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**Oncogenic Signaling from the Hematopoietic Growth Factor Receptors
c-Kit and Flt3**

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Abstract

Signal transduction in response to growth factors is a strictly controlled process with networks of feedback systems, highly selective interactions and finely tuned on-and-off switches. In the context of cancer, detailed signaling studies have resulted in the development of some of the most frequently used means of therapy, with several well established examples such as the small molecule inhibitors imatinib and dasatinib in the treatment of chronic myeloid leukemia. Impaired function of receptor tyrosine kinases is implicated in various types of tumors, and much effort is put into mapping the many interactions and downstream pathways.

Here we discuss the hematopoietic growth factor receptors c-Kit and Flt3 and their downstream signaling in normal as well as malignant cells. Both receptors are members of the same family of tyrosine kinases and crucial mediators of stem-and progenitor-cell proliferation and survival in response to ligand stimuli from the surrounding microenvironment. Gain-of-function mutations/alterations render the receptors constitutively and ligand-independently activated, resulting in aberrant signaling which is a crucial driving force in tumorigenesis. Frequently found mutations in c-Kit and Flt3 are point mutations of aspartic acid 816 and 835 respectively, in the activation loop of the kinase domains. Several other point mutations have been identified, but in the case of Flt3, the most common alterations are internal tandem duplications (ITDs) in the juxtamembrane region, reported in approximately 30% of patients with acute myeloid leukemia (AML).

During the last couple of years, the increasing understanding of c-Kit and Flt3 signaling has also revealed the complexity of these receptor systems. The impact of gain-of-function mutations of c-Kit and Flt3 in different malignancies is well established and shown to be of clinical relevance in both prognosis and therapy. Many inhibitors of both c-Kit or Flt3 or of their downstream substrates are in clinical trials with encouraging results, and targeted therapy using a combination of such inhibitors is considered a promising approach for future treatments.

Signaling by Receptor Tyrosine Kinases

In humans, protein kinase genes correspond to approximately 2% of the genome where 90 of the total 518 genes encode tyrosine kinases, and 58 of these are receptors [1], further divided into 20 subfamilies [2]. Receptor tyrosine kinases (RTKs) can be

grouped into subfamilies according to their ligand affinity, amino acid sequences and structural homology. All possess a glycosylated extracellular ligand binding domain, a short transmembrane segment and a cytoplasmic region containing the kinase domain [3]. The tyrosine kinase domain is the most conserved region among RTKs, and is in subclass III and IV split in two by a kinase insert [4]. Mutational analysis of Y751 of the PDGFR revealed a role of the kinase insert region in substrate binding and regulation [5], and other RTKs such as Flt3 and c-Kit have since then proven to contain important phosphorylation sites within this sequence as well.

The impact of receptor autophosphorylation on substrate specificity was established after the discovery of SH2 domains. These were identified from sequence similarities of Src-related proteins and found to specifically bind to phosphotyrosine residues [6]. SH2 domains are highly conserved regions of approximately 100 amino acids and are functional as isolated subunits in associating with tyrosine-phosphorylated proteins [7]. Furthermore, different SH2 domains are associated with different binding motifs and one well recognized example is the pYEEI motif as being optimal for the Src SH2 domain [8]. Proteins often contain more than one SH2 domain [9], which may be a mechanism for enhancing signal strength and specificity by phosphorylation at multiple sites (reviewed in ref. [10]).

The discovery of the Stem Cell Factor receptor and its ligand

In the early 1900s, the *W* (dominant White spotting) locus in mice was first identified and described by the presence of a white spot on the bellies of mice carrying mutations at this locus. These mice also suffered from defects in mast cell development, gametogenesis and hematopoiesis, indicating that the main function of the gene was in the proliferation and differentiation of stem cells. The Kit gene was first identified as the viral oncogene v-Kit of the Hardy-Zuckerman 4 feline sarcoma virus (HZ4-FeSV) [11], an acute transforming virus originating from the transduction of feline c-Kit sequences with Fe leukemia virus [12, 13]. It was not until 1988 that the gene product of the *W* locus was identified as the tyrosine kinase c-Kit [14, 15]. DNA sequence analysis indicated that c-Kit showed structural similarities to PDGFR and the CSF-1 receptor [15] and was later designated a member of this type III family of RTKs (reviewed in [16]).

