Src is cortactin, a protein involved in the actin dynamics [229]. Src-mediated phosphorylation regulates the activity of cortactin towards the cytoskeleton and thereby might contribute to the changes in actin dynamics and regulate cellular motility [96]. Additionally, hyperactive Src encoded by the v-src gene phosphorylates R-Ras, a relative of H-Ras involved in maintaining integrin activity. Mutant R-Ras, which can not be phosphorylated by Src, inhibits v-Src mediated decrease in cellular adhesion [246]. This finding suggests a possibility that Src can act through R-Ras to inhibit cellular adhesion and stimulate cellular motility.

In adherent junctions, Src stimulates ubiquitination and degradation of E-Cadherin via E3 Ubiquitin ligase Hakai [67]. E-Cadherin is a component of adherent junctions that forms homotypic interactions with E-Cadherin

molecules on neighboring cells, and its disruption leads to the disassembly of adherent junctions and a decrease in cell-cell adhesion. Besides E-Cadherin, Src phosphorylates p120-catenin, a protein which associates with E-Cadherin and regulates cell-cell adhesion [136].

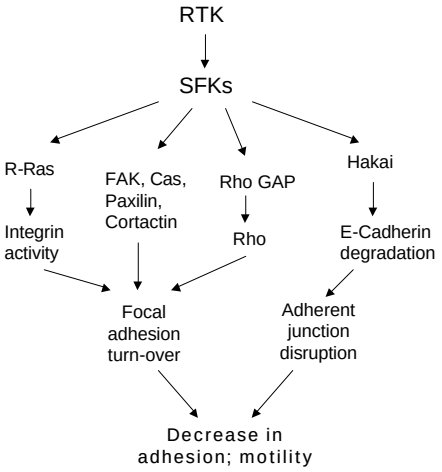


Figure 1.11: A model of SFK-mediated cytoskeletal effects downstream of RTKs. Possible mechanisms of SFK-mediated cytoskeletal effects downstream of RTKs are outlined in a simplified manner. SFKs phosphoryalate and modulate the activity of the components of focal adhesions and proteins that regulate their integrity: FAK, p130^{Cas}, paxilin, cortactin. SFKs promotes the inactive state of Rho by activating Rho GAP, thereby contributing to the turn-over of focal adhesions. In addition to the effects on focal adhesions, SFKs mediate proteasomal degradation of E-Cadherin, which leads to the disruption of adherent junctions and consequently intracellular contacts. SFK-mediated effects on focal adhesions and adherent junction decrease cellular adhesion and stimulate motility.

1.4.8 SFK-mediated modulation of RTKs

SFKs not only serve as signal transduction molecules downstream of RTKs, but also modulate the activity of RTKs. Therefore the synergy between SFKs and RTKs is bidirectional. By modulating the activity of RTKs, SFKs can take part in feed-back mechanisms regulating RTKs or participate in cross-talk between various RTKs [190] and between RTKs and other cell surface receptors [237]. The mechanisms of SFK-dependent modulation of RTKs are described below and are outlined in Figure 1.12.

SFK-mediated phosphorylation of RTKs

As described above, ligand-stimulated auto-phosphorylation of RTKs on tyrosine residues is required for their full activation and generation of the binding sites for various signaling molecules. In addition to auto-phosphorylation, RTKs such as the insulin growth factor-1 receptor (IGF-1R), EGFR, PDGFR, and Kit can be phosphorylated by intracellular tyrosine kinases including

SFKs. These findings are described below.

Src phosphorylation sites vary among different RTKs. In case of the EGFR, they include Tyr 845 in the A-loop and Tyr 1101 in the C-terminus of the receptor [15]. In case of the PDGFR and Kit, Src induces phosphorylation of a homologous tyrosine residue located in the kinase domain of these RTKs (Tyr 934 in the PDGFR and Tyr 900 in Kit) [83], [117]. For all three receptors, Src phosphorylation sites are different from auto-phosphorylation sites, suggesting a unique role for Src-mediated phosphorylation in receptor signaling. In contrast, Src phosphorylation sites on the IGF-1R correspond to auto-phosphorylation sites, implying the role of SFKs in the enhancement of the ligand-mediated phoshorylation [28]. These findings suggest that in contexts of different receptors, SFK-mediated phosphorylation might have different consequences for RTKs signaling.

Src-mediated phosphorylation of the EGFR induces catalytic activity of this receptor and is required for its full mitogenic response [15]. Similarly, in case of the PDGFR, Src phosphorylation positively modulates mitogenic response to PDGF stimulation [83]. However, receptor-mediated signaling leading to cell motility is reduced when PDGFR is phosphorylated by Src [83]. In case of the Kit receptor, SFK-mediated phosphorylation creates binding sites for adaptor protein CrkII, and is also involved in Kit-mediated cellular response which leads to cellular proliferation [117]. To summarize, SFK-mediated phosphorylation can positively regulate biological responses of RTKs leading to cellular proliferation either via an increase in their catalytic activity or by generating binding sites for other signaling molecules. In addition, SFK-mediated phosphorylation can direct RTK signaling towards a particular pathway and thereby modulate biological responses of RTKs.

SFK-mediated endocytosis of RTKs

As described in 1.2.4, activated RTKs are removed from the cell surface by endocytosis. Various studies demonstrate the ability of SFKs to promote this process. The PDGFR mutant, which is not able to activate SFKs (PDGFR Y579F), undergoes a reduced rate of the ligand-stimulated endocytosis of the PDGFR, suggesting that SFK positively regulate endocytosis of the PDGFR [153]. However, another study shows that SFKs suppress endocytosis of the PDGFRα when cells are not stimulated by PDGF [8]. Internalization of the Kit receptor in lymphoid cells lacking Lyn (DT33) is impaired as compared with cells expressing Lyn (DT40) [31]. This suggests that Lyn plays a positive role in the regulation of the Kit endocytosis. Similarly, internalization of CSF-1R, which contains a substitution of Tyr 559 with phenylalanine, and therefore defective in the ability to associate with

SFKs, is decreased [181]. The mechanisms involved in SFK-mediated regulation of receptor endocytosis in these cases are not understood. Some studies suggest that SFKs modulate the components of the endocytic machinery. For example, SFKs co-localize with clathrin in platelets and when activated phosphorylate clathrin's heavy chain [211], [140]. Furthermore, Src activated by the EGFR induces phosphorylation and redistribution of clathrin to the cell periphery, thereby promoting endocytosis of the EGFR [232]. Dynamin is another substrate of Src associated with endocytosis which is phosphorylated by Src upon EGFR stimulation [3]. Src-mediated dynamin phosphorylation induces self-assembly of dynamin and increases in its GTPase activity, both of which are required for clathrin-mediated endocytosis [3]. Some evidence also points to the potential role of SFKs in regulating the intracellular vesicular transport [105]. However, it is not known whether this function of SFKs is relevant to the endocytosys of RTKs. Additionally, SFKs regulate ubiquitination of RTKs, which was shown to mediate endocytic events (see below).

SFK-mediated ubiquitination of RTKs

In addition to phosphorylation, another reversible post-translational modification of RTKs regulated by SFKs is ubiquitination, an addition of a 76 amino acid peptide ubiquitin. Ubiquitination is mediated by a series of enzymatic reactions including activation of ubiquitin by ubiquitin-activating enzyme, E1, transfer of ubiquitin from E1 to ubiquitin-conjugating enzyme E2, and finally ligation of ubiquitin to γ-amino group of a Lys in the target protein by ubiquitin ligase E3. Depending on the attachment of a single ubiquitin moiety or a chain consisting of multiple ubiquitin polypeptides branched at internal lysines within ubiquitin, two types of ubiquitinations are defined: mono-ubiquitination and poly-ubiquitination. Proteins can also be mono-ubiquitinated at multiple sites (multi-ubiquitination). Unlike poly-ubiquitination of cytoplasmic and nuclear proteins usually associated with proteasomal degradation (at least when ubiquitin chains are branched at internal Lys 48), mono- and multi-ubiquitination of RTKs is involved in endocytosis of cell surface receptors [78]. However, exact function of ubiquitination in endocytosis is not known. Some studies show that ubiquitination induces endocytosis, while other studies suggest that the main function of RTK ubiquitination is the regulation of the endosomal sorting of the internalized RTKs [138].

Ubiquitin ligases that mediate the transfer of ubiquitin to RTKs are multi-domain proteins that belong to the Cbl family [138]. In particular, a protein encoded by proto-oncogene c-cbl, has been extensively studied with respect to ubiquitination of RTKs [220]. Molecular mechanism of c-Cbl action includes direct binding to RTKs via its PTB domain as well as re-

cruitment of ubiquitin-conjugating enzyme, E2, and mediation of ubiquitin ligase activity by RING finger domain [102], [220]. c-Cbl-mediated ubiquitination of RTKs is generally linked to their endocytosis. SFKs associate with proline-rich domain of c-Cbl via their SH3 domains [218]. Additionally, c-Cbl PTB domain specifically binds to phosphorylated tyrosine residue in the A-loop of SFKs which leads to an induction of ubiquitin ligase activity of c-Cbl and degradation of SFKs via proteasomal pathway [191]. However, relationship of SFKs and c-cbl is bi-directional. Active SFKs also phoshorylates c-Cbl, which leads to ubiquitination and proteasomal degradation of the latter [9], [240]. SFK-dependent destruction of c-Cbl was shown to play a role in RTK-mediated signal transduction. Stimulation of cells by EGF leads to the activation of SFKs which induces phosphorylation, ubiquitination and destruction of c-Cbl [9]. Destruction of c-Cbl decreases EGFR ubiquitination and leads to prolonged EGFR signaling [9]. Similar to the EGFR, ubquitination of the mutant CSF-1R, which is unable to bind SFKs, is decreased. This correlates with a reduced level of c-Cbl phosphorylation and its reduced association with the mutant CSF-1R [181]. In PDGF-stimulated cells, c-Cbl is also a direct substrate of Src. Treatment of cells with SFK inhibitor SU6656 inhibits phosphorylation of c-Cbl [19]. However, it is not clear whether similar to the EGFR, SFK-mediated phosphorylation of c-Cbl leads to its degradation and subsequent reduction of the PDGFR ubiquitination. In summary, these studies suggest that RTK-mediated activation of SFKs can positively regulate RTKs signaling by targeting c-Cbl for degradation. However, at least in the case of the PDGFR, SFKs can also positively regulate c-Cbl-mediated degradation of RTKs [184]. Therefore, further analysis of the interplay between SFK, c-Cbl and ubiquitination of RTKs is required to understand the role of SFKs in the ubiquitination of RTKs.

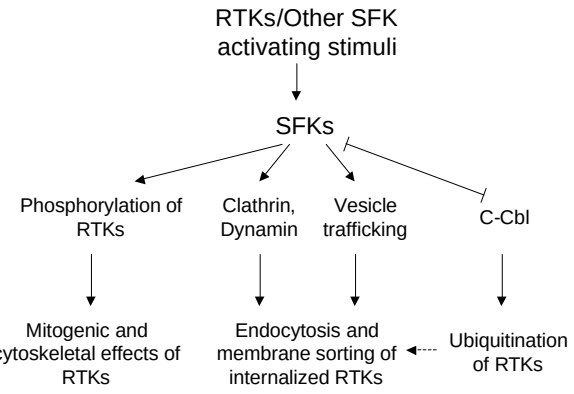


Figure 1.12: Mechanism of SFK-mediated modulation of RTKs. Possible mechanisms of of SFK-mediated modulation of RTKs are outlined in a simplified manner. Phosphorylation of RTKs by SFKs modulates their signaling leading to mitogenesis and cytoskeletal effects. Additionally, SFK-mediated phosphorylation of the components of the endocytic machinery, including clathrin and dynamin, and possibly their effects on vesicular trafficking influence endocytosis and membrane sorting of internalized RTKs. SFKs also reduce ubiquitination of RTKs by phosphorylating and promoting degradation of c-Cbl. However, c-Cbl can also target SFKs for degradation. Ubiquitination status of RTKs mediates their endocytosis and membrane sorting after receptor internalization.

1.5 Thesis proposal

Flt3 is the most recently discovered member of the class III of RTKs. Its expression profile and functional analysis, as well as the phenotype of Flt3 knock-out animals suggest a predominantly hematopoietic role. Mutations of the Flt3 gene are the most common alterations in AML patients. Of these mutations, internal tandem duplications in the JM region of Flt3 are the most frequently occurring type. For this reason, Flt3 is an attractive target for tyrosine kinase inhibitors, and a number of such inhibitors are currently being tested in clinical trials. However, preliminary data from these and other studies with tyrosine kinase inhibitors suggest that more effective therapies require cocktails of inhibitors targeted at multiple molecular lesions underlying the development of a particular malignancy.

A Src kinase family constitutes the largest family of non-receptor tyrosine kinases in human cells. Over-expression and over-activation of SFKs has been associated with the development and progression of various cancers, including several types of leukemia. These observations prompted the development of specific inhibitors of SFKs, which could serve as therapeutic agents against these malignancies. Six out of nine SFKs are expressed exclusively or predominantly in hematopoietic cells and at least two (Hck and Lyn) are found to be activated in AML patient samples. SFKs play key roles in signaling of RTKs, including those of class III. These data suggest the possibility that SFKs could be involved in Flt3-mediated intracellular

events and participate in leukemogenesis associated with activation of Flt3.

Chapter 2

Materials and Methods

2.1 Equipment

- **-80^oC Freezer,** Heraeus, Hanau, Germany
- **0.22 μm sterile filters,** Millipore, Bedford, MA, USA
- **40 μm sterile cell strainers,** BD Biosciences,
- **Automatic pipets,** Gilson, Middleton, WI, USA
- **Cell counting chambers,** Neubauer, Germany
- **Cell freezing containers,** Nalgene, Neerijse, Belgium
- **Centrifuges,** Eppendorf, Hamburg
- **CO_2 Incubators,** WTB Binder, Tuttlingen, Germany, Forma Scietific
- **Electrophoresis cells and blotting systems,** Biorad, Hercules, CA, USA
- **Flow cytometer, Coulter EPICS XL-4,** Beckman Coulter GmbH, Krefeld, Germany
- **Heating blocks,** Techne, Cambridge, UK
- **Laminar flow hoods,** BDK, Sonnenbuehl, Germany
- **Liquid nitrogen tank,** Cryoson, Schoellkrippen, Germany
- **Microscopes,** Zeiss, Oberkochen, Germany
- **Modular Flow cytometer (MoFlo),** Cytomation, Fort Collins, USA
- **pH-meter,** WTW, Weilheim, Germany
- **Spectrophotometer,** Eppendorf, Hamburg)
- **Sterile cryotube vials, and cell scrapers,** Sarstedt, Newton, USA
- **Sterile tissue culture plastic pipets, tubes, dishes, flasks and cell scrapers,** Sarstedt, Newton, USA
- **Thermocycler,** Perkin Elmer, Norwalk, USA
- **Vacuum driven disposable filtration systems,** Millipore, Bedford, MA, USA
- **Vortex,** Heidolph, Kelheim, Germany
- **Water bath,** Koettermann, Haenigsen, Germany

2.2 Reagents

- **2-Mercaptoethanol,** Merck, Darmstatdt, Germany
- **30% Acrylamide and 0.8% bisacrylamide stock solution,** Roth, Karlsruhe, Germany
- **Acetic Acid 96%,** Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- **Agarose (electrophoresis grade),** Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- **Ammonium Persulfate,** Bio-rad, Hercules, CA, USA
- **Calcium chloride,** Sigma-Aldrich Chemie GmbH, Steinheim, Germany

- **EDTA,** Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- **Ethanol,** Merck, Darmstatdt, Germany
- **Ethidium Bromide (10 mg/ml),** Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- **Gelatine,** Merck, Darmstatdt, Germany
- **Glycerol,** Merck, Darmstatdt, Germany
- **Glycine,** Merck, Darmstatdt, Germany
- **Isopropanol,** Merck, Darmstatdt, Germany
- **LB medium,** Invitrogen, Karlsruhe, Germahy
- **Luria agar,** Gibco, Eggenstein, Germany
- **Methanol,** Merck, Darmstatdt, Germany
- **Nonidet P-40,** ICN Biomedicals, Aurora, Ohio, USA
- **Phosphatase inhibitor cocktail II for tyrosine protein phosphatases, Acid and Alkaline Phosphatases,** Sigma, Saint Louis,USA
- **Ponceau S**, Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- **Protease inhibitor cocktail,** Sigma, Saint Louis, Missouri, USA
- **Protein A/G-Agarose,** Roche, Mannheim, Germany
- **SDS (electrophoresis grade),** Merck, Darmstatdt, Germany
- **SDS-PAGE loading buffer Roti Load (4X concentrate)**, Roth, Karlsruhe, Germany
- **SDS-PAGE Molecular Weight Standarts, Broad Range,** Bio-Rad, Hercules, CA, USA
- **Silver Nitrate,** Merck, Darmstatdt, Germany
- **Skim Milk Powder (blotting grade),** Roth, Karlsruhe, Germany
- **Sodium Orthovanadate,** Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- **TEMED,** Roth, Karlsruhe, Germany
- **Tween 20,** Sigma-Aldrich Chemie GmbH, Steinheim, Germany

2.3 Kits and other research products

- **Annexin V - PE Apoptosis detection kit,** BD Pharmingen, San Diego, USA
- **BCA Protein assay reagent kit,** Pierce, Rockford, IL, USA
- **DNA isolation and purification kits,** Qiagen, Hilden, Germany
- **ECL Western Blotting Detection System,** Amersham Biosciences, Buckinghamshire, England, UK
- **Effectene Transfection Reagent,** Qiagen, Hilden, Germany
- **Gel blotting paper,** Schleicher & Schuell, Dassel, Germany
- **High performance chemiluminescence film,** Amersham Biosciences, Buckinghamshire, England, UK
- **Immobilon-P PVDF Transfer Membrane,** Millipore, Bedford, MA, USA

- **Mycoplasma detection kit for conventional PCR,** Minerva Biolabs, Berlin, Germany
- **Primers,** Metabion, Martinsried, Germany
- **QuikChange Site-Directed Mutagenesis Kit,** Stratagene, La Jolla, CA, USA
- **Restriction endonucleases and DNA modifying enzymes,** New England Biolabs, Beverly, MA, USA

2.4 Antibodies

- **Anti-mouse IgG from sheep, peroxidase conjugated,** Amersham Biosciences, Buckinghamshire, England, UK
- **Anti-rabbit IgG from goat, peroxidase conjugated,** Sigma, St. Louis, USA
- **c-Src (N-16),** Santa Cruz Biotechnology, Santa Cruz, CA, USA
- **Erk (C-16),** Santa Cruz Biotechnology, Santa Cruz, CA, USA
- **Flt3 conjugated with PE (clone SF 1.340),** Immunotech, Marseille, France
- **Flt3 (S-18),**Santa Cruz Biotechnology, Santa Cruz, CA, USA
- **Fyn,** Santa Cruz Biotechnology, Santa Cruz, CA, USA
- **Hck,** BD Biosciences, San Jose, CA, USA
- **IgG_1 from mouse, conjugated with PE,** Immunotech, Marseille, France
- **Lyn,** Santa Cruz Biotechnology, Santa Cruz,
- **myc (9E10),** Roche, Mannheim, Germany
- **phospho-tyrosine (PY99),** Santa Cruz Biotechnology, Santa Cruz, CA, USA
- **phospho-Akt, Ser 473,** Cell Signaling Technology, Beverly, MA,
- **phospho-Erk, Thr 202/Tyr 204 (E10),** Cell Signaling, Technology, Beverly, MA, USA
- **phospho-STAT5, Tyr 694,** Cell Signaling Technology, Beverly, MA, USA
- **phospho-Src Family kinases, Tyr 416,** Cell Signaling Technology, Beverly, MA, USA
- **STAT 5 (C-17),** Santa Cruz Biotechnology, Santa Cruz, CA, USA

2.5 Plasmids

2.5.1 Vectors

- **pApuro** [113], gift of Dr. Seth Corey, Pitsburgh, USA
- **pCDNA3.1/His© A,** Invitrogen, Karlsruhe, Germahy
- **pCDNA6A,** Invitrogen, Karlsruhe, Germahy

- **pMSCV-IRES-EGFP,** gift of Dr. Van Etten, Tufts-New England Medical Center, Boston, MA, USA
- **ecopack,** gift of Dr. Van Etten, Tufts-New England Medical Center, Boston, MA, USA
- **MIY,** gift of Dr. C. Buske, University of Munich, Germany
- **pMSCV neo,** Clontech, Palo Alto, USA
- **pCDNA3 GFP,** gift of Dr. K. Foster, University of Munich, Germany

2.5.2 All other plasmids used for this work

- **pApuro Hck wt,** gift of Dr. Seth Corey, Pitsburgh, USA
- **pCDpuro Hck K269R,** gift of Dr. M. Warmuth, University of Munich, Germany
- **pApuro Hck Y501F,** gift of Dr. M. Warmuth, University of Munich, Germany
- **pApuro Lyn,** gift of Dr. M. Warmuth, University of Munich, Germany
- **pApuro Fyn,** gift of Dr. M. Warmuth, University of Munich, Germany
- **pApuro c-Src,** gift of Dr. M. Warmuth, University of Munich, Germany
- **pCDpuro Hck K269R R150L,** generated by mutagenesis
- **pCDpuro Hck K269R W93A ,** generated by mutagenesis
- **pApuro Hck G2A,** generated by mutagenesis
- **pCDNA3.1/His© A Hck wt.** To produce this plasmid PCR fragment generated using pApuro Hck wt as a template and the following primers: 5'-GAA TGT GAA TTC ATG GGG TGC ATG AAG TCC AAG-3' and 5'-CGG GGT ACC TGG CTG CTG TTG GTA CTG G-3', was sub-cloned into EcoRI/KpnI sites of pCDNA3.1/His© A vector.
- **pCDNA3.1/His© A Hck K269R.** To produce this plasmid PCR fragment generated using pCDpuro Hck K269R as a template and the following primers: 5'-GAA TGT GAA TTC ATG GGG TGC ATG AAG TCC AAG-3' and 5'-CGG GGT ACC TGG CTG CTG TTG GTA CTG G-3', was sub-cloned into EcoRI/KpnI sites of pCDNA3.1/His© A vector.
- **pCDNA3.1/His© A Hck K269R R150L.** To produce this plasmid PCR fragment generated using pCDpuro Hck K269R R150L as a template and the following primers: 5'-GAA TGT GAA TTC ATG GGG TGC ATG AAG TCC AAG-3' and 5'-CGG GGT ACC TGG CTG CTG TTG GTA CTG G-3', was sub-cloned into EcoRI/KpnI sites of pCDNA3.1/His© A vector.
- **pCDNA3.1/His© A Hck K269R W93A.** To produce this plas-

mid PCR fragment generated using pCDpuro Hck K269R W93A as a template and the following primers: 5'-GAA TGT GAA TTC ATG GGG TGC ATG AAG TCC AAG-3' and 5'-CGG GGT ACC TGG CTG CTG TTG GTA CTG G-3', was sub-cloned into EcoRI/KpnI sites of pCDNA3.1/His© A vector.

- **pCDNA3.1/His© A Hck G2A.** To produce this plasmid PCR fragment generated using pApuro Hck G2A as a template and the following primers: 5'-GCG AAT TCA TGG CCT GCA TGA AGT CCA AGT TCC-3' and 5'-CGG GGT ACC TGG CTG CTG TTG GTA CTG G-3', was sub-cloned into EcoRI/KpnI sites of pCDNA3.1/His© A vector.
- **pCDNA3.1/His© A c-Src.** To produce this plasmid PCR fragment generated using pApuro c-Src as a template and the following primers: 5'-G GAA TTC ATG GGT AGC AAC AAG AGC-3' and 5'-CCC AAG CTT GAG GTT CTC CCC GGG CTG GTA C-3', was sub-cloned into EcoRI/HindIII sites of pCDNA3.1/His© A vector.
- **pCDNA3.1/His© A Lyn.** To produce this plasmid PCR fragment generated using pApuro Lyn as a template and the following primers: 5'-G GAA TTC ATG GGA TGT ATA AAA TCA AA -3' and 5'-CGG GGT ACC AGG CTG CTG CTG GTA TTG CCC TTC C-3', was sub-cloned into EcoRI/KpnI sites of pCDNA3.1/His© A vector.
- **pCDNA3.1/His© A Fyn.** To produce this plasmid PCR fragment generated using pApuro Fyn as a template and the following primers: 5'-G GAA TTC ATG GGC TGT GTG CAA TGT-3' and 5'-CGC GGA TCC CAG GTT TTC ACC AGG TTG GTA CTG-3', was sub-cloned into EcoRI/BamHI sites of pCDNA3.1/His© A vector.
- **pMSCV IRES EGFP Hck wt.** To produce this plasmid Hck wt fragment was sub-cloned from pApuro Hck wt into EcoRI cloning site of pMSCV IRES EGFP vector.
- **pMSCV IRES EGFP Hck K269R.** To produce this plasmid Hck K269R fragment was sub-cloned from pCDpuro Hck K269R into EcoRI cloning site of pMSCV IRES EGFP vector
- **pMSCV IRES EGFP Hck Y501F.** To produce this plasmid Hck Y501F fragment was sub-cloned from pApuro Hck Y501F into EcoRI cloning site of pMSCV IRES EGFP vector.
- **pCDHF3,** gift of Dr. Hitoshi Kiyoi, Nagoya University School of Medicine, Nagoya, Japan
- **pCDNA6A hFlt3 wt,** gift of Dr. Spiekermann, University of Munich, Germany (generated by BamHI/PstI sub-cloning of Flt3 wt fragment from pCDHF3 plasmid)
- **pCDHFTD,** contains a full-length human Flt3 ITD (Mt3) cDNA cloned from pCDSRα expression vector, gift of Dr. Hitoshi Kiyoi, Nagoya University School of Medicine, Nagoya, Japan [111]
- **pCDNA6A hFlt3 ITD,** generated by BamHI/PstI sub-cloning of

Flt3 ITD fragment from pCDHFTD plasmid, gift of Dr. Spiekermann, University of Munich,Germany

- **pCDNA6A c-kit,** gift of Dr. Spiekermann, University of Munich, Germany
- **pCDNA6A hFlt3 K644R,** generated by mutagenesis
- **pCDNA6A hFlt3 Y589F,** generated by mutagenesis
- **pCDNA6A hFlt3 Y591F,** generated by mutagenesis
- **pCDNA6A hFlt3 Y589F, Y591F,** generated by mutagenesis
- **pCDNA6A hFlt3 Y589F, Y591F, Y597F, Y599F,** generated by mutagenesis
- **pCDNA6A hFlt3 K644R, Y589F, Y591F,** generated by mutagenesis
- **pCDNA6A hFlt3 K 644R, Y589F, Y591F, Y597F, Y599F,** generated by mutagenesis
- **pCDNA6A hFlt3 ITD K644R,** generated by mutagenesis
- **pCDNA6A hFlt3 ITD Y589F,** generated by mutagenesis
- **pCDNA6A hFlt3 ITD Y591F,** generated by mutagenesis
- **pCDNA6A hFlt3 ITD Y589F, Y591F,** generated by mutagenesis
- **MIY hFlt3 wt.** To produce this plasmid BamHI/PstI fragment encoding hFlt3 wt was sub-cloned into HpaI site of MIY vector.
- **MIY hFlt3 ITD.** To produce this plasmid BamHI/PstI fragment encoding hFlt3 ITD was sub-cloned into HpaI site of MIY vector.

2.6 Bacterial strains

- **DH5α,** gift of Dr. A. Obermeier, Sirenade AG, Martinsried, Germany
- **XL-1 Blue**, Stratagene La Jolla, USA
- **XL-10 Gold**, Strategene, La Jolla, USA

2.7 Mammalian cell lines

- **HEK-293,** gift of Dr. H. Buening, Gene Center, Munich, Germany.
  This cell was obtained after transformation of human embryonic kidney cells with adenovirus type 5 DNA [74].
  Cells were maintained in DMEM supplemented with 10% FBS, 100 Units/ml of penicillin and 0.1 mg/ml of streptamycin. Cells were split 1:5 to 1:7 every 2-3 days using trypsin/EDTA.
- **COS-7,** DSMZ, Braunschweig, Germany.
  This cell line was derived from a simian cell line CV-1 by transformation with an origin-defective mutant of SV-40.
  Cells were maintained in DMEM supplemented with 10% FBS, 100 Units/ml of penicillin and 0.1 mg/ml of streptamycin. Cells were split 1:3 to 1:5 every 2-3 days using trypsin/EDTA.

- **32D clone 3 (32D cl.3),** gift of Dr. Van Etten, Tufts-New England Medical Center, Boston, MA, USA.
  This cell line was established from Friend murine leukemia retrovirus infected continuous bone marrow culture derived from C3H/HeJ mouse. Cell growth is strictly dependent on the presence of IL-3 in the culture medium. Cells were maintained at 0.1-1x10^6 cells/ml in RPMI supplemented with 10% FBS, 10% supernatant of WEHI-3B cells as source of IL-3, 100 Units/ml of penicillin and 0.1 mg/ml of streptamycin.
- **WEHI-3B,** DSMZ, Braunschweig, Germany.
  This is a macrophage-like cell line derived from a BALB/c mouse treated for tumor induction. The cells secrete IL-3 into the culture medium.
  Cells were maintained in RPMI supplemented with 10% FBS, 100 Units/ml of penicillin and 0.1mg/ml of streptamycin. For splitting cells were loosened by incubation in PBS (without Mg^{2+} or Ca^{2+}) and detached mechanically using cell scrapers.
- **THP-1,** gift of Dr. Spiekermann, University of Munich, Germany.
  This cell line was established from the peripheral blood of a 1-year old boy with acute monocytic leukemia at relapse.
  Cells were maintained at 0.1-1 X 10^6 cells/ml in RPMI supplemented with 10% FBS, 100 Units/ml of penicillin/ml and 0.1 mg/ml of streptamycin. Cells were split 1:2-1:3 every 3-4 days.
- **MV4-11,** gift of Dr. Spiekermann, University of Munich, Germany.
  This cell line was established from peripheral blood of a 10-year old boy with acute monocytic leukemia. The Flt3 gene in this cell line contains an ITD mutation.
  Cells were maintained in RPMI supplemented with 10% FBS, 100 Units/ml of penicillin and 0.1 mg/ml of streptamycin.
- **MM6,** gift of Dr. Spiekermann, University of Munich, Germany.
  This cell line was established from the peripheral blood of a 64-year old man with relapsed acute monocytic leukemia.
  Cells were maintained at 0.3-1 X10^6 cells/ml in RPMI supplemented with 10% FBS, 100 Units/ml of penicillin and 0.1 mg/ml of streptamycin.

2.8 Molecular biological techniques

2.8.1 Introduction of plasmid DNA into bacteria

Exposure of bacterial cells to high concentrations of certain divalent ions makes a small fraction of cells permeable to foreign DNA by a mechanism not yet understood. Bacteria that incorporated plasmid DNA, which carries antibiotic resistance gene, are selected by plating the cells on dishes containing nutrient agar and appropriate antibiotic. Methods for preparation and

transformation of competent *E.coli* used in this work are described below.

Generation of competent bacterial cells

$CaCl_2$ Solution for generation of competent E.coli

60 mM	*$CaCl_2$*
15%	*Glycerol*
10 mM	*PIPES, pH 7.0*

filter sterilized

Overnight bacterial cultures were diluted 1:10 with fresh LB-medium and grown at 37^{o}C with shaking at 250 rpm until cell density reached an OD_{590} of 0.4. Cells were then aliquoted into prechilled 50 ml tubes and left on ice for 5-10 minutes. Cells were centrifuged for 7 minutes at 1600g, 4^{o}C. Centrifuge was allowed to decelerate without breaks. Supernatant was poured out, and each cell pellet was resuspended in 10 ml of ice cold $CaCl_2$ solution. Cells were then centrifuged for 5 minutes at 1100g, 4^0C. Each cell pellet was resuspended in 10 ml of ice cold $CaCl_2$ solution and left on ice for 30 minutes. Cells were pelleted down by centrifugation for 5 minutes at 1100g, 4^{o}C. Supernatant was poured out and each cell pellet was resuspended in 2 ml of ice cold $CaCl_2$ solution. Cells were dispensed into prechilled sterile 1.5 ml tubes and frozen immediately either at -80^{o}C or in liquid Nitrogen.

Transformation of competent bacteria

An aliquot containing 50-150 μl of competent bacteria was thawed on ice. 0.5-5 μl of DNA was added to bacteria, and tubes were swirled gently to mix bacteria and DNA. The mixture was incubated on ice for 30 minutes. Tubes were then placed into 42^{o}C water bath for 45 seconds. Immediately afterwards tubes were returned on ice for 2 minutes. 500 μl of LB medium was added to each tube. Tubes were incubated at 37^{o}C with shaking at 250 rpm for 1 hour. Bacteria was pelleted down by centrifugation at 7000g and plated on LB plates containing the appropriate antibiotic. Plates were incubated at 37^{o}C overnight.

2.8.2 Production of recombinant DNA plasmids

Production of DNA fragments of interest and their insertion into the plasmid DNA is a first step in the generation of multiple copies of a plasmid DNA (plasmid DNA cloning). The techniques for the generation of recombinant plasmid DNA used in this work are described below.

Digestion of plasmid DNA with restriction endonucleases

Restriction endonucleases recognize short DNA sequences and cleave double-stranded DNA at specific sites within or adjacent to the recognition sequence.
Restriction nuclease cleavage was accomplished by incubating the enzyme(s) with the DNA in the appropriate reaction conditions according to enzyme manufacturer's instructions.

De-phosphorylation of 5'-phosphate residues from DNA using calf intestine phosphatase (CIP)

When cDNA fragments resulted from cleavage with a single restriction endonuclease had to be sub-cloned into a vector linearized using the same restriction endonuclease, linearized vector DNA was treated with CIP to remove 5'-phosphate. The presence of 5'-phosphate residue is necessary for formation of phosphodiester bonds catalyzed by DNA ligases. Therefore, de-phosphorylation of 5'-phosphate residues prevents ligation of vector on itself and reduces the amount of false positive colonies obtained after transformation of ligation mixture into the bacteria.
De-phosphorylation of 5'-phosphate residues from DNA was accomplished by incubating CIP with the DNA in the appropriate reaction conditions according to enzyme manufacturer's instructions.

Modification of sticky ends of DNA into blunt ends using T4 DNA polymerase

When direct sub-cloning of DNA containing compatible cohesive ends was not possible, cohesive ends were converted into blunt ends and blunt-end cloning was used. 3'-recessed DNA ends were filled in and 3'-overhanging ends were removed using T4 DNA polymeraze, which possess both 5'→3' DNA polymerase and 3'→5' exonuclease activities.
DNA was dissolved in 1X T4 DNA Polymerase reaction buffer, supplemented with 100 mM dNTPs and 50 μg/ml BSA, and incubated with 0.2 units/μl of T4 DNA polymerase at 12^{o}C for 20 minutes. Reaction was stopped by incubating the tubes at 75^o C for 10 minutes. DNA was then purified using PCR purification kit (Qiagen).

Ligation using T4 DNA Ligase

T4 DNA Ligase catalyzes the formation of phosphodiester bonds between juxtaposed 5'-phosphate and and 3'-hydroxyl termini in duplex DNA.
To join restriction fragments having either cohesive or blunt ends, DNA was dissolved in 1X T4 DNA Ligase reaction buffer (50 mM Tris-HCl, pH 7.5, 10

mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μ/ml BSA) and incubated with 40 Units/μl (for cohesive end cloning) or 200 Units/μl (for blunt end cloning) of T4 DNA Ligase at 16^0C for 3 hours. Ligation mixture was used directly to transform competent *E. coli*.

Amplification of DNA fragments by PCR

PCR was used to amplify DNA fragments necessary for production of recombinant DNA plasmids. Optimal parameters for the PCR reaction, such as primer annealing temperature, elongation temperature and time were chosen individually for each reaction.
Composition of a standard PCR mix is presented below.

Standard PCR mix

1 μM	*primers, each*
0.5 mM	*dNTPs mixture (each)*
10pg - 0.2 μg	*template DNA*
*1*X	*10*X *Taq Polymerase Reaction Buffer*
1 u	*Taq Polymerase*
variable	*sterile deionized H_2O*

Purification of DNA fragments after enzymatic reactions

QIAquick PCR Purification kit and QIAquick Gel Extraction kit were used to purify DNA fragments after enzymatic reactions and agarose gel electrophoresis. In both kits spin-columns with silica membranes are used. At high concentration of chaotropic salts and optimal pH, DNA adsorbs to silica membranes while contaminants pass through. DNA is then eluted from the columns using a buffer with low salt concentration and basic pH.

2.8.3 Site-directed PCR-based mutagenesis

To introduce point mutations into cDNAs, Stratagene's QuikChange Site-Directed mutagenesis kit or its components were used. Suprecoiled double stranded DNA vector and two oligonucleotide primers, which are complementary to the opposite sites of the vector and contain the desired mutation were used for PCR reaction. During temperature cycling with high fidelity Pfu Turbo DNA polymerase, mutated plasmids containing staggered nicks were generated. The presence of PCR reaction product was confirmed by analyzing 10 μl of PCR mixture on the agarose gels. Parental DNA was digested with DpnI endonuclease, which is specific for metylated and hemi-metylated DNA. Plasmids produced in dam^+ *E. coli* strains were

used as template DNA, therefore the parental strands contained methylated adenosine. In contrast the PCR-synthesized daughter strands did not contain 6-methyl-adenosine and were resistant to cleavage by DpnI. For DpnI digestion, 1 μl of DpnI endonuclease was added to 40 μl of the PCR mixture, and restriction was carried out at 37^{o}C for 1 hour. 5 μl or PCR mixture treated with DpnI was transformed into XL-10 Gold or DH5α bacterial strains. Clones, containing correct plasmids were identified by restriction analysis. Presence of only desired mutations was confirmed by the sequence analysis of the whole cDNA. Composition of a PCR mix for site-directed mutagenesis is presented below. Parameters used for generation of Flt3 and Hck point mutants are described in Table 2.1.

Standard PCR mix for site-directed mutagenesis

1 μM	*primers, each*
200 μM	*dNTPs mixture (each)*
0.1 - 1 μg	*template DNA*
3μl	*QuikChange Solution*
*1*X	*10*X *Pfu Turbo Reaction Buffer*
2.5 u	*Pfu Turbo DNA polymerase*
	sterile deionized H_2O to 50 μl

cDNA[a]	Mutation	Sense and antisense primers	Thermocycling[b]		
			T_{ann} (^{o}C)	t_{ann} (sec)	n
hFlt3	K644R	5' CAA TCC AGG TTG CCG TCC GAA TGC TGA AAG AAA AAG CAG ACA GC 3' 3' GTT AGG TCC AAC GGC AGG CTT ACG ACT TTC TTT TTC GTC TGT CG 5'	60	50	18
hFlt3	Y589F Y591F	5' G GTG ACC GGC TCC TCA GAT AAT GAG TTC TTC TTC GTT GAT TTC AGA G 3' 3' C CAC TGG CCG AGG AGT CTA TTA CTC AAG AAG AAG CAA CTA AAG CTC C 5'	60	50	18
hFlt3 Y589F, Y591F	Y597F Y599F	5' C GTT GAT TTC AGA GAA TTT GAA TTT GAT CTC AAA TGG GA 3' 3' G CAA CTA AAG TCT CTT AAA CTT AAA CTA GAG TTT ACC CT 5'	55 60	50 55	3 17
Hck K269R	R150L	5' CTG GGC TCC TTC ATG ATC CTG GAT AGC GAG ACC ACT AAA GG 3' 3' GAC CCG AGG AAG TAC TAG GAC CTA TCG CTC TGG TGA TTT CC 5'	55 60	50 55	4 16

Table 2.1: Parameters used for the generation of Flt3 and Hck point mutants by PCR-based site-directed mutagenesis. All cycling protocols started by a 3 min. denaturation step and were concluded by a 7 min. extension step at 68^{o}C. The individual cycles contained a 50 sec. denaturation step at 95^{o}C and were concluded by an extension step at 68^{o}C for 21 min.[a]: templates used for Flt3 and Hck, respectively: pcDNA6-Flt3wt(ITD) and pCDpuro-Hck. [b]: T_{ann}: annealing temperature, t_{ann}: time for annealing, n: cycle number.