Another mutation in mice, designated Steel (*Sl*) was identified and the observed phenotype turned out to be practically identical to that of the *W* locus. *Sl/Sl* homozygotes are deficient in germ cells and coat pigment and die perinatally due to macrocytic anemia. Three separate groups identified the protein encoded by the *Steel* locus in parallel, and named it according to the source of the discovery as mast cell growth factor [17], stem cell factor [18] and c-Kit ligand [19], respectively. Due to the very similar characteristics of the mice with *Sl* and *W* mutations, the groups hypothesized it to be a ligand-receptor relation and further showed that this protein was able to specifically interact with, and activate c-Kit.

Stem cell factor (SCF) is a multipotent hematopoietic growth factor for early progenitor cells with strong synergistic activities with other factors such as G-CSF, IL-3 and erythropoietin. SCF regulates the differentiation, proliferation and migration of mast cells and injection of SCF results in increased numbers of mast cells. The levels of SCF in myeloid leukemia cells have been analyzed and found to be an important mediator of proliferation [20]. As a result of alternative splicing, SCF exists in a membrane-bound form, that can be proteolytically cleaved to release SCF, and soluble form, which is present to a large extent in human serum [21]. The membrane-bound isoform is associated with a more persistent activation of the receptor, thus resulting in a delayed “turn-off” signal and different biological outcomes [22]. Mice deficient of the membrane-bound SCF display similar defects as *Sl/Sl* mutant mice which further indicates a distinct role for the membrane-bound SCF [23].

The structure and function of c-Kit

Members of the type III family of RTKs (PDGFRs, Flt3, c-Kit and CSF-1R) all share the common structure of five extracellular immunoglobulin-like domains, a single transmembrane segment, a juxtamembrane (JM) domain and a split cytoplasmic kinase domain. In the extracellular part of c-Kit, SCF is thought to bind to the second and the third domains whereas the other domains are crucial for receptor dimerization [24]. Human c-Kit consists of 976 amino acids which results in a size of 140 and 155 kD depending on the extent of N-linked glycosylation [13, 25]. Several isoforms of c-Kit have been described in humans, and exist as a result of alternative splicing. The two best characterized splice transcripts result in the presence or absence of the amino acid sequence GNNK in the extracellular domain [26]. Recent studies have shown differential signaling capacities from the two isoforms, where the absence of GNNK enhances the overall c-Kit signaling, which also leads to qualitatively different signaling outcome [27, 28]. A number of phosphorylation sites in the c-Kit receptor have been identified and are summarized, together with the corresponding interacting proteins, in Table 1. A summary of some of the most important signal transduction pathways downstream of c-Kit is shown in Fig. 1.

As the alternative name for c-Kit implies, Stem cell factor receptor refers to its initially identified role in survival, self renewal and differentiation of hematopoietic stem cells. Mast cells and hematopoietic progenitor cells express c-Kit on their surface whereas the more differentiated cells lack c-Kit expression. Primitive hematopoietic cells depend on several cytokines in synergy, including SCF for growth and survival. Other areas that require c-Kit activity is in the proliferation of germ cells [29], the migration of melanocytes from the neural crest to the dermis during development [30] and the function of interstitial cells of Cajal (ICCs) in the gut [31].

The role of c-Kit signaling in disease

Defects of c-Kit in humans are associated with a rare genetic disease known as the piebald trait, arising in heterozygous individuals and resulting in non-pigmented patches similar to that observed in mice [32]. c-Kit is involved in several types of malignancies, either through mutations that render the receptor constitutively active, for example in mast cell leukemia [33], germ cell tumors [34] and gastrointestinal tumors (GISTs) [35], or via autocrine loops where the tumor cells produce both SCF and c-Kit. Examples of such tumors are small cell lung carcinomas [36], colorectal carcinoma [37], gynecological tumors [38] and certain types of melanoma [39]. Moreover, evidence has been presented that c-Kit is involved in the proliferation of human leukemia cells [40]. Wild-type c-Kit is expressed in most human AML cells, and is in many cases constitutively phosphorylated. SCF has been shown to stimulate proliferation of AML cells and also to be co-expressed by some AML blasts [20, 41]. In addition, activating mutations of c-Kit have been detected in the core-binding factor (CBF) subtypes of AML samples [42].