2.9 Mammalian cell culture techniques

2.9.1 Transfection of plasmid DNA into mammalian cells

Transfection of plasmid DNA into HEK-293 cells using Calcium Phosphate method

In this approach precipitate containing calcium phosphate and DNA is formed by mixing HEPES buffered saline solution (HBS) with a solution containing calcium chloride and DNA. The precipitate adheres to the surface of the cells and is taken up by the cells via yet unknown mechanism.

Solutions

HBS buffer

50mM Hepes
280mM NaCl
*1.5mM NaP**

filter sterilized

pH is adjusted with NaOH to 7.2
**NaP is 1:1 mixture of equal molar amounts of Na_2HPO_4 and NaH_2PO_4.*

pH range of HBS solution optimal for transfection is very narrow. Therefore, 5 aliquots of HBS solution with pH ranging from 6.6 to 7.8 with the step of 0.3 were prepared and tested using pCDNA3 GFP for the highest transfection efficiency.

$CaCl_2$ Solution for transfection

250 mM $CaCl_2$ in H_2O
filter sterilized

250 mM $CaCl_2$ Solution was prepared shortly before transfection by diluting 2.5 M $CaCl_2$ stock solution using sterile endotoxin-tested water (Sigma, USA).

HEK-293 cells were sub-cultured as described in 2.7. The day before transfection cells were seeded at 1.7 X 10^6 cells per 6 cm tissue culture plate. 10 μg of DNA was diluted in 119 μl of 250 mM $CaCl_2$ solution. Components were mixed by vortexing briefly at low speed. 119 μl of HBS

solution was then added dropwise to DNA/ $CaCl_2$ mixture and tubes were vortexed immediately for 3-4 seconds at low speed. Culture medium was changed to 4 ml of fresh medium and DNA/ CaCl2/ HBS mixture was added to the cells dropwise within two minutes after addition of HBS buffer. 12-24 hours after the transfection, culture medium was changed to 4 ml of fresh medium. 36-48 hours after transfections cells were either lysed or supernatant containing recombinant retrovirus was harvested.

Transfection of COS-7 cells using Effectene Transfection reagent kit

In this method, DNA is condensed by interaction with Enchancer reagent of the kit in a defined buffer system. Effectene reagent, which is based on a proprietary non-liposomal lipid, is then used to coat the condensed DNA. DNA-Effectene complexes fuse with the plasma membrane of tissue culture cells resulting in both uptake and expression of the DNA.
COS-7 cells were sub-cultured as described in 2.7. The day before transfection cells were split 1:3, which resulted in about 50% confluent cellular monolayer on the day of the transfection. Right before transfection, medium was changed to 2 ml of DMEM containing 1.5 g/L of glucose, supplemented with 10% FBS, 100 Units/ml of penicillin and 0.1 mg/ml of streptamycin. 1-2 μg of DNA was mixed with 100 μl of EC buffer from the kit and 12 μl of Enchancer. The mixture was vortexed and incubated at room temperature for 5 minutes. 14 μl of Effectene Reagent was added to the mixture. The components were mixed by vortexing for 10 seconds. The mixture was then incubated at room temperature for 10 minutes. 900 μl of DMEM containing 4.0 g/L of glucose, supplemented with 10% FBS, 100 Units/ml of penicillin and 0.1mg/ml of streptamycin, was added to the mixture. Samples were mixed carefully and the mixture was added to the cells dropwise. Culture medium was changed 8-16 hours after the transfection. Cells were lysed 48 hours after the transfection.

2.9.2 Production of recombinant retrovirus using transient transfection of HEK-293 cells

Recombinant replication-incompetent retroviruses have been generated by co-transfecting HEK-293 cells with the packaging vector (ecopac) and the retroviral vector (pMSCV neo, pMSCV IRES EGFP, MIY). Packaging vector carries retroviral *gag, pol* and *env* genes that are required for replication of retrovirus. Retroviral vector carries the gene of interest, $\Psi+$ packaging signal and LTRs, which encode most of the viral control elements including promoter, enchancer, the polyadenylation signal and the integration signal. HEK-293 cells were co-transfected with ecopac and appropriated retroviral vector using calcium phosphate method. 10 μg of retroviral vector and 5 μg of ecopac were usually used per transfection of 80-90% confluent cells in 6 cm dishes. 12 hours after the transfection, medium was changed to 3

ml of fresh medium. Supernatant, containing recombinant retrovirus was harvested 36-48 hours after the transfection, aliquoted and stored at - 80^{o}C.

2.9.3 Titering retroviral stocks

Following infection and reverse transcription of the viral genome, integration of the viral DNA into the host cell DNA takes place. The number of proviral integrants into the cellular genome depends on the infection efficiency. According to Poisson statistics, the uninfected fraction is equal to e^{-l}, where l is the mean proviral copy number per cell. Therefore, when 10% of cells are infected, the mean proviral copy number per cell is -ln(0.9)=0.1. So, most infected cells have single proviruses. To generate cell lines, in which most cells have single proviruses, infections efficiency was kept at 10% or lower.

To normalize between different viral stocks, 32D cl.3 cells were infected with serial dilutions of retrovirus. Infection efficiency was measured 36-48 hours after the infection by FACS analysis of GFP or YFP expression. Dilutions, at which infection efficiency was 10% or less were used for generation of 32D cl.3-based cell lines.

2.9.4 Infection of mammalian cells with recombinant retroviruses

Infection of 32D cl.3 cells with retorovirus was achieved by incubation of retrovirus with cells in the presence of polycations (Polybrene).

Polybrene Solution

800 μg/ml of Polybrene in H_2O

filter sterilized

Fresh aliquot of the HEK-293 cells' supernatant, containing appropriate recombinant retrovirus was thawed in 37^{0}C water bath shortly before the infection. Exponentially or sub-confluently growing cultures of 32D cl.3 cells were used for infection. 5 X 10^4 cells per infection were resuspended in 250 μl of RPMI, supplemented with 10%FBS, 10% WEHI-3B supernatant, 100 Units/ml of penicillin and 0.1mg/ml of streptamycin, in 24-well plate. 16 μg/ml (2X final concentration) of polybrene was added to the cells. When necessary (see 2.9.3), viral stocks were diluted in RPMI supplemented with 10% FBS, 10% WEHI-3B supernatant, 100 Units/ml of penicillin and 0.1 mg/ml of streptamycin. 250 μl of diluted or undiluted viral stock was added to the cells. Cells were mixed gently and returned to the incubator for 3-5 hours. 1.5 ml of fresh culture medium was then added to the cells. Cells were incubated for 48 hours at 37^{0}C before subsequent analysis or fluorescence-activated cell sorting.

For infection of higher numbers of cells, the volumes of all the components were multiplied accordingly.

2.9.5 Fluorescence-activated cell sorting

Modular Flow cytometer (MoFlo), Cytomation, USA, was used for cell sorting. In flow cytometer equipped for cell sorting, fluorescent light emitted by each cell is measured and cell suspension is passed through a nozzle, which forms droplets containing at most single cells. At the time of formation, each droplet is given an electric charge proportional to the amount of fluorescence in the cell. Droplets containing electric charge which corresponds to the fluorescence of desired cells are separated by an electric field and collected.
Cells to be sorted were pelleted by centrifugation at 1000g for 5 minutes, resuspended in the small volume of culture medium to the final concentration of $5x10^7$ cells/ml. Cell suspension was passed through sterile 40 μl cell strainers to obtain single cell suspension right before sorting. Immediately after sorting cells were resuspended in the appropriated culture medium and returned to the cell culture incubators.

2.9.6 Generation of 32D cl.3 cells expressing Flt3 and Flt3 F692T using antibiotic selection

Retroviruses encoding Flt3 constructs and neomycin resistance gene were generated by transient transfection of HEK-293 cells. 32D cl.3 cells were infected with retroviruses as described. 48 hours after the transfection, cells were transfered to the medium containing 1 mg/ml of G418. Infected cells were selected in the presence of G-418 for 1 week until all cells in the uninfected control have died. Resulted cell lines were sub-cultured in the presence of 0.5 mg/ml of G-418.

2.9.7 Generation of 32D cl.3-based cell lines expressing Flt3 and Hck constructs using FACS

To generate 32D cl.3 cell lines expressing Flt3 and Hck constructs, cells were first infected with retrovirus containing Flt3-IRES-EYFP and YFP-positive cells were sorted as described in 2.9.5. Within a few days after sorting, sorted cells were infected the second time with retroviruses containing Hck-IRES-EGFP, and GFP/YFP-positive cells were sorted. Infection with retrovirusese were performed as described in 2.9.4. Infectiion efficiency was always kept at 10% or lower. Aliquots of sorted cells were frozen within a few days after sorting. Cells, which were in culture for no longer than 3 weeks were used for subsequent analysis.

2.10 Analysis of mammalian cells

2.10.1 Analysis of cells by flow cytometry

Coulter EPICS XL-4 flow cytometer, Beckman Coulter GmbH, Germany, was used for analysis of cells by flow cytometry. Individual cells expressing different molecules can be detected by flow cytometry. Expression of proteins can be detected by staining cells with specific antibodies covalently conjugated with fluorochrome. Alternatively, cells expressing fluorescent proteins, such as GFP or YFP can be detected directly. When cells are placed on flow cytometer, a stream of cells is directed through an argon laser beam that excites fluorochrome to emit light. The emitted light is then detected by a photomultiplier tube specific for an emission wavelength of fluorochrome by virtue of a set of optical filters. The signal is amplified in its own channel and is displayed in a variety of different forms: histogram, dot blot or contour display.

Analysis of GFP and YFP expression by FACS

5 X 10^5 cells were pelleted down by centrifugation at 1000g for 2 minutes, resuspended in 500 μl of fresh culture medium and used directly for flow cytometry.

Analysis of Flt3 surface expression by FACS

5X10^5 cells were pelleted down by centrifugation at 1000g for 2 minutes and washed once with PBS, containing 0.2% FBS. 10 μl of either anti-Flt3 antibody (clone SF 1.340, Immunotech, France) or isotype control antibody (mouse Ig G_1 antibody, Immunotech, France), both covalently conjugated to phycoerythrin (PE), were added to the cells. Cells were mixed by flicking the tubes and incubated with the antibodies for 15 minutes at room temperature in the dark. 4 ml of PBS containing 0.2% FBS was then added to the cells. Cells were pelleted down by centrifugation at 1000g for 5 minutes, resuspended in 250 μl of PBS containing 0.2% FBS, and analyzed by flow cytometry.

2.10.2 Determination of cell viability and numbers

When cells undergo necrosis or are at the late stages of apoptosis (secondary necrosis), they lose the integrity of the plasma membrane. These cells can be detected using exclusion dyes such as Trypan Blue. Trypan Blue stains only those cells, which have lost the membrane integrity, leaving cells with intact plasma membrane unstained.
10 μl of cell suspension was mixed with 10 μl of Trypan Blue solution and

counted using cell counting chambers. Cells which were negative for Trypan Blue staining were considered viable.

Cell growth assay with 32D cl.3 derived cell lines, expressing Flt3 ITD

Cell growth reflects the rates of cell proliferation and apoptosis. Cell growth was determined by counting viable cells over a period of time.
Cell lines which were in culture for no more than 3 weeks were used for the assay. Prior to the start of the assay, all cell lines were sub-cultured as described in 2.7 in the presence of IL-3. Concentration of viable was determined using Trypan Blue dye staining as described above. Cells were washed 3 times with culture medium without IL-3, and resuspended in the culture medium with or without IL-3 to the final concentration of 1 X 10^4 viable cells/ml. Cells were plated in triplicates in 24 well plates at 1 ml of cell suspension per well. 72 hours after plating, concentration of viable cells was determined using Trypan Blue dye staining as described above.

2.10.3 Apoptosis assay using Annexin-V apoptosis detection kit

Annexin-V is a calcilum-dependent phospholipid binding protein, that has a great affinity for phosphatidylserine. In normal cells phosphatydilserine is present in the inner leaflet of the plasma membrane, while outer leaflet contains mostly neutral phospholipids. However, as cells undergo apoptosis, loss of asymmetry in the plasma membrane phospholipids occurs. The amount of phosphatidylserine in the outer leaflet of the membrane increases. Therefore, Annexin-V binds to the surface of apoptotic, but not normal cells. Late stages of apoptosis (secondary necrosis) and necrosis, are accompanied by the loss of the integrity of the plasma membrane. Annexin-V can enter such cells and bind phosphatidylserine in the inner inner leaflet of cells. To discriminate between apoptotic and necrotic cells, staining with the vital dye 7-Amino-actinomycin (7-AAD) was performed in parallel to Annexin-V labeling.

Cells were pelleted by centrifugation, washed with PBS and resuspended in 100 μl of Annexin-V binding buffer at the concentration of 1 X 10^6 cells/ml. 2.5 μl of Annexin-V conjugated to PE and 2.5 μl of 7-AAD were added to the cells. Cells were mixed gently and incubated for 15 minutes at room temperature in the dark. 400 μl of binding buffer was then added to the cells and cells were analyzed by flow cytometry within 2-3 hours. All cells which were Annexin-V positive were considered apoptotic.

Apoptosis assay with 32D cl.3 derived cell lines expressing Flt3 wt

Cell lines which were in culture for no more than 3 weeks were used for the assay. Prior to the start of the assay, all cell lines were sub-cultured as described in 2.7 in the presence on IL-3. Concentration of viable cells was determined using Trypan Blue dye staining as described above. Cells were washed 3 times with culture medium without IL-3, and resuspended in culture medium with or without 10% of WEHI-3B supernatant as a source of IL-3 or 100 ng/ml Flt3 ligand (Promocell, Germany) to the final concentration of $1X10^5$ cells/ml. Cells were plated in triplicates in 24 well plates at 1 ml of cell suspension per well. Apoptosis was measured 24-48 hours after plating using Annexin-V apoptosis detection kit as described above.

2.11 Analysis of proteins

2.11.1 Preparation of cellular lysates

During lysis intracellular and membrane proteins are solubilized due to the presence of detergent and high salt concentration in the lysis buffer. Non-solubilized proteins are precipitated by centrifugation. Protease inhibitor cocktail is included in the lysis buffer to prevent proteolysis, phosphotase inhibitor cocktail - to maintain the phopsphorylation status of phosphoproteins, EDTA - to chelate divalent ions that are essential for metalloproteases.

Lysis Buffer

20mM	*Tris-HCl, pH 8.0*
150mM	*NaCl*
10%	*Glycerol*
1%	*NP-40*
10mM	*EDTA, pH 8.0*

filter sterilized

Protease and phosphatase inhibitor coctails were added freshly before cell lysis at 1:100 vol./vol. dilution.

Preparation of lysates of adherent cells after transient transfection

HEK-293 and COS-7 cells were transfected in 6 cm cell culture dishes as described 2.9.1. 36-48 hours after transfection, some cells were stimulated with recombinant human Flt3 ligand (FL). For stimulation, 3 ml of medium was removed and 1 ml of medium was left in each of 6 cm cell culture dishes. Fresh aliquot of Flt3 ligand (Promocell, Germany) was thawed up

shortly before stimulation. Flt3 ligand was added to the cells at the final concentration of 100 ng/ml. Cells were incubated in the presence of the ligand for 10 minutes at 37^0C. Medium was then aspirated, and cell culture dishes were placed on ice. 450 μl of lysis buffer for HEK-293 cells, and 300 μl of lysis buffer for COS-7 cells, was added to each 6 cm dish. Cells were detached from the dishes using plastic cell scrapers and transfered to 1.5 ml pre-chilled tubes. Cells were lysed at 4^o with constant rocking for 15-30 minutes. Non-solubilized proteins were pellted down by centrifugations at 20000g, 4^oC for 10 minutes . Pre-cleared lysates were then transfered to the new 1.5 ml pre-chilled tubes. Lysates were stored at -80^{o}C and thawed on ice when necessary.

Preparation of lysates of non-stimulated suspension cells

To prepare lysates of non-stimulated cells suspension cells, exponentially or sub-confluently growing cell cultures were pelleted down by centrifugation at 1000g for 5 miuntes and culture medium was discarded. Cells were washed once with ice cold PBS and resuspended in lysis buffer at the concentrations described in table 2.2. Cellular lysates were prepared further as described above for adherent cells.

Table 2.2: Concentrations of suspensions cells used for preparation of cellular lysates.

Cell line	*Cells per 1 ml of lysis buffer*
32D cl.3	*1 X 10^8*
THP-1 *MM6* *MV4-11*	*2 X 10^7*

Preparation of lysates of suspension cells stimulated with Flt3 ligand

Stimulation of 32D cl.3 cells with Flt3 ligand before lysis. Exponentially or sub-confluently growing 32D cl. 3 cells were pelleted down by centrifugation at 1000g for 5 miuntes and culture medium was discarded. Cells were washed 3 times with culture medium without IL-3 and resuspended in the same volume of culture medium without IL-3 as that of the discarded medium. Cells were returned to the incubator for about 12 hours. Cells were then pelleted down by centrifugation at 1000g for 5 miuntes and resuspended in a small volume of culture medium without IL-3 to the final concentration of about 2-4 X 10^7 cells/ml in 15 ml or 50 ml tubes. Flt3 ligand (Promocell, Germany) was added to the cells at the final concentration of 100 ng/ml. Cells were stimulated with the ligand for 10 minutes at 37^oC.

Stimulation of THP-1 cells with Flt3 ligand before lysis. Exponentially or sub-confluently growing THP-1 cells were pelleted down by centrifugation at 1000g for 5 miuntes, washed 3 times with culture medium without FBS and resuspended in the same volume of culture medium supplemented with 0.5% FBS. Cells were returned to the incubator for about 16 hours. Cells were then pelleted down by centrifugation at 1000g for 5 miuntes and resuspended in a small volume of culture medium with 0.5% FBS to the final concentration of about 4 X 10^6 cells/ml in 15 ml or 50 ml tubes. Cells were stimulated with Flt3 ligand (R&D Systems) at the final concentration of 20 ng/ml for different periods of time at 37^oC.

Lysis of stimulated suspension cells. Immediately after stimulation, tubes containing cell suspensions were filled with ice cold PBS and cells were pelleted down by centrifugation at 1000g, 4^oC for 5 minutes. PBS was discarded and appropriate amounts of lysis buffer (see table 2.2) were added to the cells. Cellular lysates were prepared as described above.

2.11.2 Determination of protein concentration in cellular lysates

The BCA Protein Assay Reagent kit, Pierce, USA, was used to measure protein concentration in cellular lysates. BCA method combines the reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium and selective colorimetric detection of the cuprous cation (Cu^{1+}) with a reagent containing bicinchoninic acid (BCA). The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous cation. This complex has a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations.

2 μl of protein lysates were diluted in 98 μl of water. Protein standards for calibration were prepared by diluting 0, 5, 15 and 30 μg of bovine serum albumin (BSA) from 2 mg/ml stock solution in water to the final volume of 100 μl. Working BCA reagent was prepared freshly by mixing reagent A (containing bicinchoninic acid, sodium carbonate, sodium bicarbonate and sodium tartrate in 0.1M sodium hydroxide) and reagent B (containing 4% cupric sulfate) from the kit at the ratio of 1:50 vol./vol.. 900 μl of working reagent was added to protein samples diluted in water and components were mixed by inverting the tubes 3-4 times. Tubes were placed in 65^oC water bath for 5 minutes. Tubes were allowed to cool down at room temperature for about 10 minutes. 562 nm absorbance of all samples was measured using spectrophotometer. Standart curve was prepared by plotting standard absorbance measurements against their protein concentrations. Equation of a linear fit curve was used to calculate protein concentration in each sample.

2.11.3 Immunoprecipitation of proteins from cellular lysates

In this approach, specific antibody is added to the cellular lysate to bind protein of interest. Antibody-protein complexes are then precipitated using solid-phase matrix. Bacterial proteins A and G which have specific binding sites for Fc-parts of antibodies, covalently coupled to cross-linked agarose, are usually used as solid-phase matrix to precipitate protein-antibody complexes.

Cellular lysates containing 0.5-1 mg of total protein were mixed with about 1 μg of antibody in pre-chilled 1.5 ml tubes on ice. Volumes of the mixture were adjusted to 200-300 μl to obtain equal protein concentrations in each sample. Tubes were incubated for 3 hours to overnight at 4^{o}C with constant rotation. 20-25 μl of Protein A or G coupled agarose was added to each sample and tubes were incubated for additional 45 minutes at 4^{o}C with constant rotation. Following incubation with Protein A or G agarose, immuno-complexes were pelleted by centrifugation at 1000g, 4^{o}C for 15 seconds and washed 3 times with ice-cold lysis buffer. Each time complexes were collected by centrifugation at 1000g, 4^{o}C for 15 seconds. 5-10 μl of the lysis buffer used for the last wash were left above the agarose pellet. 9μl of 4X SDS-PAGE loading buffer (Roti-load, Roth, Germany) were added to the samples and proteins were denatured by heating to 100^{o}C for 5 mimutes. Samples were cooled down on ice and analyzed immediately or frozen at -80^{o}C for later analysis. Protein A or G agarose was pelleted by centrifugation at 1000g for 15 seconds and supernatants, containing immunoprecipitated proteins were analyzed by SDS-PAGE.

2.11.4 Separation of proteins by denaturing discontinuous polyacrylamide gel electrophoresis

In this approach proteins in the mixture are denatured by heating in the presence of 2-mercaptoethanol and SDS. Denatured polypeptides bind SDS and become negatively charged. The amount of bound SDS is almost always proportional to the molecular weight of a polypeptide, and is independent of its sequence. Therefore proteins and protein subunits are separated according to their size during migration through the pores in the gel matrix in responce to an electrical field.

Protein samples for electrophoresis were prepared by dissolving a mixture of proteins in SDS-PAGE loading buffer Roti-Load, Roth, Germany, and heating up the samples at 100^{o}C for 5 minutes.

Separation gels with the following dimensions were used: thickness 1.0 mm, length 7.3 cm and width 8.3 cm.

Solutions

SDS Electrophoresis Buffer, 5X

0.125M	*Tris*
0.96M	*Glycine*
0.5%	*SDS*

H_2O to 1000 ml

Solutions for casting one 8% separating and one stacking gel

	Separating gel, 8%	*Stacking gel*
H_2O	*2.3 ml*	*1.4 ml*
30% Acrylamide	*1.3ml*	*0.33 ml*
1.5M Tris (pH 8.8)	*1.3ml*	-
1.5M Tris (pH 6.8)	-	*0.25 ml*
10% SDS	*0.05ml*	*0.02ml*
10% APS	*0.05 ml*	*0.02 ml*
TEMED	*0.003 ml*	*0.002 ml*

Proteins were separated at 120 V until the dye front has left the separation gel.

2.11.5 Coomassie staining of proteins in gel

Commasie blue dye is a triphenylmethane textile dye, which binds to amino acids in acidic environment. Excess dye is diffused from gel during prolonged period of de-staining.

Solutions

Staining solution (Bio-Rad)

0.25%	*Coomassie Blue R-250*
40%	*Methanol*
10%	*Acetic acid*

De-staining solution

40%	*Methanol*
10%	*Acetic acid*

SDS-PAGE gels were placed for 30 minutes in the staining solution. Gels were then distained in a large excess of de-staining solution until the

background was satisfactory clear.

2.11.6 Silver staining of proteins in gel

This method of staining proteins in gels is based on the affinity of silver ions to nucleophilic and aromatic groups. This affinity causes co-localization of silver ions at the sites of macromolecules in gel. Silver ions are reduced to metalic silver generating an image of electrophoretic band.

Solutions

Formaldehyde fixing solution

40%	*Methanol*
37%	*Formaldehyde*
0.2 g/L	$Na_2S_2O_3$ *(added freshly before use)*

Thiosulfate developing solution

30 g/L	Na_2CO_3
4 mg/L	$Na_2S_2O_3$
37%	*Formaldehyde (added freshly before use)*

Silver nitrate solution

0.1%	*Silver nitrate (prepared freshly before use)*

Drying solution

10%	*Ethanol*
4%	*Glycerol*

A gel was placed in a plastic container containing 50 ml of formaldehyde fixing solution and agitated slowly for 10 minutes on the orbital shaker. Fixing solution was then poured out and gel was washed twice with water, 5 minutes for each wash. After washing, gel was soaked for 1 minute in 50 ml of 0.1% Silver nitrate solution. Silver nitrate solution was poured out and gel was washed for 1 minute with water and then for 30 seconds with a small volume of thiosulfate developing solution. The gel was then soaked in 50 ml of thiosulfate developing solution until band intensities were adequate. 5 ml of 2.3 M citric acid solution per 100 ml of thiosulfate developing solution was added to the gel for 10 minutes to stop the reaction. Gel was then washed in

water for 10 minutes and soaked in 50 ml of drying solution for 10 minutes. For drying, gel was sandwiched between two pieces of wet dialysis membrane on a glass plate. Edges were clamped with the notebook clamps and the gel was dried overnight at room temperature.

2.11.7 Transfer of proteins to PVDF-membrane

Proteins separated by SDS-PAGE were transfered to PVDF membrane by direct electrophoretic transfer. Tank transfer system (Bio-Rad) was used.

Transfer Buffer

250 mM	*Tris*
200 mM	*Glycine*
20%	*Methanol*

PVDF membrane was soaked in methanol for a few minutes and then transfered to a container with transfer buffer. Gel and attached PVDF membrane were sandwiched between two pieces of Whatman 3 MM paper, two porous pads, soaked in transfer buffer, and two plastic supports. The entire construct was then immersed in an electrophoresis tank, equipped with platinum electrodes, which contains transfer buffer. PVDF membrane was placed towards the anode. Transfer was carried out at 100 V for 1-2 hours.

2.11.8 Ponceau S staining of proteins on PVDF-membrane

Staining with Ponceau S was used to provide visiual evidence that electrophoretic transfer of proteins has taken place and to locate molecular weight markers.

Ponceau S staining solution

0.5g of Ponceau S was dissolved in 1ml of glacial acetic acid and the volume was adjusted to 100 ml with H_2O

PVDF membrane was incubated for 5-10 seconds in Ponceau S staining solution and then washed with water until the protein bands became visible.

2.11.9 Analysis of proteins on PVDF membrane by immunoblotting

In this method, specific antibodies are used to identify proteins trasferred to PVDF membrane. First, membrane is immersed in blocking buffer to fill all protein binding sites with non-reactive protein. Then membrane is incubated in a solution containing antibody directed against the antigen(s)

in the protein to be detected. Primary antibody bound to the protein of interest are recognized by secondary antobody conjugated with horse radisch peroxidase (HRP). The complex containing the antigen, primary antibody and secondary antibody- horse radisch peroxidase conjugate is detected by chemiluminecsent visualization using ECL detection system (Amersham Biosciences).

Solutions

TBS-T buffer

10 mM	*Tris-HCl, pH 8.0*
150 mM	*NaCl*
0.01%	*Tween*

NET buffer, 10X

1.5M	*NaCl*
0.05M	*EDTA, pH 8.0*
0.5M	*Tris, pH 7.5*
0.5%	*Triton X-100*

Milk blocking solution

5% (w./vol.of non-fat dry milk dissolved in TBS-T buffer

G-NET blocking solution

2.5 g of gelatine was dissolved in 100 ml of 10X NET buffer and the mixture was brought to boiling. The solution was then mixed using magnetic stir bar until it became clear. The volume was adjusted to 1000 ml with water.

PVDF membranes were blocked in the blocking solution either for 1-3 hours at room temperature or overnight at 4^oC. G-NET blocking solution was used with phosphoprotein-specific antibodies and corresponding secondary antibodies, while milk blocking solution was used with all other antibodies. After blocking, membranes were transferred to the containers with primary antibody in 1% milk blocking solution or G-NET solution and incubated for either 1 hour at room temperature or overnight at 4^oC. After

incubation with primary antibodies, membranes were washed 3-4 times over 20-30 minutes with TBS-T buffer. Washed membranes were transfered to the containers with secondary antibody-HRP conjugates in 1% milk blocking solution or G-NET solution and incubated for 30 minutes at room temperature. Blots were then washed 3-4 times over 20-30 minutes with TBS-T buffer. Excess of TBS-T buffer was drained using tissue paper and 2 ml of freshly prepared ECL developing solution was added per each blot. Membranes were incubated with ECL solution for 1 minute with slow rocking. Excess of ECL solution was drained using tissue paper. Membranes were exposed to Hyperfilm (Amersham Biosciences).

Chapter 3

Results

3.1 Analysis of the specificity of STI-571

The initial goal of the work described in this section was to understand the specificity of the small molecule inhibitor STI571. However, early results, which have raised questions as to the role of SFKs in Flt3-mediated signal transduction, lead to a change the direction of the project.

3.1.1 Single amino acid substitution renders Flt3 sensitive to STI-571

The small molecule tyrosine kinase inhibitor STI-571 belongs to the 2-phenylaminopyrimidine class of pharmacophores. STI571 is used as therapeutic agent to treat CML. STI-571 selectively inhibits Abl, Kit, PDGFR and Arg kinases. Comparison of protein kinase structures and amino acid alignments of STI571-sensitive and insensitive kinases revealed that three amino acids surrounding the nucleotide binding site (amino acids at positions 315, 380 and 382 of Abl) are critical for the sensitivity of a kinase to STI-571. In general, protein kinases insensitive to STI-571 have bulkier amino acids at the positions 315 and 380. In support of this, it was shown that substitution of Thr 315 in Abl for an amino acid with a bulkier side chain rendered Abl resistant to STI-571. Analysis of the crystal structure of the catalytic domain of Abl, complexed to a variant of STI-571, also demonstrates that presence of Thr 315 is a key requirement for inhibition of Abl by STI-571 [195]. To further understand the high specificity of STI-571, it was investigated whether the presence in Flt3 of Phe 692 at the position corresponding to Thr 315 of Abl is the only cause for insensitivity of Flt3 to STI-571.

Phe 692 in Flt3 was substituted with threonine and the sensitivity of the modified kinase to STI-571 was tested. 32D cell lines expressing Flt3 wt and Flt3 ITD F692T were generated as described in 2.9.6. Cells were incubated with STI-571 and the inhibitors of SFKs PP1 and CGP76030 as controls for 10 minutes. Flt3 was immuno-precipitated using Flt3 specific antibodies and the phosphorylation status of the receptor was analysed by immunoblotting with phospho-tyrosine specific antibodies. As shown in Figure 3.1, phosphorylation of Flt3 wt or Flt3 ITD was not changed upon incubation of cells with STI571, confirming insensitivity of Flt3 to inhibition by STI-571. However, when Phe 692 was substituted by threonine, incubation of cells with STI-571 led to a significant decrease in Flt3 phosphorylation as compared with the phosphorylation of Flt3 F692T in the absence of the inhibitor. This result demonstrates that the presence of phenylalanine in position 692 causes insensitivity of Flt3 kinase to STI-571 and its substitution by threonine renders Flt3 sensitive to STI-571. Similar results were also obtained independently by another group [21].

Substitution of Phe 692 with threonine also led to a decrease in the kinase activity of Flt3. In contrast to Flt3 ITD, expression of Flt3 ITD F682T in 32D cl. 3 was not sufficient to transform these cells to IL-3-independent growth (data not shown).

3.1.2 Inhibition of Flt3 phosphorylation by inhibitors of Src kinases.

High specificity of STI-571 is not only affected by the special amino acid composition in the ATP binding pocket of the sensitive kinases, but also by their special conformation in the inactive state, to which the compound specifically binds [195]. For this reason SFKs, which have a high homology of amino acids essential for the binding of STI-571, are still insensitive to this inhibitor. SFKs differ from the STI-571-sensitive kinases in the position of the A-loop, which in the inactive state of the kinase blocks the access of STI-571 to its binding pocket. A high degree of homology in amino acids surrounding the nucleotide binding site between SFKs and Abl explains why most of the SFK inhibitors, including PP1 and CGP76030, also inhibit STI-571 sensitive kinases such as Abl, PDGFR and Kit. STI-571, PP1 and CGP76030 share overlapping binding sites in these kinases, as suggested by studies demonstrating that mutation of Thr 315 makes Abl insensitive not only to STI-571, but also to PP1 and CGP76030 [228]. Together, these observations suggest that the presence of phenylalanine in the position corresponding to Thr 315 of Abl would be incompatible with sensitivity of a kinase to SFK inhibitors such as PP1 and CGP76030. Surprisingly though, Figure 3.1 shows that incubation of 32D cells expressing Flt3 wt or Flt3 ITD with SFK inhibitors PP1 and CGP76030 leads to a decrease in the phosphorylation of Flt3 regardless of the presence of F692T mutation. This observation is in contradiction with the assumption mentioned above and suggest that SFKs phosphorylate Flt3. Stimulated by this finding, the possibility that SFKs phosphorylate Flt3 and modulate Flt3-mediated signal transduction was investigated, and the results are presented in the following sections of this chapter.

3.2 Functional interaction of Hck and Flt3 in HEK-293 cells

3.2.1 Hck interferes with maturation of Flt3

Detection of mature and immature forms of Flt3 wt and Flt3 ITD in HEK-293 cells

As described in 1.3.2, Flt3 is N-glycosylated and differentially glycosylated forms can be detected in cellular lysates by Western blot analysis. Consistent with these studies, Figure 3.2 shows that two forms of Flt3 can be detected

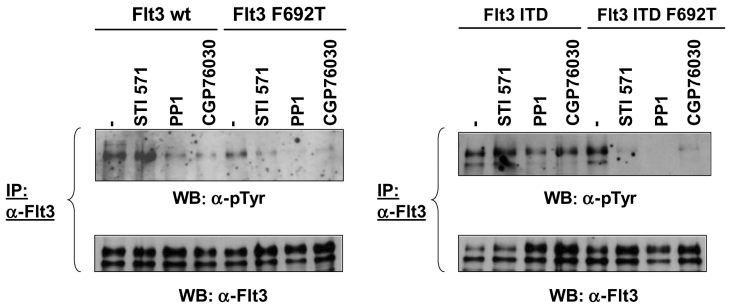


Figure 3.1: Single amino acid substitution renders Flt3 sensitive to inhibition by STI-571. SFK inhibitors inhibit Flt3 phosphorylation.. 32D cells expressing Flt3 constructs were incubated either in the absence of the inhibitors or in the presence of 2.5 μM of STI-571, 5 μM of CGP76030 or 50 μM of PP1 for 10 minutes. Flt3 immunoprecipitates were analyzed with either anti-phospho-tyrosine or anti-Flt3 antibodies.

in the lysates of HEK-293 cells by Western blotting. According to previously published studies (described in detail in 1.3.2), the high molecular weight form represents mature fully glycosylated Flt3 receptor, which is present on the cell surface, while a lower molecular weight form corresponds to the immature receptor that contains high mannose glycosaccharides, which is inside the cell. Figure 3.2 also shows that the mature form of Flt3 wt receptor is predominantly detected in HEK-293 cell lysates, whereas equal amounts of mature and immature forms are present in the cells expressing Flt3 ITD. This finding suggests that the presence of an ITD mutation interferes with maturation of Flt3.

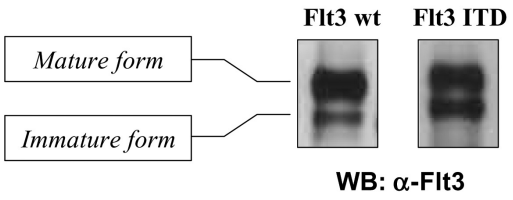


Figure 3.2: Detection of mature and immature forms of Flt3 in HEK-293 cell lysates by Western blot. Flt3 wt and Flt3 ITD were over-expressed in HEK-293. Flt3 was detected in cellular lysates by immuno-blotting with Flt3 specific antibodies.

Co-expression of kinase active Hck interferes with maturation of Flt3

To analyze interaction of Flt3 with Src kinases, Src kinase Hck was chosen. Expression of Hck is restricted to the cells of myeloid origin and its expression and activation was found in patients with AML. Therefore, Hck is likely to be present in the same cell type as Flt3 in normal and malignant hematopoiesis.

HEK-293 cells were used as an over-expression system to study the interaction of Flt3 and Hck. Figure 3.3 (a) shows that the mature form of wild type Flt3 receptor is expressed at a higher level than the immature form. However, upon co-expression of Flt3 with Hck, the amount of mature form of the receptor decreases and more of the immature form of receptor can be detected. Figure 3.3 (b) demonstrates that constitutively active Flt3 containing an ITD mutation is also sensitive to Hck-mediated interference with maturation. Similar to Flt3, co-expression of Flt3 ITD with Hck leads to a decrease in the amount of the mature form and to an increase in the amount of the immature form of the receptor, as compared to Flt3 ITD expression alone. This finding suggests that co-expression of Hck interferes with maturation of wild type and the ITD mutant Flt3 receptors resulting in accumulation of immature receptor inside the cell.

To investigate whether kinase activity of Hck is required for Hck-mediated interference with maturation of Flt3 receptor, a kinase inactive mutant of Hck was employed. In the Hck K269R mutant (Hck KR), lysine 269, which is essential for the positioning of α- and β- phosphate groups of ATP for catalysis [99], is substituted with Arginine. As a result, Hck K269R is not capable of binding ATP, while substrate binding is not affected. Figure 3.3 shows that in contrast to Hck wt, co-expression of Hck KR with Flt3 does not interfere with maturation of the receptor: the relative amounts of mature and immature forms of Flt3 are unchanged in case of Flt3 co-expression with Hck KR. Similar to Flt3 wt, kinase activity of Hck is required for interference with maturation of Flt3 containing an ITD mutation (Figure 3.3 (b)). Thus, co-expression of kinase active, but not kinase-inactive, Hck interferes with maturation of Flt3 wt and Flt3 ITD.

Over-activation and over-expression of Hck promote Hck-mediated Flt3 maturation block

Both over-activation and over-expression of Src kinases can contribute to cellular transformation. Hyperactivation of Hck could be achieved by substitution of the negative-regulatory tyrosine in the C-terminus of the kinase with phenylalanine. The resulting Hck Y501F mutant (Hck YF) is unable to form an intramolecular interaction between the C-terminal phosphorylated Tyr 501 and the SH2 domain of Hck and is therefore constitutively active. To analyze the effects of the activity and expression levels of Hck on the maturation of Flt3, different amounts of wild type, kinase inactive and constitutively active Hck were co-expressed with Flt3 in HEK-293 cells.