Gain-of-function mutations in c-Kit occur either in the JM domain or in the kinase domain, and both groups cause constitutive activation of the receptor. Moreover, both groups of mutations induce transformation of cytokine-dependent hematopoietic cell lines and drive tumorigenesis when introduced in mice [43]. Although some features are similar, the kinase domain mutants of c-Kit show a more pronounced activation of the receptor and are considered more potent as oncogenes. The most commonly found kinase domain mutation is a substitution of D816 in the activation loop of c-Kit, which has been described in different types of tumors, in mast cells from patients with mastocytosis and in about 1% of patients with CBF AML [44]. A deletion, insertion or point mutation in the JM domain of c-Kit is the most frequently occurring alteration in GISTs [35], a tumor form in the digestive tract originating from the ICCs, known to express c-Kit. These tumors generally respond well to the tyrosine kinase inhibitor imatinib mesylate (STI-571, Gleevec) [45].

The hematopoietic growth factor receptor Flt3 and its ligand

The Flt3 receptor was isolated by two separate groups in 1991. The first group used a hybridization technique with a DNA probe from the M-CSF receptor (c-Fms) to isolate a closely related DNA sequence which was named Fms-like tyrosine kinase 3 (Flt3) [46, 47]. The second group designed oligonucleotides based on conserved regions within the tyrosine kinase domain of RTKs, and used a PCR-based approach to isolate a receptor fragment from murine fetal liver stem cells. This was further used to isolate a full length receptor given the name Fetal liver kinase-2 (Flk-2) [48]. In the same study, Flk-2 was found to be expressed in hematopoietic stem cells and primitive uncommitted progenitors and absent in the more differentiated cells. Other organs such as the nervous system and gonads also have Flt3 expression, but the function here is not well studied [46, 49].

Human Flt3 is 993 amino acids in length and exists as a less glycosylated immature form of about 130-140 kD and a more glycosylated and plasma membrane expressed form of 155-160 kD [50]. Knock-out mice of Flt3 are viable and fertile but exhibit hematological defects with reduced numbers of B-cell precursors in the bone marrow [51] with abnormalities in the generation of dendritic cells, most evident in the periphery where Flt3 is crucial for the expansion of dendritic cells in lymphoid organs [52].

The soluble form of the ligand for Flt3 (Flt3 ligand or FL) was purified and sequenced in 1994 [53]. By using an affinity column of Flt3 the group was able to purify FL from conditioned thymic stromal cell medium. In parallel, an array of cell lines was screened in order to find a ligand on the cell surface capable of binding to the soluble Flt3 [54]. FL is a homodimer and exists both as a membrane-bound form as well as a soluble form as a result of alternative splicing and/or proteolytic cleavage [55, 56]. It is homologous to SCF and M-CSF [53] and functions as a multipotent cytokine affecting a broad range of hematopoietic cell expansion [57].

The role of Flt3 signaling in cancer

Over the past couple of years, the impact of Flt3 signaling in the development of hematopoietic malignancies has become evident, and *Flt3* is recognized as one of the most frequently mutated gene in AML. The most common alteration identified is termed internal tandem duplication (ITD) and refers to a duplication of a sequence of amino acids in the JM domain. ITDs vary in both length and position in different patients but regardless of this render the receptor constitutively activated. Approximately 30% of patients with AML carry at least one copy of Flt3 ITD, which has a strong correlation to a worse prognosis with a poor drug response and larger risk for relapse [58, 59].

The most frequent kinase domain mutation is a substitution of aspartic acid 835 to tyrosine, but it can also be other amino acids such as valine, histidine or glutamate. An additional class of Flt3 mutants constitutes a deletion or substitution of I836 [60]. The clinical outcome of patients with tyrosine kinase domain (TKD) mutations differs from those with ITDs, with regards to the overall survival which is significantly higher in the Flt3 TKD proportion [61]. Furthermore, the ITD receptor is capable of inducing a myeloproliferative syndrome in a murine bone marrow transplantation model [62] whereas TKD mutants are not, but instead induce a lymphoid disorder [63].