Figure 3.4 (left panel) shows that at high concentrations both wild type and hyperactive Hck interfere with maturation of Flt3. When high amounts

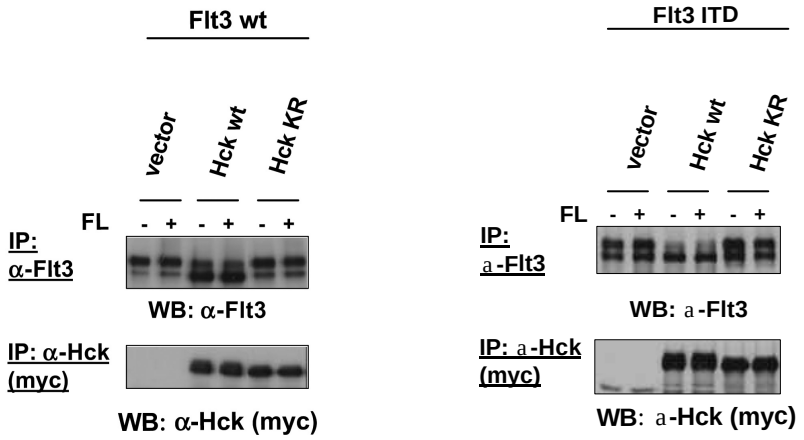


Figure 3.3: Co-expression of Hck interferes with maturation of Flt3 wt (a) and Flt3 ITD (b). Flt3 and Hck constructs were expressed in HEK-293 cells as indicated. Where indicated, cells were stimulated with FL for 10 minutes prior to lysis. Flt3 and Hck were immuno-precipitated from lysates and analyzed by immuno-blotting.

of hyperactive Hck were co-expressed with Flt3, almost no mature form of Flt3 was detected. Co-expression of similar amounts of wild type Hck resulted in a decrease, but not disappearance of the mature form of Flt3. Figure 3.4 (right panel) shows that much lower amounts of hyperactive Hck were required to interfere with Flt3 maturation, as compared to Hck wt: while no effect of Hck wt was observed when 0.25 μg of Hck encoding plasmids were transfected, Hck YF still strongly inhibited maturation of Flt3. All together, Figure 3.4 demonstrates that Hck-mediated effect on maturation of Flt3 correlate with the dose of Hck activity.

Association of Hck with its SH2 or SH3 domain binding partners is not required for Hck-mediated Flt3 maturation block

Many of the biological activities of Src kinases depend on their ability to physically interact with binding partners via their SH2 and SH3 domains (described in detail in 1.4.4). To test whether association of Hck with its binding partners is required for Hck-mediated interference with Flt3 maturation, Hck R150L (Hck RL) and Hck W93A (Hck WA) mutants were used. The strictly conserved arginine in the SH2 domain of SFKs (Arg 150 in Hck) is required for the association of the SH2 domains of SFKs with the phosphate groups of the phosphorylated tyrosine residues in the SH2 domain binding partner. Its substitution with leucine leads to the disruption of the SH2 domain-mediated protein-protein interactions of SFKs [215], [26]. The conserved tryptophane in the SH3 domains of SFKs (Trp 93 in Hck) forms part of the ligand binding surface in the fold of the SH3 domain. Its substi-

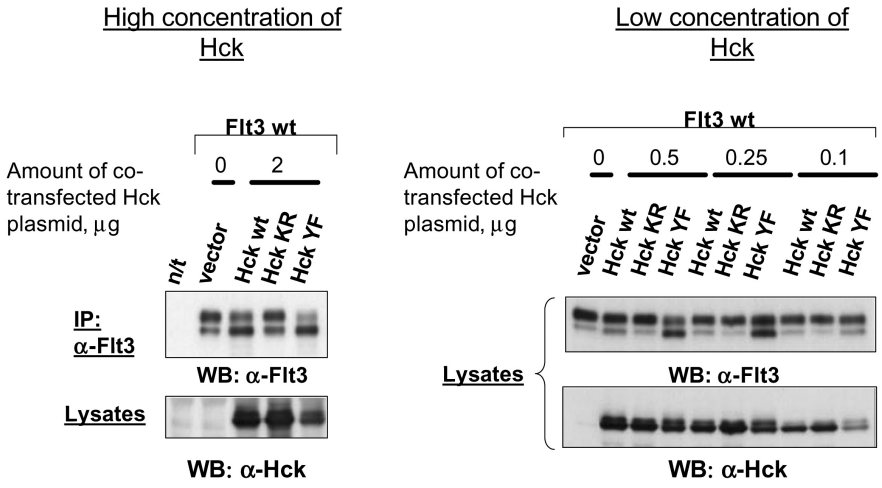


Figure 3.4: Interference with maturation of Flt3 by Hck depends on the kinase activity and concentration of Hck. Flt3 and Hck constructs were co-expressed in HEK-293 cells as indicated. Expression of Flt3 and Hck was analyzed by immunoblotting of cellular lysates or immuno-precipitated proteins.

tution with alanine results in the disruption of SH3 directed protein-protein interactions of SFKs [60].

As shown in Figure 3.5, similar to Hck wt, co-expression of Hck R150L and Hck W93A with Flt3 interferes with maturation of Flt3 receptor. These results suggest that the binding of SH2 or SH3 domain interacting proteins is not required for Hck-mediated interference with Flt3 maturation.

Due to the disruption of negative regulatory intramolecular interactions, kinase activity of Hck RL and Hck WA is higher than that of the wild type Hck (data not shown). Considering the essential role of Hck kinase activity for interference with Flt3 maturation, amounts of wild type Hck and Hck R150L and Hck W93A mutants corresponding to equal kinase activities should be tested to completely exclude the involvement of SH2 and SH3 domains in Hck-mediated maturation block of Flt3.

Binding of interacting proteins to SH2 or SH3 domain leads to the activation of Src kinase activity. Therefore, it can not be excluded that under certain circumstances binding of SH2 or SH3 domain binding partners via activation of the kinase activity of Hck contributes to Hck-mediated block in maturation of Flt3.

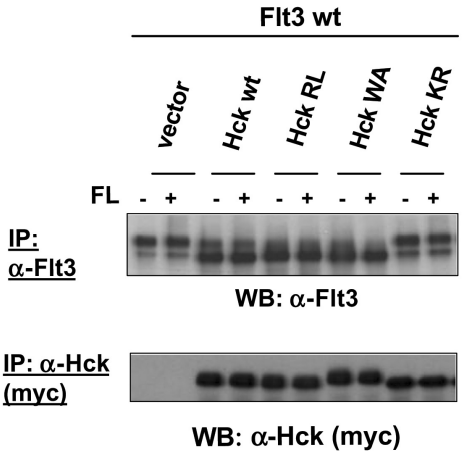


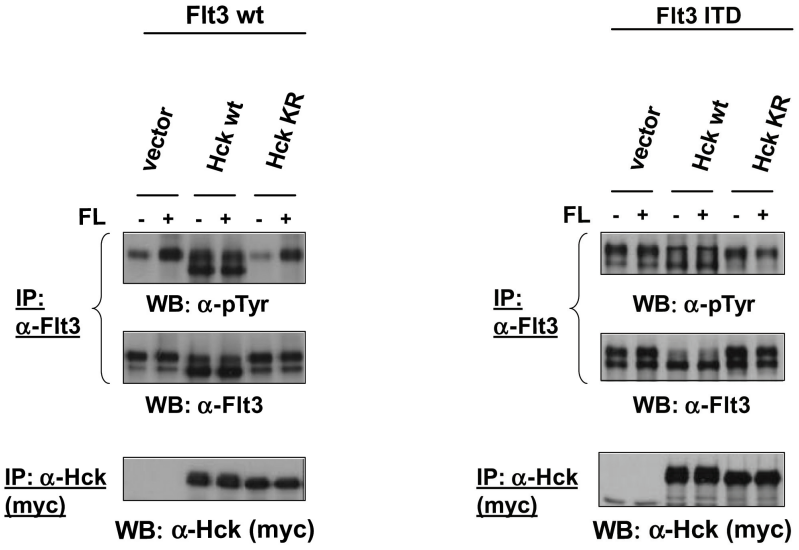
Figure 3.5: Association of Hck with its SH2 or SH3 domain binding partners is not required for Hck-induced Flt3 maturation block. Flt3 and Hck constructs were co-expressed in HEK-293 cells as indicated. Where indicated, cells were stimulated with FL for 10 minutes prior to lysis. Flt3 and Hck were immuno-precipitated from lysates and analyzed by immuno-blotting.

3.2.2 Hck phosphorylates Flt3

Co-expression of Hck increases phosphorylation level of Flt3

In accordance with previous publications [111], Figure 3.6 demonstrates that stimulation of wild type Flt3 receptor with Flt3 ligand (FL) results in the increase of Flt3 phosphorylation (left panel), while Flt3 containing an ITD mutation is constitutively phosphorylated in a ligand-independent manner (right panel). Stimulation of the wild type Flt3 receptor with FL leads to the phosphorylation of predominantly mature form of Flt3, while in case of Flt3 ITD both mature and immature forms of Flt3 are phosphorylated.

Figure 3.6 shows that co-expression of wild type Hck with Flt3 wt or Flt3 ITD leads to the increase in phosphorylation of Flt3 regardless of FL stimulation. In case of the Hck co-expression, both the mature and immature forms of Flt3 are phosphorylated. In contrast to cells expressing Flt3 wt only, stimulation of cells co-expressing Flt3 wt and Hck wt with FL did not induce a further significant increase in phosphorylation of the receptor. In contrast to Hck wt, co-expression of kinase inactive Hck mutant with Flt3 wt or Flt3 ITD did not result in the elevated phosphorylation of the receptor and did not inhibit FL stimulated Flt3 wt phosphorylation. Moreover, the basal level of tyrosine phosphorylation of Flt3 was decreased when Hck KR was co-expressed, as compared with Flt3 in the absence of Hck co-expression. This finding suggests that Hck KR acts as a dominant negative mutant preventing endogenously expressed SFKs from phosphorylating the Flt3 receptor.



(a) (b)

Figure 3.6: Co-expression of Hck increases phosphorylation of Flt3. Wild type Flt3 (a) or Flt3 ITD (b) and Hck were co-expressed in HEK-293 cells as indicated. Where indicated, cells were stimulated with FL for 10 minutes prior to lysis. Flt3 and Hck were immuno-precipitated from lysates and analyzed by immuno-blotting.

Hck induces phosphorylation of kinase inactive Flt3

The Hck-mediated increase in Flt3 phosphorylation described above could be explained either by enhancement of autophosphorylation of Flt3 or by direct phosphorylation of Flt3 by Hck or by another kinase activated by Hck. To discriminate between these possibilities, a kinase inactive mutant of Flt3 was employed. Similarly to Hck K269R, Lys644 in the N-lobe of Flt3 kinase domain was substituted by arginine. As shown in Figure 3.7 (a), in contrast to Flt3 wt, Flt3 K644R was not phosphorylated on tyrosine residues even upon stimulation with FL. Similarly, Flt3 ITD containing the K644R mutation (Flt3 ITD KR) was not constitutively phosphorylated compared to Flt3 ITD (figure 3.7 (b)).

Figure 3.7 demonstrates that co-expression of Flt3 K644R or Flt3 ITD K644R with kinase active Hck results in a significant increase in phosphorylation of the receptor. This finding suggests that Hck phosphorylates Flt3 directly without prior stimulation of the Flt3 kinase activity.

Tyrosine residues in the JM region of Flt3 are the sites for phosphorylation by Hck

It was previously demonstrated that SFKs associate with phosphorylated tyrosine residues in the JM region of class III RTKs [154], [137], [6], [119].

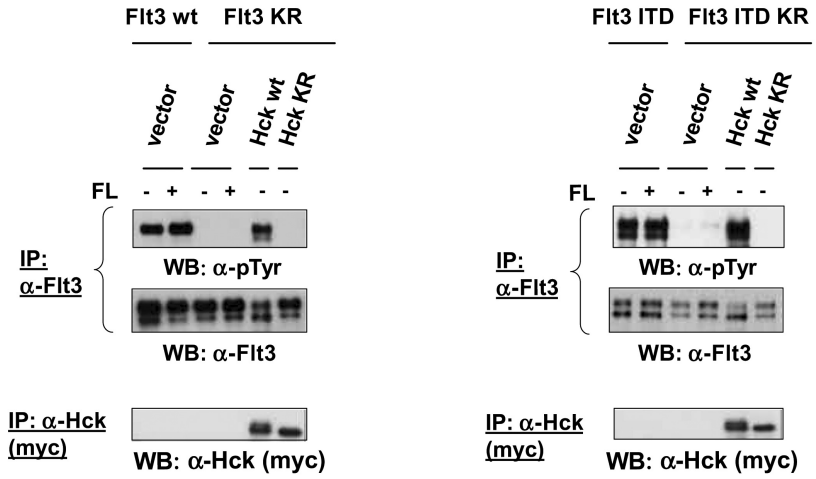


Figure 3.7: Hck phosphorylates Flt3 wt (a) and Flt3 ITD (b). Flt3 and Hck were co-expressed in HEK-293 cells as indicated. Where indicated, cells were stimulated with FL for 10 minutes prior to lysis. Flt3 and Hck were immuno-precipitated from lysates and analyzed by immuno-blotting.

A similar binding mode is described here for Flt3 (see 3.3). As Src kinase phosphorylation and binding sites often overlap, the possibility that tyrosines in the JM region of Flt3 could serve as phosphorylation sites for Hck was investigated.

To analyze whether Hck phosphorylates tyrosine residues in the JM region of Flt3, all four tyrosines present in this region were substituted with alanines in the kinase inactive Flt3 receptor. The resulting Flt3 K644R, Y589F, Y591F, Y597F, Y599F (Flt3 KR 4F) mutant was co-expressed with different amounts of wild type Hck in HEK-293 cells and the receptor phosphorylation level was compared with that of Flt3 KR with all tyrosine residues present in the JM region. Figure 3.8 demonstrates that compared to Flt3 KR, phosphorylation of Flt3 KR 4F by Hck was significantly reduced albeit not completely abolished. Further analysis demonstrates that Hck-mediated phosphorylation of Flt3 containing mutation of only two tyrosines in the JM region, Tyr 589 and Tyr 591, is also significantly reduced (data not shown). This suggests that tyrosine residues in the JM region of Flt3 serve as the the major phosphorylation sites for Hck.

3.2.3 Several SFKs phosphorylate Flt3 and interfere with its maturation

The Src family of tyrosine kinases comprises nine members with different patterns of tissue expression in humans. However, expression profiles of Src family kinases often overlap and several Src kinases with overlapping

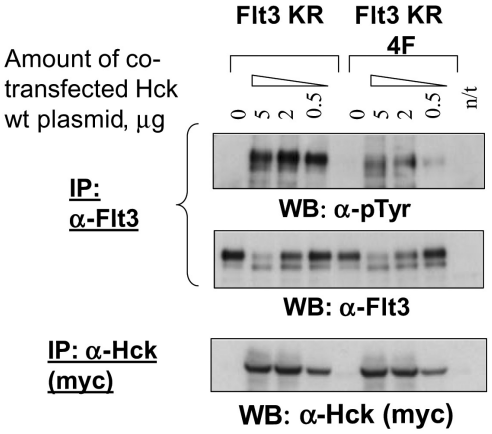


Figure 3.8: Tyrosine residues in the JM region of Flt3 are the major sites for phosphorylation by Hck. Kinase inactive Flt3 with intact JM region (Flt3 KR) and containing substitutions of four tyrosines in the JM region with Phenylalanines (Flt3 KR 4F) were co-expressed with different amounts of wild type Hck in HEK-293 cells as indicated. Flt3 and Hck were analyzed by immuno-blotting of immuno-precipitated proteins or of cellular lysates.

functions are usually expressed in the same cell type (described in detail in 1.4.2). To test whether interference with maturation and phosphorylation of Flt3 is a unique function of Hck or a more general feature of SFKs, two other Src family members, Lyn and Fyn, were tested. Lyn and Fyn are expressed in hematopoietic cells and, similar to Hck, their transcripts were found in bone marrow of healthy donors and AML patients, as well as in all tested myeloid cell lines (data not shown).

To test whether Lyn and Fyn interfere with Flt3 maturation and phosphorylate Flt3, these SFKs were co-expressed with wild type and kinase inactive Flt3 in HEK-293 cells. Ratios of immature and mature forms and phoshphorylation of the receptor were analyzed by immuno-blotting of immuno-precipitated Flt3. Figure 3.9 shows that Lyn and, to a lesser extent, also Fyn co-expression with Flt3 interferes with Flt3 maturation (Figure 3.9 (a)). Similar to Hck, Lyn and Fyn also induce phosphorylation of kinase inactive Flt3 receptor (Figure 3.9 (b)). These results suggest that other SFKs members besides Hck do also interfere with Flt3 maturation and induce Flt3 phosphorylation on tyrosine residues.

3.2.4 SFKs phosphorylate the Kit receptor and interfere with its maturation

RTKs of class III share structural homology and all undergo a maturation process, with glycosylation being one of the major accompanying chemical

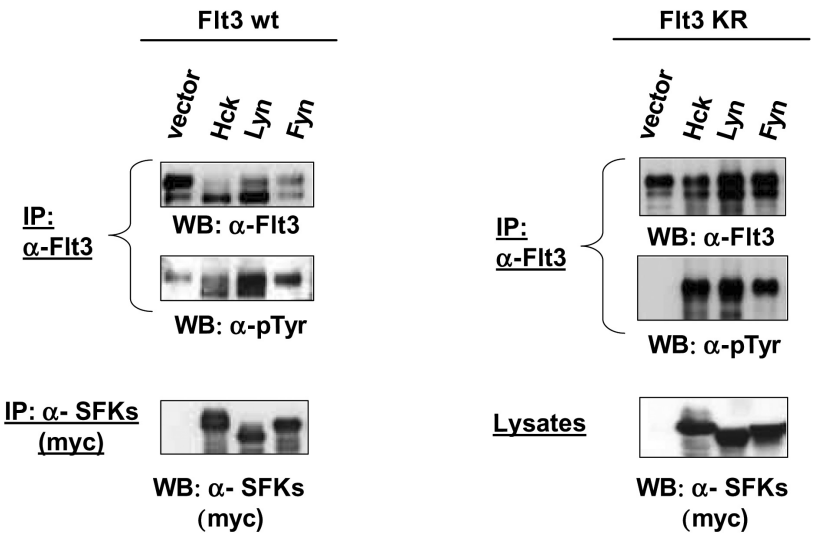


Figure 3.9: Several SFKs phosphorylate Flt3 and interfere with its maturation. SFKs were co-expressed with wild-type Flt3 (a) or kinase inactive Flt3 mutant (b) in HEK-293 cells. Expression and phosphorylation of the proteins were analyzed by immuno-blotting.

modification. It is therefore likely that cellular mechanisms regulating and affecting maturation of this class of receptors would be conserved among different family members. To analyze whether SFK-mediated interference with maturation and phosphorylation of Flt3 could represent a general phenomenon, effects of Hck, Lyn and Fyn on maturation and phosphorylation of the class III RTK Kit were investigated.

As shown on Figure 3.10 (a), similar to Flt3, two species of the Kit receptor with lower and higher molecular weights could be detected in HEK-293 cells, representing the immature intracellular and mature cell surface forms of the receptor, respectively. Similarly to Flt3, the higher molecular weight form of Kit was expressed to a larger extent than the lower molecular weight form. Figure 3.10 shows that, by analogy with Flt3, co-expression of Hck with Kit leads to an increase in the amount of lower molecular weight species of Kit and a decrease in the amount of the higher molecular weight species. Figure 3.10 (a) also demonstrates that Hck-mediated interference with c-kit maturation is dependent on kinase activity of Hck, as co-expression of the kinase inactive Hck mutant did not induce any changes in the amounts of immature and mature forms of Kit.

Figure 3.10 (b) shows that similarly to Flt3, co-expression of Lyn and to a lesser extent Fyn also interfere with maturation and phosphorylate Kit. However, to discriminate between direct Hck phosphorylation and stimula-

tion of Kit autophosphorylation activity, phosphorylation of kinase inactive mutant of Kit by SFKs would have to be analyzed.