Signaling downstream of c-Kit and Flt3

The general mechanism for activation of RTKs is via binding of the respective ligand to two receptor monomers which induces dimerization followed by transphosphorylation of specific tyrosines which ultimately stabilizes the active conformation of the receptor. For c-Kit, crystal structures have revealed that autophosphorylation of the tyrosines in the JM domain occurs first [64], as opposed to several other RTKs such as insulin-like growth

factor receptors where the activation loop tyrosine is the first to be phosphorylated [65]. Table 2 summarizes our present knowledge regarding autophosphorylation sites in Flt3 and their corresponding binding partners. Some of the major signal transduction pathways downstream of Flt3 are summarized in Fig. 2.

The JM domain is considered to sterically hold the activation loop in an inactive conformation as shown in the case of c-Kit by Mol *et al.* [66]. Crystal structures of the JM domain from the closely related c-Fms receptor kinase revealed a hydrophobic site adjacent to the ATP binding pocket, held in place by JM interaction and thus locking the receptor in an autoinhibited state [67]. Flt3 was shown to be inhibited by a similar mechanism [68]. Upon phosphorylation of tyrosines 589 and 591 the phosphate group interrupts the autoinhibited complex and the introduction of ITDs in Flt3 is thought to act in a similar manner, making this autoinhibition “leaky” [69].

The MAP-kinase signaling pathway

The mitogen-associated protein kinase (MAPK)-pathway was one of the first kinase cascades to be elucidated. In mammals, five MAPK families have been found; Erk-1 (p44) and Erk-2 (p42), the Jnk (c-Jun N-terminal kinase) family, the p38 MAPK family, Erk-3 and Erk-5 (reviewed in [70]). Erk-1 and Erk-2 are both activated upon phosphorylation of threonine and tyrosine residues. Erks in turn activate a number of both cytoplasmic and nuclear proteins by phosphorylation of serine and threonine residues. Examples of downstream targets are transcription factors such as c-Fos, Rsk and Elk-1. Although Erk1 and Erk2 have previously been thought to be functionally redundant, recent data suggest that they have distinctive functions [71]. The identification of MAPKs further led to the discovery of upstream activators of Erk-1 and -2, referred to as MAPK activators [72] and later also shown to be kinases (MAPK kinases, MAPKKs). MAPKKs, also named Mek, is a family of serine/threonine kinases, and the members Mek-1 and -2 are the activators of Erk-1 and -2. Contrary to popular belief, recent data suggest that they are not functionally redundant [73].

Another serine/threonine kinase, Raf-1 was then reported to activate Mek upon growth factor stimulation [74]. Mammalian Raf proteins include Raf-1, A-Raf and B-Raf and the main function is thought to be in the coupling of Ras to the MAPK pathway [75].

Ras proteins belong to a family of small GTPases including H-Ras, N-Ras and K-Ras, and are localized to the plasma membrane by prenylation of the C-terminus. Ras proteins bind GDP or GTP and are active in the GTP-bound state, as a response to guanine nucleotide exchange factors (GEF's) which release GDP from Ras. Upon RTK activation, Ras associates with Sos, a GEF existing in complex with the adaptor protein Grb2, which in turn is recruited to the phosphorylated YXN motif of proteins via its SH2 domain. In addition, Ras is activated by another GEF, Vav in response to SCF in

hematopoietic cells [76]. Contrary to this, GTPase activating proteins (GAP's) act as negative regulators of the Ras proteins by favoring the dephosphorylation of GTP.

Other means of Erk phosphorylation has been suggested to occur via its association with the tyrosine phosphatase Shp2, which in most RTK systems is required for the activation of Erk [77]. From studies on Shp2 mutant fibroblasts, a decreased Erk phosphorylation was reported and suggested to be due to the direct interaction between Shp2 and Erk [78]. Recent data suggest that the direct binding of Grb2 to Shp2 is not important, but rather the phosphatase activity of Shp2. Several substrates of Shp2 have been described that can be linked to Erk activation. Some studies suggest that Sprouty can be dephosphorylated by Shp2, leading to the release of Grb2 to activated Ras [79]. It has also been described that Shp2 by acting on Csk, blocks Src kinase activity. This in turn leads to decreased phosphorylation of phospholipase C- γ that serves as an activator of Ras in the endomembranes [80]. Additional substrates of Shp2 of important for Erk activation most likely add to the effect (reviewed in [81]). Moreover, a cross-talk between PI3K and Ras on many levels has been suggested in several studies. It is known that Ras mediated activation of the catalytic subunit p110 of PI3-kinase is kept in control by the p85 subunit. Binding of p85 to phosphorylated tyrosine residues releases this inhibition, leading to Ras mediated activation of p110 [82]. Conversely, PI3-kinase has also been shown to activate Ras [83].