These findings suggest that co-expression of SFKs interferes with maturation and can also induce phosphorylation of another member of RTKs of class III, Kit. Thus, these findings above functional interaction between Flt3 and Hck appear to be more general and do at least apply to other SFKs on the one hand and other class III RTKs on the other hand.

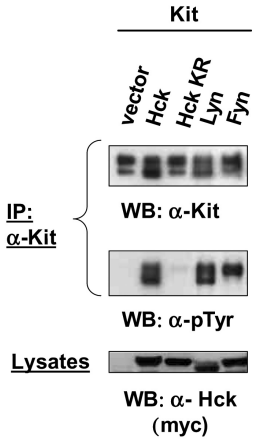


Figure 3.10: SFKs phosphorylate the Kit receptor and interfere with its maturation. Hck interferes with maturation and induces phosphorylation of Kit in a kinase-dependent manner. Lyn and Fyn also interfere with maturation and phosphorylate Kit. SFKs and Kit were co-expressed in HEK-293 cells as indicated. Expression and phosphorylation of proteins were analyzed by immuno-blotting.

3.3 Physical interaction of Hck and Flt3 in HEK-293 cells

Previous publications have demonstrated that SFKs associate with different members of class III RTKs. A chimeric Flt3 receptor, consisting of an extracellular domain of CSF-1R and transmembrane and cytoplasmic domains of murine Flt3 receptor, was also shown to co-immunoprecipitate with Src and Fyn upon CSF-1 stimulation [53]. Results of detailed analysis of physical association of human Flt3, as well Flt3 ITD with Hck are presented in this section.

3.3.1 Flt3 receptor activated by Flt3 ligand or by an ITD-type mutation recruits Hck via its SH2 domain

Flt3 activated by FL or by an ITD-type mutation associates with kinase inactive Hck

Firstly, the association of wild type Hck with Flt3 was investigated. Figure 3.11 (a) shows that, regardless of FL stimulation, almost no Flt3 was detected in immuno-precipitates of wild type Hck. The following possibilities or their combination could explain the lack of detectable association between Flt3 and wild type Hck: co-expression of Hck prevents Flt3 maturation and therefore its exposure to the ligand, phosphorylation of Flt3 by Hck does not create high affinity binding sites for Hck and/or creates high affinity binding sites for other proteins whose binding to Flt3 is competitive with Hck. Following these assumptions, a kinase-inactive Hck mutant, Hck KR, which neither interferes with maturation nor phosphorylates Flt3 (described in 3.2) should associate with Flt3. Indeed, Figure 3.11 (a) demonstrates that in contrast to Hck wt, kinase inactive Hck co-immunoprecipitated with Flt3 in the FL-dependent manner. Similar observations were made for Flt3 containing an ITD mutation. Figure 3.11 (b) shows that Flt3 ITD associates with kinase inactive and not with wild type Hck. However, association of Flt3 ITD with Hck KR did not depend on FL stimulation, correlating with the constitutive activation of Flt3 ITD independently of the ligand. These results suggest that Hck is inducibly recruited to wild type Flt3 stimulated with FL, as well as to Flt3 containing an ITD mutation.

Association of Flt3 with Hck is mediated by SH2 domain of Hck

Activation of RTKs results in their dimerization and trans-phosphorylation of receptor monomers. This phosphorylation creates binding sites for SH2 domain-containing proteins. To analyze whether the described association of Hck and Flt3 is mediated by the SH2 domain of Hck, a R150L mutation which results in the disruption of SH2 domain directed protein-protein interactions of Hck (described in 3.2.1) was introduced into the kinase inactive Hck. Association of the resulting double mutant (Hck KR RL) was analyzed by co-immunoprecipitation. Figure 3.11 (a) demonstrates that in contrast to Hck KR, almost no Flt3 was detected in immuno-precipitates of Hck KR RL even after FL stimulation. This result suggests that Hck is recruited to wild type Flt3 activated by FL via its SH2 domain. Similarly to wild type Flt3 receptor, Flt3 ITD did not associate with Hck KR RL (Figure 3.11 (b). These findings suggest that activation of Flt3 by FL stimulation or an ITD mutation results in the generation of binding sites for the SH2 domain of Hck.

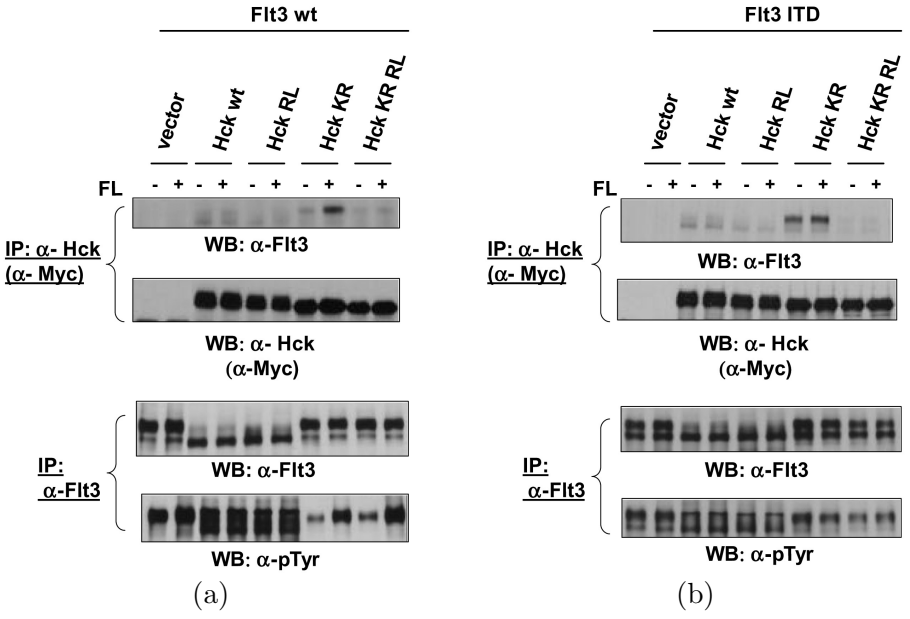


Figure 3.11: Flt3 activated either by Flt3 ligand (a) or by an ITD-type mutation (b) recruits Hck via its SH2 domain. Flt3 and Hck constructs were co-expressed in HEK-293 cells as indicated. Hck and Flt3 were immuno-precipitated using myc or Flt3 specific antibodies, respectively. Expression and phosphorylation of proteins were analyzed by immuno-blotting.

3.3.2 Tyrosines 589 and 591 in the JM region of Flt3 serve as Hck binding sites.

Studies of class III RTKs have demonstrated that tyrosines in the JM region of these receptors serve as binding sites for SFKs [154], [137], [6], [119]. Considering the high homology between class III RTKs in the JM region, similar binding mode of SFKs to Flt3 could be expected.

To test whether tyrosines in the JM domain of Flt3 serve as binding sites for Hck, Tyr 589 and Tyr 591 were replaced by phenylalanines. In Flt3 ITD, Tyr 589 and Tyr 591 in the wild type, but not in the duplicated sequence were substituted with Phenylalanines. Figure 3.12 (a) shows that FL inducible association of Hck KR with Flt3 Y589F, Y591F was decreased, as compared with Flt3 wt, but not completely abrogated. Figure 3.12 (b) demonstrates that similarly to Flt3 wt, less of Flt3 ITD Y589F, Y591F was detected in Hck KR immuno-precipitates, as compared with Flt3 ITD. These data suggest that Tyr 589 and Tyr 591 in the JM region of Flt3 serve as binding sites for Hck, although other binding sites for Hck exist on Flt3.

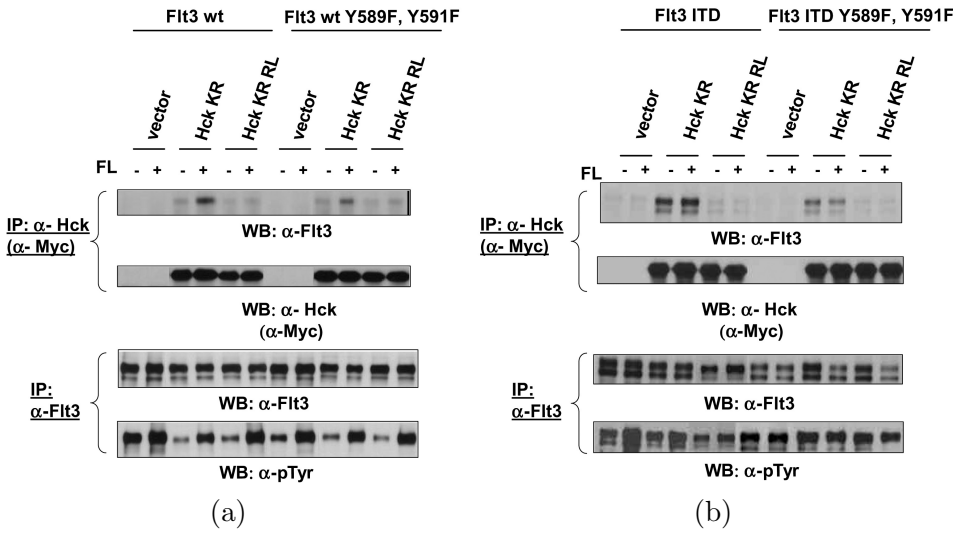


Figure 3.12: Tyr 589 and Tyr 591 in the JM region of Flt3 serve as Hck binding sites. Flt3 and Hck constructs were co-expressed in HEK-293 cells as indicated. Hck and Flt3 were immuno-precipitated using anti-myc or anti-Flt3 antibodies, respectively. Proteins were analyzed by immuno-blotting.

Hck associates with Flt3 in intact cells

To test whether association between Hck and Flt3 takes place inside viable cells or after cellular lysis during immuno-precipitation (*in vitro*), different co-immunoprecipitation conditions were used. Had the association been taking place *in vitro*, high concentration of proteins in the immuno-precipitation reaction (small volume of immuno-precipitation reaction), as well as longer times of immuno-precipitations would shift the equilibrium towards the associated couples of proteins. Figure 3.13 shows that the amount of co-immunoprecipitated Flt3 only slightly increases with the increase in the time of immuno-precipitation. This slight increase most likely reflects the increase in the amount of the directly immuno-precipitated Hck. In addition, the ratios of the amounts of co-immunoprecipitated Flt3 to immuno-precipitated Hck stayed roughly the same when low and high volumes of reaction were used. These findings suggest that association of Flt3 and Hck takes place in viable cells before cellular lysis. However, formation of complexes containing Flt3 and Hck within a very short period of time after cellular lysis can not be excluded.

3.4 Biological function of Hck in Flt3-mediated cellular responses

To test whether the described functional and physical association of Hck with Flt3 affects biological responses mediated by the Flt3 receptor, 32D cl. 3 cell lines which express Flt3 wt or Flt3 ITD and Hck constructs with

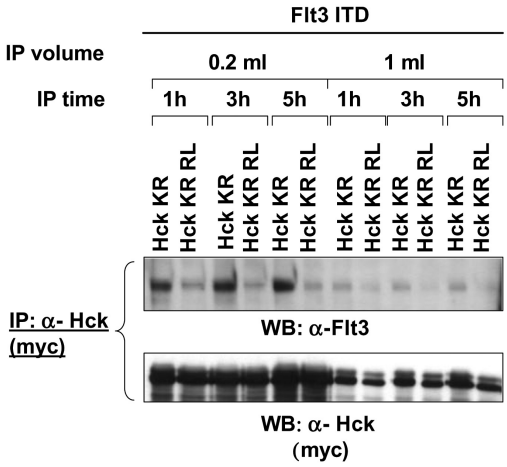


Figure 3.13: Association of Flt3 and Hck takes place in intact cells. Flt3 and Hck constructs were co-expressed in HEK-293 cells as indicated. Immuno-precipitations were performed in the indicated volumes for indicated periods of times (additional 45 minutes for incubation with protein A/G agarose are not included). Precipitated proteins were analyzed by immuno-blotting.

different kinase activities were used. 32D cl. 3 cells are strictly dependent on the presence of IL-3 for growth. However, as previously described [146], over-expression of the Flt3 receptor in these cells and its stimulation with FL leads to sustained survival of the cells in the absence of IL-3. Over-expression of Flt3 containing an ITD mutation abrogates the requirement of IL-3 for growth [147] and blocks G-CSF-mediated differentiation of these cells into monocytes [243]. These characteristics make 32D cl. 3 cells a commonly used model for studying Flt3-mediated signaling events leading to resistance to apoptosis and to proliferation.

3.4.1 Expression of Flt3 and Hck in 32D cl.3 cells

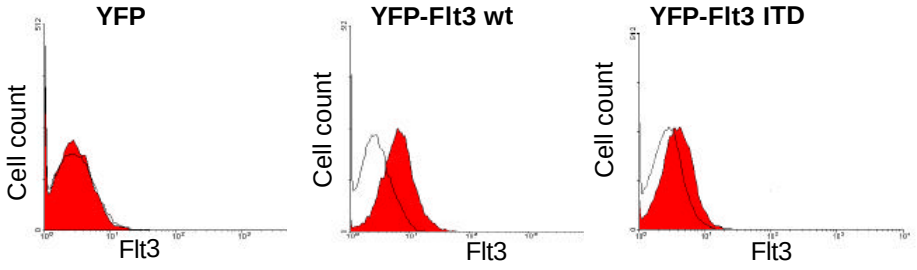
To generate 32D cl. 3 cell lines expressing Flt3 and Hck mutants, cells were infected with recombinant retroviruses containing Flt3 or Hck connected via IRES sequence to EYFP or EGFP respectively. Retroviruses were generated by transfection of the appropriate constructs in HEK-293 cells. Titers of all retroviral stocks were normalized by infecting 32D cl. 3 cells with several dilutions of retroviruses. To generate 32D cl. 3-based cell lines, in which most of the cells contain a single copy of provirus, dilutions for which infection efficiency was 10% or lower were used.

First, cells were infected with EYFP-IRES-Flt3 wt or EYFP-IRES-Flt3 ITD-containing viruses and YFP positive cells were sorted using FACS. YFP positive cells were then infected the second time with EGFP-IRES-Hck-

containing viruses and double positive YFP+/GFP+ cells were sorted by FACS.

Analysis of Flt3 surface expression in 32D cl. 3 cells lines by FACS

To confirm that YFP positive cells indeed express Flt3 on their cell surface, cells were stained with Flt3 specific antibodies conjugated to phycoerythrin and analyzed by flow cytometry. Figure 3.14 shows that cells infected with control EYFP-containing virus do not express Flt3 on their surface (figure 3.14 (a)), while cells infected with retroviruses containing EYFP-IRES-Flt3 wt (Figure 3.14 (b)) and EYFP-IRES-Flt3 ITD (Figure 3.14 (c)) constructs do express Flt3. Level of Flt3 surface expression is higher in the case of Flt3 wt than in the case of Flt3 ITD. Impaired maturation is likely to account for the lower Flt3 ITD surface expression in 32D cl. 3 cells compared with Flt3 wt.



(a) (b) (c)

Figure 3.14: Expression of Flt3 on the surface of 32D cl. 3 cells. Cells infected with EYFP control virus (a), EYFP-IRES-Flt3 wt-containing virus (b) and EYFP-IRES-Flt3 ITD-containing virus (c) were stained with Flt3 specific antibodies conjugated to phycoerythrin and analyzed by flow cytometry.

Analysis of Flt3 and Hck expression in 32D cl. 3 cells by immuno-blotting

Expression of Hck and Flt3 constructs in all generated cell lines was also analyzed by immuno-blotting. Figure 3.15 (a) shows that equal amounts of Flt3 were detected in cells expressing either Flt3 wt only and in cells co-expressing Flt3 wt and EGFP or Hck constructs. Similar results were obtained for cell lines expressing Flt3 ITD (Figure 3.15 (b). These results suggest that no loss of Flt3 expression took place after second infection and second sorting steps.

To confirm that all cells express Flt3 ITD after sorting, 32D cl. 3 cell lines expressing Flt3 ITD were also analyzed after withdrawal of IL-3 for 3 days. Only cells expressing Flt3 ITD are able to grow with IL-3. Therefore,

presence of Flt3 ITD negative cells in the cell lines before IL-3 withdrawal would result in the increase in the amount of Flt3 detected by immunoblotting from the lysates containing equal amounts of total protein after cells have been deprived of IL-3 for three days. Figure 3.15 (b) shows that expression level of Flt3 ITD did not change after IL-3 has been withdrawn from the culture medium for three days. This result suggests that all cells in Flt3 ITD expressing cell lines are Flt3 ITD positive.

Figure 3.15 also shows expression levels of Hck constructs in 32D cl. 3 cells. Kinase inactive Hck mutant was expressed at the higher level than wild type or constitutively active Hck. This is in agreement with previous publications showing that activated forms of Src are less stable due to their degradation via the proteosomal pathway [85].

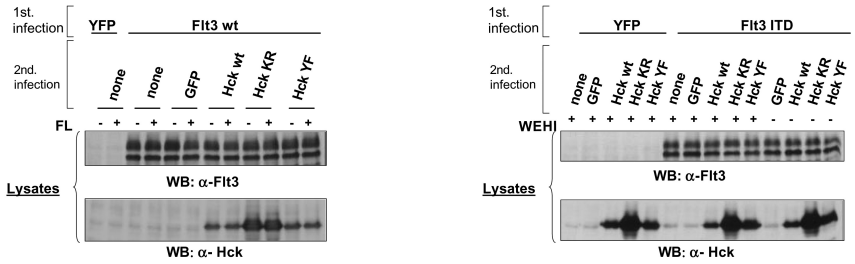


Figure 3.15: Analysis of Flt3 and Hck expression in 32D cl. 3 cells by immunoblotting. 32D cl. 3 cells, expressing indicated proteins were lysed and lysates containing equal amounts of total protein were analyzed by immuno-blotting. Cells expressing Flt3 wt were stimulated with FL for 10 minutes where indicated (a). Cells expressing Flt3 ITD were either sub-cultured in the presence of 10% WEHI-3B supernatant as source of IL-3 or deprived of IL-3 for 3 days prior to lysis (b).

3.4.2 Hck is not involved in Flt3 signal transduction leading to cell survival

To investigate whether Hck interferes with anti-apoptotic signalling mediated by Flt3 wt, apoptosis was compared in 32D cl. 3 cells co-expressing Flt3 wt and EGFP and cells co-expressing Flt3 and wild type, kinase inactive or constitutively active Hck after stimulation with Flt3 ligand.

Influence of retroviral infection on apoptosis in 32D cl. 3 cells

Figure 3.16 shows that in the absence of cytokine stimulation, the percentage of apoptotic cells infected with retrovirus twice and therefore over-expressing two proteins (EYFP and EGFP or Flt3 wt and EGFP) was lower than the percentage of apoptotic cells infected with retrovirus once and therefore over-expressing only one protein (EYFP or Flt3). This finding suggests that, as

compared single retrovial infection, double infection of 32D cl. 3 cells with retrovirus leads to the activation of anti-apoptotic signalling in these cells. Single infection of 32D cl. 3 cells with higher titers of retrovirus (more than 10% infection efficiency) also resulted in increased cell growth in the absence of cytokines (data not shown). This effect was especially pronounced when cells were sub-cultured for more than 2 weeks after sorting or were incubated in the absence of cytokine for more than 3 days (data not shown). Together these results suggest that the presence of more than one proviral copy per cell leads to activation of anti-apoptotic pathways.

Sustained survival of 32D cl. 3 cells expressing Flt3 wt in IL-3 free medium upon FL stimulation

Figure 3.16 shows that stimulation of Flt3 wt expressing cells with FL reduced the proportion of apoptotic cells: about 20% of the cells underwent apoptosis in the presence of FL, while about 70% of apoptotic cells were detected when no cytokine was added to the medium. In contrast, when cells expressing EYFP only were analyzed, only a small reduction in the number of apoptotic cells was detected (about 70% of apoptotic cells in the presence of FL versus about 80% of apoptotic cells in the absence of cytokines). This slight effect of FL probably reflects stimulation of endogenously expressed Flt3 in 32D cl. 3 cells. Stimulation of Flt3 wt expressing cells with FL could not, however, fully substitute conditioned medium: about 20% of Flt3 expressing cells underwent apoptosis in the presence of FL, as opposed to less than 2% in the presence of conditioned medium. In accordance with previous publications [146], these data demonstrate that Flt3 wt activates anti-apoptotic signals in 32D cl. 3 cells which leads to their sustained survival.