Both c-Kit and Flt3 activate the MAPK pathway, and many components for this have been suggested. The interactions with Grb2/Sos to tyrosines 703 and 936 of c-Kit and 768, 955 and 969 of Flt3 result in Erk phosphorylation, as does the association between Grb2 and Gab2 which recruits Shp2 [84, 85]. Moreover, when mutating the Src binding sites of c-Kit and Flt3, a reduced Erk phosphorylation was observed [86, 87], confirming that RTKs activate the MAPK pathway in multiple ways.

The PI3-kinase signaling pathway

The phosphoinositide 3 (PI3)-kinase pathway is evolutionarily conserved from yeast to mammals and regulates various cellular processes such as metabolism, proliferation and apoptosis. PI3-kinases are a family of lipid kinases that phosphorylate the 3'-hydroxyl group of phosphoinositides. Class I PI3Ks all phosphorylate the membranebound lipid phosphatidylinositol-4, 5-bisphosphate (PIP₂) to phosphatidylinositol-3, -4, 5-trisphosphate (PIP₃), but are further divided into class 1A and 1B based on their activation by RTKs or by G protein-coupled receptors, respectively. The members of class 1A exist in a heterodimeric complex composed of a 110 kD (p110 α , β or δ)-catalytic subunit and a 85 kD (p85 α , β or p55 γ)-regulatory subunit which contains two SH2 domains [88]. The non-p110-bound p85 subunits exist in a molar excess in cells and bind tyrosine phosphorylated proteins via their SH2 domains, suggesting a more complex function. Specific p85 knock-out cells have revealed a disrupted regulation of PI3K where the p85 subunit both inhibited and activated the p110 subunit depending on the

context (reviewed in [89]). PIP₃ recruits PH-domain containing proteins to the membrane. The best known downstream target is the serine/threonine kinase Akt (also referred to as protein kinase B), a mediator of survival and proliferation [90].

Upon activation of PI3K, Akt is localized to the plasma membrane and phosphorylated on T308 by 3-phosphoinositide-dependent kinase-1 (PDK1) [91]. The Mammalian target of rapamycin complex 2 (mTORC2) is responsible for a second phosphorylation at S473, which locks Akt in an active conformation. Phosphorylation of both residues is required to activate Akt, which in turn activates a vast amount of different substrates such as Foxo proteins, p27, Bim, Tuberous sclerosis complex 2 (TSC2), Bad, glycogen synthase kinase 3 (GSK3), murine double minute-2 (Mdm2) as well as nuclear factor-kappa B (NF-κB) (reviewed in [92]). As mentioned earlier, crosstalk between Ras and PI3K signaling pathways adds another level of complexity where receptors lacking a direct p85 binding site are able to activate PI3K either through Gab1, Gab2 or via Ras. Such RTKs, for example the receptors for EGF and FGF as well as Flt3, are to some extent dependent on this interaction for their normal functions. In a mouse model, disruption of the EGF and FGF-mediated direct interaction between p110 subunit of PI3K with Ras blocked the ability to drive tumor formation [93].

SCF induces association with the SH2 domain of the p85α subunit of PI3-kinase with Y721 in c-Kit [94] and a number of studies have revealed that this interaction contributes to, but is not absolutely required for c-Kit-mediated biological responses. One exception was shown using transgenic mice expressing c-Kit with a Y721F mutation where the PI3K signaling by c-Kit was suggested to be required in the process of gametogenesis [95, 96]. However, recruitment of PI3-kinase to Y721 of c-Kit is not the only route for activation of PI3-kinase. Several studies have implicated Gab2 as an important mediator of SCF-induced PI3-kinase activation [28, 97]. Furthermore, the transforming capacity of the oncogenic D816V c-Kit receptor has been demonstrated to be dependent on PI3K activity [98]. In the case of Flt3, the human receptor lacks a direct binding motif for the p85α subunit of PI3K, whereas the murine form contains this motif and has been verified to directly associate with p85α [99]. Human Flt3 was found to phosphorylate Gab1 and Gab2, thus suggested to activate the PI3K pathway indirectly through these intermediates [100], which is in line with the identification of tyrosines 768, 955 and 969 as Grb2/Gab2 association sites and mediators of downstream signaling via PI3K and Akt [85].

Src family kinases

The discovery of the c-Src proto-oncogene was based on years of studies on the Rous sarcoma virus (RSV) (reviewed in [101]). Src is a member of the Src family kinases (SFKs), consisting of Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk and Yrk [102]. The conserved domain structure of SFKs is composed of a myristoylated or palmitoylated N-terminal region that differs between the family members, an SH3 domain, an SH2 domain and a tyrosine kinase domain followed by a short C-terminal tail. Phosphorylation of the C-terminal Y527 by carboxyterminal Src kinase (Csk) keeps the protein in an inactive

conformation by the interaction with the SH2 and further strengthened by SH3 domain interactions with prolines close to the active site. The protein is released into an active state upon dephosphorylation of Y527 or displacement with another stronger binder. The activity of Src is also dependent on the phosphorylation status of Y416 in the activation loop [103]. Thus, phosphorylation of these two residues have opposing effects on the Src activation [104].

The role of SFKs in leukemia was recently established in an ALL mouse model, showing that Src kinases (Lyn, Hck and Fgr) were required for the proliferation of Bcr-Abl expressing pre-B cells but not Bcr-Abl expressing myeloid cells [105]. This exemplifies the diverse and highly cell type-specific functions of SFKs and suggests that SFKs would be good targets in Bcr-Abl positive ALL but not in CML (reviewed in [106]). Mice with ALL induced by Bcr-Abl resistant to inhibition displayed a significantly prolonged survival when treated with dasatinib as opposed to imatinib treatment. Dasatinib inhibits both Bcr-Abl and SFKs whereas imatinib does not target SFKs, which may further enhance the overall response observed when using dasatinib.

SFKs associate with human c-Kit through Y568 while phosphorylated Y570 contributes to binding by providing an acidic amino acid residue [107] and have a role in the both positive and negative regulation of the receptor via phosphorylation of Shc and via ligand-induced internalization and activation of the E3 ubiquitin ligase c-Cbl, respectively [108, 109]. v-Kit, the transforming version of c-Kit, lacks the binding sites for SFKs as well as for APS and Cbl [110], and it is hypothesized that it is subsequently not downregulated appropriately, which might contribute to the transforming activity of v-Kit. In addition to these residues, Y900 of c-Kit is proposed to be a Src phosphorylation site and a Y-to-F mutation of this residue resulted in decreased SCF-induced DNA synthesis in NIH3T3 cells as compared to cells expressing wild-type c-Kit [111].

Several studies show that Src is phosphorylated by Flt3, and Src inhibitors were found to block the transforming effects of Flt3 mutants in AML [112]. Tyrosines 589, 591 and 599 are suggested to be Src binding sites [87], in line with findings indicating that Hck associates with JM tyrosines of Flt3 [113]. Y589 and Y591 are homologous to Y568 and Y570 in c-Kit, but the second association site, Y599, is unique to Flt3. In a recent study, Lyn was found selectively and constitutively phosphorylated downstream of the mutant Flt3 ITD when compared to the wild-type Flt3, proposing a specific target for Flt3 ITD expressing AML cells [114].

JAK/STAT signaling

The function of Janus kinases (JAKs) was first described downstream of interferon receptors as kinases able to transmit signals from receptors lacking intrinsic kinase activity. The JAK family consists of four members; Jak1, Jak2, Jak3 and Tyk2 and encode a group of unique domains termed JAK homology domains (JH1-JH7). The C-

terminal JH1 and JH2 are highly homologous to the tyrosine kinase domains but only JH1 appears to be functional. The JH2 domain is considered a pseudokinase domain as it lacks critical amino acids and has no kinase activity. The name Janus kinase is derived from the Roman god with two faces, referring to the concept of JAKs having both a kinase and a pseudokinase domain. Activated JAKs phosphorylate sites on receptors which further allow binding of proteins like STATs, SFKs, phosphatases and adaptor proteins (reviewed in [115]).

Aberrant JAK signaling has been reported in hematological malignancies, and a ETV6-Jak2 fusion product was detected in patients with T-cell ALL and also some types of myeloid leukemias (reviewed in [115]). Constitutive activation of Jak2, in most cases as a result of a V617F substitution, is found in hematological malignancies with the highest mutation frequency in myeloproliferative disorders like polycythemia vera and essential thrombocythemia [116].