Prolonged survival of 32D cl. 3 cells in IL-3 free medium upon co-expression of the hyperactive Hck

When cells co-expressing EYFP and EGFP were compared with cells co-expressing EYFP and wild type Hck or kinase inactive Hck, no significant differences in the percentage of apoptotic cells upon cytokine withdrawal were detected. In contrast, apoptosis was decreased in cells co-expressing EYFP and hyperactive Hck mutant after cytokine withdrawal (Figure 3.16). This result is in accordance with a previous publication [59], and shows that over-expression of hyperactive Hck mutant in 32D cl. 3 cells prolongs survival in the absence of IL-3, while over-expression of wild type or inactive Hck has no effect on apoptosis.

Over-expression of Hck does not influence Flt3-mediated signal transduction resulting in resistance to apoptosis

When cells over-expressing Flt3 wt and wild type Hck or Flt3 wt and kinase inactive Hck were compared with cells over-expressing Flt3 wt and EGFP, no differences in the percentage of apoptotic cells were observed when cells were incubated in the presence of FL. In addition, co-expression of wild type Flt3 and hyperactive Hck did not result in either an additive or a synergistic effect with respect to reduction of apoptosis. The percentage of apoptotic cells in case of Flt3 wt and Hck YF co-expression was about the same as in case of Flt3 wt and EGFP co-expression or EYFP and Hck YF co-expression upon stimulation with FL. Combined, these observations suggest that Hck does not influence any signals downstream of Flt3 with respect to apoptosis.

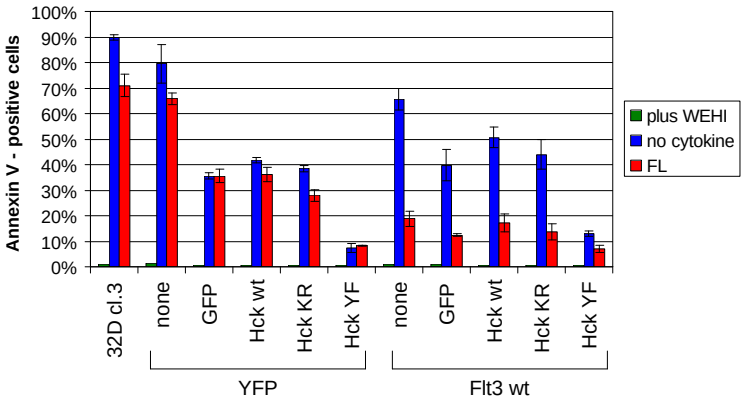


Figure 3.16: Hck is not involved in Flt3 signal transduction leading to cell survival. All cell lines were sub-cultured in the presence of IL-3 prior to the start of the assay. Cells were washed three times with IL-3 free medium and seeded at $1X10^5$ cells/ml in the medium containing either 10% of WEHI-3B supernatant as source of IL-3 (green), no cytokine (blue) or 100 ng/ml of FL (red). Apoptosis was measured 36 hours after plating using Annexin-V apoptosis detection kit. All Annexin-V positive cells were considered apoptotic.

3.4.3 Hck is not involved in Flt3 ITD signal transduction leading to cell growth

To investigate whether Hck influence on Flt3 ITD-mediated cell growth, growth rates of 32D cl. 3 cells co-expressing Flt3 ITD and GFP were compared with growth rates of cells co-expressing Flt3 ITD and wild type, hyperactive or kinase inactive Hck.

Over-expression of Flt3 ITD strongly reduces IL-3 requirement of 32D cl.3 cells for growth

In accordance with the previous publications [147], Figure 3.17 shows that cells over-expressing Flt3 ITD could grow in the absence of IL-3 while cells over-expressing EYFP could not. Growth rate of cells over-expressing Flt3 ITD was higher in the presence of IL-3 than in the absence of the cytokine from the medium. Possible explanations of this difference include: lack of Flt3 ITD expression in some cells, lower expression levels of Flt3 ITD in some cells, which are not sufficient to confer IL-3-independent growth, counteraction of Flt3 ITD signalling by the action of tumor-suppressor genes in some cells and, finally, potentiation of Flt3 ITD signalling by IL-3 stimulation. The first possibility could be excluded on the basis of the previous finding that equal amounts of Flt3 ITD are expressed in cells incubated for three days in the presence and in the absence of IL-3 (3.4.1 and Figure 3.15).

Over-expression of hyperactive Hck increases growth rate of 32D cl. 3 cells in the absence of cytokine

Figure 3.17 demonstrates that in the absence of IL-3, growth of cells expressing hyperactive Hck was increased compared to the growth of cells expressing EGFP, wild type or kinase inactive Hck. The number of cells expressing Hck YF and EYFP did not change three days after the start of the assay, as compared to the number of seeded cells. However, if cells were kept in culture for more than three days, the growth of all double-infected cells, including cells co-expressing Hck YF and EYFP was increased (data not shown). Therefore, it is not clear whether hyperactive Hck contributes to anti-apoptotic signaling only or it also promotes proliferation.

Over-expression of Hck does not affect Flt3 ITD-dependent cell growth

When cells over-expressing Flt3 ITD and wild type, kinase inactive or hyperactive Hck were compared with cells over-expressing Flt3 ITD and EGFP, no significant differences in cell growth were observed after IL-3 withdrawal (Figure 3.17). The lack in the increase in growth rate of cells over-expressing Flt3 ITD and Hck YF is surprising and could be explained by a subtle negative effect of Hck YF on Flt3 ITD-mediated cell growth. Combined, these results suggest that Hck does not play any significant role in Flt3 ITD-mediated cell growth.

3.5 Interaction of Hck and Flt3 in 32D cl. 3 cells

As described in 3.2, Hck co-expression with Flt3 in HEK-293 cells interferes with receptor maturation and induces its phosphorylation. In addition, as

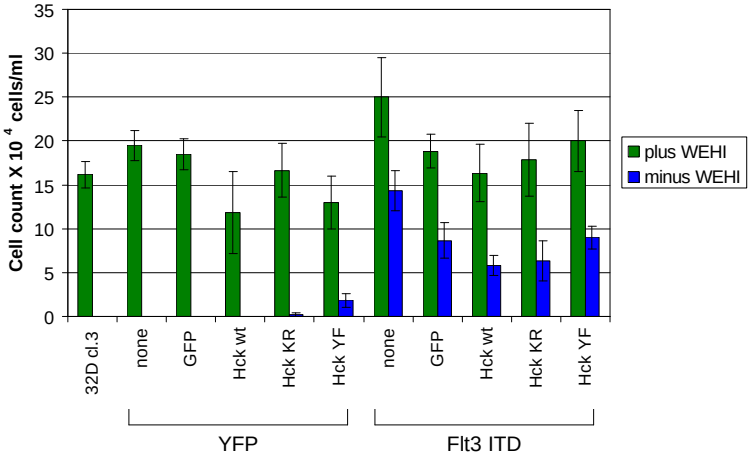


Figure 3.17: Hck is not involved in Flt3 ITD signal transduction leading to cell growth. All cell lines were sub-cultured in the presence of IL-3 prior to the start of the assay. Cells were washed three times with IL-3 free medium and seeded at 1 X 10^4 cells/ml in the medium containing either 10% of WEHI-3B supernatant as source of IL-3 (green) or no cytokine (blue). Viable cells were counted 3 days after seeding using Trypan Blue exclusion assay. All Trypan blue negative cells were considered viable.

described in 3.3, Flt3 physically associates with Hck in the SH2/phosphotyrosine-dependent manner. Results presented below describe the analysis of functional and physical interaction between Hck and Flt3 in 32D cl. 3 cells and provide a potential explanation for the lack of any detectable biological effect of Hck on Flt3-mediated cellular responses in 32D cl.3 cell lines.

3.5.1 Hck does not interfere with maturation and does not phosphorylate Flt3 in 32D cl. 3 cells

Figures 3.15 and 3.20 show that in contrast to the findings made when using HEK-293 cell system, co-expression of neither wild type nor hyperactive Hck mutant with Flt3 in 32D cl. 3 cells interferes with the maturation of Flt3 or leads to its increased phosphorylation. The following possibilities could account for the differences observed between the two cell lines: restriction of functional interaction of human Hck and Flt3 to cell lines of human origin (unlike HEK-293, 32D cl. 3 is a murine cell line), high level of phosphatase activity counteracting the activity of SFKs in 32D cl. 3 cells, as compared with HEK-293 cells, and/or lower expression levels of Hck in 32D cl. 3 cells than in HEK-293 cells. The latter possibility is investigated below.

As described in 3.2.1, Hck-mediated interference with Flt3 maturation depends on kinase activity and expression level of Hck. Therefore, the possibility that lower expression levels of Hck in 32D cl. 3 cells, as compared with HEK-293 cells, account for the lack of Hck-mediated Flt3 maturation

block and phosphorylation was analyzed. Hck expression levels in 32D cl. 3 cells were compared with Hck expression levels in HEK-293 cells in which maturation of Flt3 was affected to various degrees dependent on Hck expression levels. Figure 3.18 shows that expression levels of wild type and hyperactive Hck in 32D cl. 3 cells are lower than those at which maturation block is still observed in HEK-293 cells. In addition, Figure 3.18 shows that expression levels of wild type and hyperactive Hck in 32D cl. 3 cells correspond to those in HEK-293 cells, at which almost no Hck-mediated increase in phosphorylation of wild type Flt3 is observed. These results suggest that lower Hck expression levels in 32D cl. 3 cells, as compared with HEK-293 cells, account for the lack of both Hck-mediated block in maturation and increase in phosphorylation of Flt3 in 32D cl. 3 cells. However, contribution of other factors mentioned above can not be excluded. For example, when hyperactive Hck is expressed in HEK-293 and 32D cl.3 cells at the similar levels, phosphorylation of Hck is higher in HEK-293 cells as compared with 32D cl.3 cells (Figure 3.18, lanes 10 and 14). These data suggest the possibility that in addition to the lower Hck expression levels, activation and subsequent auto-phosphorylation of Hck might be decreased in 32D cl.3 cells as compared with HEK-293 cells.

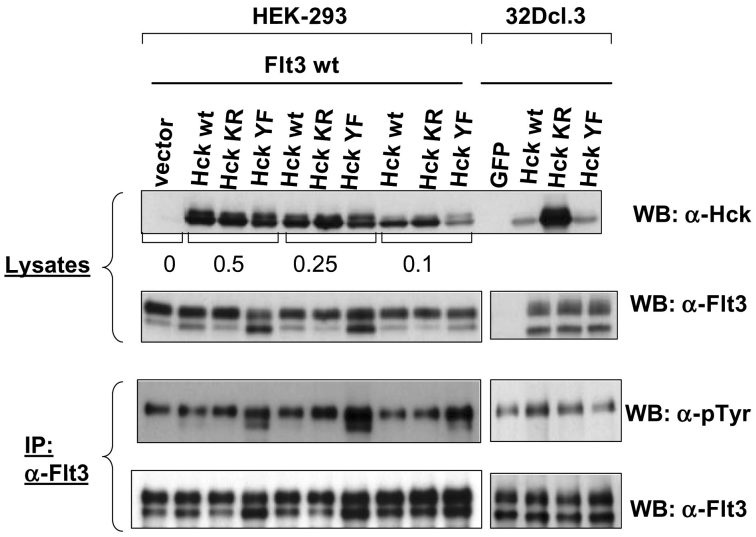


Figure 3.18: Lower Hck expression level could account for the lack of Hck-mediated block in maturation and increase in phosphorylation of Flt3 in 32D cl. 3 cells. HEK-293 cells were transfected with different amounts of wild type, kinase inactive and hyperactive Hck (0.5, 0.25 and 0.5 μg of Hck encoding plasmid per transfection, as indicated). Equal amounts of proteins from cellular lysates of HEK-293 and 32D cl. 3 cells were analyzed by immuno-blotting with Hck and Flt3 specific antibodies. Flt3 was immuno-precipitated from cells using Flt3 specific antibodies and its phosphorylation was analyzed using phospho-tyrosine specific antibodies.

3.5.2 Flt3 does not detectably associate with Hck in 32D cl. 3 cells

As described in 3.3, Flt3 inducibly associates with Hck in HEK-293 cells. To investigate whether Flt3 associates with Hck in 32D cl. 3 cells, cells were starved in the absence of IL-3 for 12 hours and then stimulated with FL for 10 minutes to induce Flt3 autophosphorylation. Hck KR was immunoprecipitated from cellular lysates using Hck specific antibodies. Flt3 was detected in immuno-precipitates by immunobloting with Flt3 specific and phospho-tyrosine specific antibodies. As a control, HEK-293 lysates containing equal amounts of protein were used. Figure 3.19 (a) shows that no Flt3 was detected in Hck immuno-precipitates from 32D cl. 3 cells even when more sensitive phospho-tyrosine specific antibodies were used for detection. In contrast, Flt3 could be easily detected by both Flt3 and phosphotyrosine specific antibodies in Hck KR immuno-precipitates from HEK-293 cell lysates containing equal amount of protein. No Flt3 could be detected in immuno-precipitates of wild type and hyperactive Hck from 32D cl. 3 lysates either (data not shown). The following possibilities could account for the lack of association between Hck and Flt3 in 32D cl. 3 cells: 1) interference of a protein expressed in 32D cl. 3, but not HEK-293 cells, which binds to Hck or Flt3 competitively preventing Hck-Flt3 interaction, 2) restriction of the interaction of human Hck and Flt3 to the cell lines of human origin, 3) high activity of phosphatases de-phosphorylating Hck binding sites on Flt3 in 32D cl. 3 cells as compared with HEK-293 cells, and 4) lower levels of over-expressed Hck in 32D cl. 3 cells than in HEK-293 cells.

To test whether lower levels of Hck KR expression could account for the lack of association between Hck KR and Flt3 in 32D cl. 3 cells, HEK-293 cells were transfected with lower amounts of Hck KR and Hck KR RL as a negative control and association of the two proteins was analyzed by co-immunoprecipitation. Figure 3.19 (b) shows that FL inducible association between Flt3 and Hck KR could be detected in HEK-293 cells, even when significantly lower Hck KR amounts were expressed. Considering only a slight difference in the expression of Hck KR between 32D cl. 3 and HEK-293 cells, these data suggest that lower Hck KR expression levels in 32D cl. 3 cells do not account for the lack of association between Hck and Flt3 in these cells.

3.5.3 Hck does not have any effect on Flt3-mediated phosphorylation of key signal transducing molecules

Cellular response to Flt3 activation mainly relies on the activation of the MAPK, PI3K-Akt and STAT-5 signaling pathways [86], [147], [241]. Full activation of the latter, however, requires aberrant signalling induced by Flt3 ITD while the activation by wild type Flt3 is not sufficient to induce

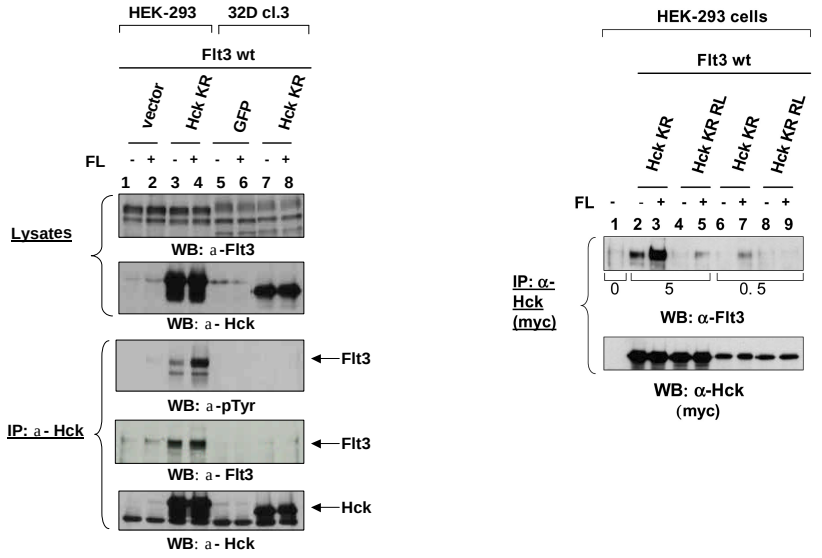


Figure 3.19: Flt3 does not detectably associate with Hck in 32D cl. 3 cells. Lysates from HEK-293, expressing myc-tagged Hck and Flt3 constructs, and 32D cl. 3 cells, expressing non-tagged Hck and Flt3 constructs, which contain equal amounts of proteins were used for immuno-blotting and for co-immunoprecipitation with anti-Hck antibodies. The lowest band on the bottom panel reflect non-specific reaction of antibodies (a). Different amounts of Hck (0, 5 and 0.5 μg of Hck encoding plasmid per transfection, as indicated) were transfected into HEK-293 cells and co-immunoprecipitations were performed using anti-Hck (myc) antibodies (b).

transcription of STAT-5 target genes [148].

To analyze whether Hck interferes with Flt3-mediated signaling events, activation of signal transduction molecules was compared in cells co-expressing Flt3 wt and EGFP and Flt3 wt and wild type, kinase inactive and hyperactive Hck. Cells were starved in the absence of IL-3 for 12 hours and then stimulated with Flt3 ligand for 10 minutes. Cellular lysates containing equal amounts of total protein were analyzed by immuno-blotting with antibodies specific for activation of key signal transduction molecules. To analyze activation of MAPK-dependent pathway, anti-pErk antibodies, which are specific for Erk 1/2 phosphorylation on Thr 202 and Tyr 204, were used. To detect activation of PI3K-dependent pathways, antibodies specific for the downstream Serine/Threonine kinase Akt, phosphorylated on Ser 473, were used. STAT-5 activation was analyzed by immuno-blotting of lysates with antibodies specific for an active form of STAT-5 which is phosphorylated on Tyr 694.

As shown on Figure 3.20, activation of Erk proteins and Akt was induced by stimulation of Flt3 wt expressing cells with FL. Activation of STAT-5 was detected in Flt3 wt expressing, but not in control EYFP expressing

cells. However, in contrast to Erk and Akt, STAT-5 activation was not dependent on the ligand stimulation. No changes in the activation of Erk, Akt or STAT-5 were detected in cells co-expressing Flt3 wt and Hck wt, Hck KR or Hck YF mutants, as compared to cells co-expressing Flt3 wt and EGFP. This finding suggests that co-expression of Hck does not effect Flt3-mediated activation of key signal transduction molecules.

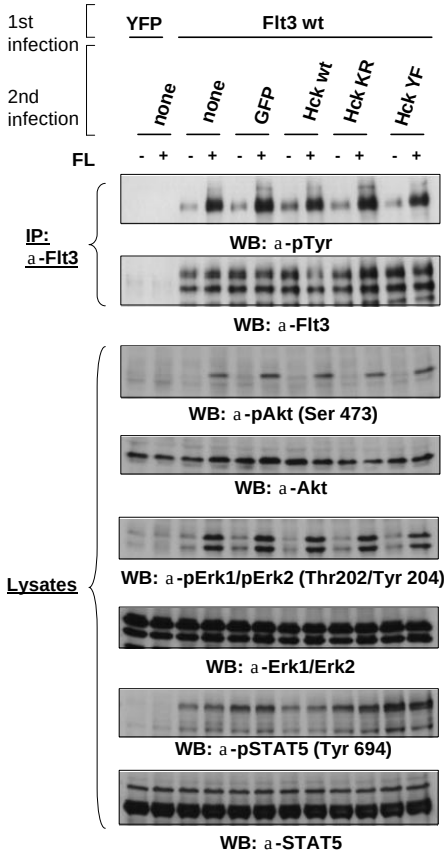


Figure 3.20: Flt3-mediated activation of key signal transducing molecules is not affected by co-expression of Hck in 32D cl. 3 cells. Cells expressing Flt3 wt and indicated Hck constructs were starved for 12 hours and then stimulated with FL for 10 minutes where indicated. Flt3 was immuno-precipitated from lysates and its activation and expression were analyzed by immuno-blotting. Lysates containining equal amounts of total protein were analyzed by immuno-blotting with antibodies specific for activation of signaling molecules and corresponding control antibodies.

Chapter 4

Discussion

4.1 Hck-mediated interference with maturation of Flt3

4.1.1 Possible mechanisms of Hck interference with Flt3 maturation

SFKs modulate signaling mediated by RTKs in various ways. SFK-mediated phosphorylation increases the kinase activity of the receptors and generates binding sites for phospho-tyrosine binding proteins (described in 1.4.8). In addition, SFKs participate in endocytosis and ubiquitination of RTKs. This work shows that besides these activities, SFKs interfere with the maturation of class III RTKs in a kinase-dependent manner. While mostly mature form of the Flt3 RTK is present in HEK-293 cells in the absence of Hck co-expression or when kinase inactive Hck is co-expressed, predominantly the immature form of the receptor is detected in cells which have high activity of Hck due to its over-expression or hyperactivation (Figure 3.3). Other studies have shown that the immature form of the receptor contains high mannose oligosaccharides and is located intracellularly, while the mature form contains complex oligosaccharides and is present on the cell surface [131], [139]. Glycoproteins that carry high mannose glycans have not undergone terminal glycosylation in the Golgi complex and in most cases are present in the ER or ER-Golgi transition [88]. Therefore relative abundance of the immature high mannose oligosaccharide-containing receptor suggests its retention in the ER and/or block of its terminal glycosylation in the Golgi complex. Similar results are also shown here for the Kit receptor, suggesting that SFK-mediated block in maturation is not restricted to Flt3, but rather respresents a general effect of SFK activation on the maturation of class III RTKs.

The mechanism underlying the SFK-mediated block of Flt3 and other RTKs' maturation is not clear. This study shows that in addition to interfering with the maturation of class III RTKs, SFKs also phosphorylate these receptors. It is possible that SFK-mediated phosphorylation of RTKs is responsible for the maturation block. In support of this hypothesis, this work shows that the dose of Hck activity (expression and activation level) correlates with the strength of interference with maturation and the extent of Flt3 phosphorylation (Figure 3.18). Moreover, results presented in this thesis and in other studies show that independently of SFK-mediated effects, the maturation of Flt3 ITD is impaired as compared with Flt3 wt (described in 3.2.1, and shown in figures 3.3, 3.6, 3.11, 3.15, [147], [198]). This difference may also arise from the relatively high basal (ligand-independent) phosphorylation level of Flt3 ITD as compared with Flt3 wt (Figure 3.6, [147]). Another indirect evidence that phosphorylation of RTKs in the secretory pathway might interfere with their maturation is the impaired maturation of the constitutively active murine Kit receptor [68]. The latter study shows that the immature form of the Kit receptor is predominantly observed in

cells expressing activated Kit containing a substitution of aspartic acid 814 to valine, while a mature form of Kit is predominant in cells expressing wild type Kit. The work presented in this thesis also shows that SFKs interfere with the maturation of Kit in a SFK-kinase-dependent manner.

Assuming that SFK-mediated phosphorylation of RTKs interferes with their maturation, what could be a mechanism that inhibits proper maturation of phosphorylated RTKs? Maturation of N-glycosylated proteins and their movement along the secretory pathway is controlled by a number of regulatory mechanisms. First, all proteins in the secretory pathway undergo a so called primary ER quality control (QC) [56]. Proteins that participate in primary QC control include ER resident chaperons (e.g the Calnexin-Calreticulin cycle, BiP) and folding factors (e.g ERp57). These factors assist in protein folding and retain or dispatch misfolded proteins for destruction into the cytosol [56]. Binding of the immature proteins to these factors prevents their forward transport to the Golgi complex [57]. One possibility is that phosphorylation of RTKs at this stage interferes with their proper folding, which leads to an increased binding of the receptors to one of the factors participating in the primary ER QC and therefore to their retention in the ER. It is not clear, however, how phosphorylation which takes place on the cytosolic side of the ER membrane could cause the folding defect that is sensed by the residing in the ER lumen chaperons. Phosphorylation could either result in conformational changes in the lumenal part of the receptor or, alternatively, transmembrane ER chaperones could recognize the conformational changes in the cytosolic part of the receptors. Another possibility is that phosphorylation of RTKs in the secretory pathway does not affect their folding, but instead inhibits the inclusion of protein into the ER exit sites or another step in the exit of proteins from the ER.

Besides direct phosphorylation of RTKs, other mechanism could contribute to the retention of RTKs in the secretory pathway when active Src kinases are present in the cell. For example, a number of studies point to the role of SFKs in membrane trafficking. Src associates with proteins in the synaptic vesicles [63], [101] and is involved in the secretion of catecholamine by bovine adrenal chromaffin cells [58]. Additionally, a recent study suggests that SFKs are required for the the integrity of the Golgi complex [10]. In this respect it would be interesting to know if active SFKs interfere with the maturation of other transmembrane or secreted proteins which can not be phosphorylated in the SFK-dependent manner.

4.1.2 Biological relevance of Hck-mediated interference with Flt3 maturation

The data presented in this work show that co-expression of kinase inactive Hck does not potentiate the maturation of RTKs, even in case of already maturation-impaired Flt3 ITD receptor. The lack of a dominant negative effect of the kinase inactive Hck with respect to maturation of Flt3 (Flt3 ITD) is surprising because this mutant does induce a dominant negative effect with respect to phosphorylation of Flt3. It is possible that, while the activity of the endogenous SFKs in HEK-293 cells is sufficient to induce phosphorylation of Flt3, and therefore can be blocked by co-expression of dominant negative kinase-inactive Hck, endogenous SFKs do not interfere with maturation of the receptor because a certain threshold of SFK activity level (above the endogenous level) has to be reached to affect maturation of Flt3.

If SFKs interfere with the maturation of class III RTKs only when they are over-expressed or hyperactivated, is this process relevant to a physiological situation? Further work is required to answer this question. Cells derived from Src knock out animals or agents that specifically block the expression or the kinase activity of SFKs could be used to analyze the maturation and the biological responses of class III RTKs. SFK-mediated interference with the maturation of glycoproteins could contribute to the transformation of cells by over-expression or over-activation of SFKs. SFK activity is associated with advanced stages of metastatic tumors [239] and activated SFKs contribute to the metastatic spread of carcinoma cells [25]. It is known that cell surface carbohydrates affect tumor cell interaction with endothelial cells and extracellular matrix during metastatic cell growth. Therefore, the SFK-mediated influence on maturation of glycosylated proteins could be a general mechanism that contributes to Src-mediated cancer progression. However, further research is required to test this hypothesis.

4.2 Phosphorylation of Flt3 by Hck

4.2.1 Hck-dependent phosphorylation of Flt3 and Hck association with Flt3

This work shows that Hck directly phosphorylates Flt3. As autophosphorylated Flt3 also binds Hck via its SH2 domain, it is possible that Hck binding to Flt3 activates Hck and results in additional phosphorylation of Flt3 by Hck. It might be technically difficult to prove this point, though. Hck that can not engage in SH2-dependent protein-protein interactions due to a point mutation in the SH2 domain (Hck RL) still phosphorylates Flt3. However, this Hck mutant should be more active than the wild type Hck, so the absence of an increased phosphorylation of Flt3 by Hck RL (Figure 3.11)

suggests that binding of Hck to Flt3 promotes the phosphorylation of Flt3 by Hck. Kinase inactive Hck reduces the basal phosphorylation of Flt3 wt and ITD obviously acting in a dominant negative fashion. This finding suggests that endogenous SFKs phosphorylate Flt3. It is surprising, however, that the kinase inactive Hck reduces a basal level of Flt3 phosphorylation even when it can not participate in SH2 domain-dependent interactions (Hck KR RL). Therefore it is possible that in the absence of ligand stimulation, Hck activated independently of binding to Flt3 or any other SFK-SH2 domain binding partner(s) also phosphorylates Flt3. One interesting possibility emanating from this is that SFKs activated by one receptor can modulate the signaling by another receptor and therefore participate in cross-talk between different receptors in a cell.

4.2.2 Consequences of Hck-mediated phosphorylation of Flt3

Flt3 is not the only RTK that serves as a substrate for SFKs. As described in 1.4.8, SFKs phosphorylate EFGR, PDGRF and IGF-1R to name only a few, and SFK-mediated phosphorylation can modulate signaling events downstream of these RTKs and/or increase their catalytic activity. So, what are the possible consequences of SFK-dependent phosphorylation of Flt3? This work shows that SFKs phosphorylate Flt3 on tyrosine residues located in the JM region, including Tyr 589 and Tyr 591. As described in 1.3.4, phosphorylation of these two tyrosine residues plays a critical role in the autoinhibition of the catalytic activity of Flt3 by the JM region. The crystal structure of autoinhibitted Flt3 suggests that phosphorylation of one of these tyrosine residues releases a part of the JM from its binding site within the catalytic domain of Flt3 and relieves an autoinhibition imposed by the JM region [75]. In light of this model, Hck-mediated phosphorylation of Flt3 on Tyr 589 and 591 should promote the activation state of Flt3 and positively regulate Flt3 signaling. Substitution of the tyrosines in the JM region of Flt3 by phenylalanines does not completely abolish Hck-mediated phosphorylation of Flt3 suggesting that Hck phosphorylates Flt3 on additional tyrosine residues outside of the JM region. The determination of Hck phosphorylation and autophosphorylation sites of Flt3 and their role in Flt3-mediated signaling should help to clarify the function of Hck-mediated phosphorylation of Flt3. As described above, phosphorylation of Flt3 by Hck in the secretory pathway might cause retention of Flt3 in the secretoty pathway and block its maturation. This suggests that Hck-mediated phosphorylation might have different consequences depending on the localization of Flt3.

4.3 Association of Flt3 and Hck

4.3.1 Mechanism of recruitment of Hck to Flt3

This work shows that in addition to phosphorylating tyrosine residues in the JM region of Flt3, Hck also binds to these tyrosine residues in a SH2-dependent manner. However, Hck-mediated phosphorylation of Flt3 is not sufficient for Flt3-Hck association because Flt3 does not bind strongly to Hck wt that induces its phosphorylation and blocks maturation of the receptor. It appears that auto-phosphorylation, but not Hck-mediated phosphorylation of Flt3 creates high affinity binding sites for Hck. In this respect the interference by Hck with receptor maturation and plasma membrane exposure and the consequence that FL can not bind such intracellularly retained Flt3 and induce its autophosphorylation might be a reason for the largely reduced association of Hck wt with Flt3 as compared with the association of Flt3 with kinase-inactive Hck KR. In line with this is the finding that the substitution of Tyr 589 and Tyr 591 by phenylalanines decreases the binding of Flt3 to Hck, but does not abrogate it completely, suggesting that besides Tyr 589 and Tyr 591 Hck also binds to other sites on auto-phosphorylated receptor. However, this hypothesis does not explain why Flt3 ITD which is auto-phosphorylated in the absence of ligand stimulation, before its final transport to the plasma membrane, does not bind to kinase active Hck. However, Flt3 ITD's auto-phosphorylation pattern may differ from the ligand-induced auto-phosphorylation of Flt3 wt. Additional factors may contribute to the weakness of the association between Hck and Flt3 when the latter is phosphorylated by Hck and is impaired in its maturation. For example, membrane localization of both proteins might be required for a stable interaction. Furthermore, Hck-induced phosphorylation of Flt3 could create high affinity binding sites for other phospho-tyrosine binding proteins, whose binding interferes with the binding of Hck to Flt3.

4.3.2 Consequences of Hck recruitment to Flt3

Whatever the reason is, it is clear that auto-phosphorylated Flt3 localized in the plasma membrane recruits Hck via its SH2 domain. This scenario most likely takes place only when the activity of SFKs in the cell is relatively low. Under these conditions maturation of Flt3 is not impaired and its phosphorylation is mostly a consequence of auto-phosphorylation.

Binding of tyrosine-phosphorylated proteins to SFKs leads to their activation due to the disruption of intramolecular interactions, though activation of Hck or other SFKs upon stimulation of Flt3 was not proven directly. However, SFKs were shown to be activated when recruited to other class III RTKs, strongly suggesting that recruitment to Flt3 would also lead to the activation of SFKs. Further studies are required to determine activation of

SFKs downstream of Flt3 and analyze the substrates phosphorylated by the active SFKs upon their recruitment to Flt3. This work shows that besides their commonly accepted role in promotion of signaling of RTKs, activated SFKs might have an additional function of interfering with the maturation of the receptors which may prevent their own binding to these receptors.

4.4 Interaction of Flt3 with Hck in different cell lines

To understand the biological role of functional and physical interaction of Hck with Flt3, 32D cl. 3 cell lines co-expressing Flt3 wt (Flt3 ITD) with kinase inactive, wild type and hyperactive Hck were used in this study. Despite a strong effect of Hck on the maturation of Flt3 in HEK-293 cells, over-expression of either wild type Hck or the hyperactive Hck mutant does not detectably interfere with the maturation of Flt3 in 32D cl.3 cells. The data presented in this work suggest that the lower Hck expression levels in 32D cl. 3 cells, as compared with HEK-293 cells, account for the lack of Hck-mediated interference with Flt3 maturation in 32D cl. 3 cells. This result is in accordance with the hypothesis that relatively high activity of SFKs in the cell is required to interfere with the maturation of Flt3 (see above). Relatively low Hck expression level may also account for the lack of phosphorylation of Flt3 by Hck in 32D cl. 3 cells. It is surprising though, that rather high levels of kinase inactive Hck in 32D cl.3 cells did not have a dominant negative effect on Flt3 phosphorylation in 32D cl.3 cells (Figure 3.18).

Another factor that could account for the lack of Hck-mediated interference with Flt3 maturation and its phosphorylation by Hck in 32D cl.3 cells involves decreased activity of Hck in 32D cl.3 cells as compared with HEK-293 cells. In line with this hypothesis is the finding that Hck phosphorylation, which usually reflects an increase in the kinase activity, is decreased in 32D cl.3 cells as compared with HEK-293 cells.

In contrast to the lack of functional interaction between Hck and Flt3, the lack of association is not likely to be due to the lower Hck expression level in 32D cl. 3 cells. It is possible that due to a different pool of pre-existing signaling molecules in 32D cl. 3 cells, Hck binding sites on Flt3 are occupied by other proteins that bind Flt3 with higher affinity. Additionally, the activity of phosphatases that de-phosphorylate phospho-tyrosines involved in Hck binding could be significantly higher in 32D cl.3 than in HEK-293 cells.

4.5 Concluding remarks

In conclusion, the work presented in this thesis shows that SFKs interfere with the maturation of Flt3 in a kinase-dependent manner. SFKs also interfere with the maturation Kit, suggesting that this function of SFKs generally applies to class III RTKs. This work also shows that SFKs phosphorylate Flt3 and Kit. In case of Flt3, tyrosine residues in the JM region are the major phosphorylation sites for Hck. According to the crystal structure of auto-inhibited Flt3, phosphorylation of these sites promotes an active conformation of Flt3 by relief of auto-inhibition imposed by the JM region.

It is very likely that SFK-mediated phosphorylation is a reason for the impaired maturation of Flt3. Further along that line, another group has recently shown that premature auto-phosphorylation of Flt3 in Flt3 ITD impairs its maturation and tyrosine phosphatase SHP-1 promotes maturation of the receptor [198]. In accordance with these findings, this work suggests a regulatory role of SFKs in the control of receptor maturation mediated by phosphorylation of the latter. This is the first study that involves SFKs in the maturation process of class III RTKs.

This work confirms the finding that SFKs associate with the cytoplasmic part of Flt3 upon auto-phosphorylation of the receptor. However, in contrast an earlier study [53], which used chimeric receptor, this work shows that Flt3 wt as well as Flt3 ITD, associated with the development of AML, recruit SFKs. Additionally, this work identifies Tyr 589 and Tyr 591 in the JM region of Flt3 as the binding sites for a Src kinase Hck. This finding correlates with the binding mode of SFKs to other class III RTKs, which also recruit SFKs to tyrosines located in their JM regions.

It would be very interesting to understand the mechanism underlying SFK-mediated interference with the maturation of RTKs and to further investigate the biological relevance of this novel function of SFKs.

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Abbreviations

The standard IUPAC nucleotide and amino acid codes were used

2-ME	2-mercaptoethanol
A-loop	Activation loop
AML	Acute myeloid leukemia
AP	Assembly particle
APC	Antigen presenting cell
APL	Acute promyelocytic leukemia
APS	Ammonium-persulfate
ATP	Adenosine triphosphate
B-ALL	B cell acute lymphoblastic leukemia
BCA	Bicinchoninic acid
BCR	B cell receptor
Blk	B lymphoid kinase
BSA	Bovine serum albumine
CaMK	Calmodulin-sensitive kinase
CCV	Clathrin-coated vesicle
cDNA	Complementary deoxyribonucleic acid
Chk	Csk-homologous kinase
CIP	Calf intestine phosphatase
CML	Chronic myeloid leukemia
CNX	Calnexin
CRT	Calreticulin
CSF-1R	Colony stimulation factor-1 receptor
Csk	C-terminal Src kinase
DC	Dendritic cell
DG	Diacylglycerol
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
DTT	Dithiotreithol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine-tetra acetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum

Erk	Extracellular regulated kinase
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
FGFR	Fibroblast growth factor receptor
Fgr	Gardner-Rasheed feline sarcoma
FL	Fms like tyrosine kinase-3 ligand
Flt3	Fms like tyrosine kinase-3
Fyn	Fgr/Yes-related novel gene product
GAP	GTPase-activating protein
GDP	Guanosine 5'-Diphosphate
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guanosine 5'-Triphosphate
Hck	Hematopoietic cell kinase
HEK	Human embryonic kidney
HRP	Horse radish peroxidase
Ig	Immunoglobulin
IGF-1R	Insulin growth factor-1 receptor
IL	Interleukin
Inst	Inositol
IRES	Internal ribosomal entry site
ITAM	Immunoreceptor tyrosine-based activation motif
ITD	Internal tandem duplication
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
IUPAC	International Union of Pure and Applied Chemistry
JAK	Janus kinase
JM	Juxtamembrane
LB	Luria-Bertani
Lck	Lymphocyte-specific protein tyrosine kinase
Lyn	Lck/Yes related novel tyrosine kinase
M-CSF	Macrophage colony stimulating factor
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
mRNA	Messenger ribonucleic acid
MVB	Multivesicular body
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
NO	Nitric oxide
PAGE	Polyacrylamid gel electrophoresis
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PE	Phycoerythrin

PH	Pleckstrin homology
PI3-K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PML	Promyelocytic leukemia
PtdInst	Phophoinositol
PTP	Protein tyrosine phosphatase
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SFK	Src family kinase
SH	Src homology
SHIP	SH2 domain containing inositol polyphosphate 5-phosphatase
SHP	SH2 domain phosphatase
siRNA	Small interfering ribonucleic acid
Src	Raus sarcoma virus proto-oncogene product
STAT	Signal transducers and activators of transcription
SV-40	Simian virus 40
TCR	T cell receptor
TEMED	N,N,N',N'-Tetra-methylethylenediamine
TKD	Tyrosine kinase domain
TM	Transmembrane
UDP	Uridine diphosphate
wt	Wild type
Yes	Yamaguchi 73 and Esh avian sarcoma
YFP	Yellow fluorescent protein
Yrk	Yes-related kinase

Curriculum Vitae

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Publications

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