The signal transducers and activators of transcription (STATs) were originally described as ligand-induced transcription factors in interferon-treated cells [117]. Normally, STATs are located in the cytoplasm and upon phosphorylation dimerize and get translocated to the nucleus. Activated STATs then bind to specific Gamma-interferon-activated sequences (GAS) in the DNA to initiate transcription of target genes. Crosstalk between the MAPK and the STAT signaling pathways are exemplified by a C-terminal conserved serine residue (S727) of the Stat1 and Stat3 proteins which is phosphorylated by MAP kinases in order to enhance the transcriptional activity [118], and the regulation of Stat3 activity by MEKK1 (Mek kinase) [119]. One of the general STAT target genes is the cytokine-inducible SH2-containing protein (CIS) which is induced by cytokines such as GM-CSF, erythropoietin and IL-3 and act to reverse the STAT activation. Stat3 in specific activates the *c-myc* gene and Stat5 has been associated with induced gene expression of *Bcl-xL* and *pim-1* (reviewed in [120]).

Stat5, originally referred to as mammary gland factor (MGF), was isolated from mouse mammary gland tissue and linked to the prolactin signaling pathway. Stat5a and Stat5b are encoded by two separate genes but show a 96% sequence similarity but their main sites of expression and transcriptional activities display a distinction between the two (reviewed in [121]). However, in individually mutated mouse models, the functions of Stat5a and Stat5b appeared relatively redundant in mediating GH and prolactin functions [122], and Stat5a/b $-/-$ mice but not Stat5a or Stat5b knock-outs alone were associated with a decrease in lymphocytes.

Upon disruption of Stat5 expression in Ba/F3 cells, the induction of *cis*, *pim-1*, *Id-1*, *osm* and *c-fos* was inhibited and the IL-3 dependent growth was reduced [123]. This

was later verified in a study where a constitutively active form of Stat5 promoted cell proliferation and transformation in Ba/F3 cells [124] and was furthermore shown to be present and essential for the transformation in Bcr-Abl expressing K563 cells [125]. In a study performed in CML cell lines, Stat1 and Stat5 were phosphorylated in the absence of JAKs suggesting that Bcr-Abl signaling alone was enough for the activation [126]. Constitutively activated Stat5 downstream of deregulated RTKs as well as the V617F mutant of Jak2 is known to contribute in the development of AML and polycythemia vera, respectively [127]. A potential role for STATs in AML was first observed as constitutive DNA binding of Stat3 and Stat5 in leukemic cells of AML patients [128] and is now considered one of the main signal transduction pathways downstream of mutated Flt3 for driving transformation.

There has been conflicting data regarding the ability of c-Kit to activate STATs. Some studies have shown that Jak2 as well as Stat3 and Stat5 associate with c-Kit and that SCF activates JAK-STAT signaling [129-131], but there are other groups reporting no observed activation of JAK-STAT by SCF [132, 133]. Most likely, these signaling pathways are highly cell type- as well as time- and context-specific, and the outcome may therefore differ depending on the experimental setup. The findings with Flt3 are more consistent, as the ITD receptor, but not the wild-type Flt3 is able to activate Stat5.

Shp1 and Shp2

The Src homology-2 domain-containing phosphatases (SHPs) is comprised of two members; Shp1 (encoded by *PTPN6*) and Shp2 (encoded by *PTPN11*). Shp1 (also referred to as PIP1C or SH-PTP1) is suggested to be a negative regulator of RTKs and was shown to interact with c-Kit at Y568 [134]. This protein has a negatively regulatory role in the cell adhesion and migration of hematopoietic cells [135]. Shp2 (also referred to as PTP1D or Syp) was shown to be involved in cell migration and adhesion as demonstrated by fibroblasts lacking functional Shp2 which were impaired in cell motility [136]. The main role for Shp2 however, is considered to be in the positive regulation of Ras in response to growth factors, via dephosphorylation of inhibitory sites. Shp2 is recruited to the receptors, either directly as in the case of PDGFRs [137] and Flt3 [87] or via scaffolding proteins such as Gab1 and Gab2. Shp2 was shown to be of importance in the transforming ability of oncogenic versions of for example EGFR, FGFR, Ret and Bcr-Abl (reviewed in [138]). Furthermore, Shp2 dephosphorylates Ras-Gap binding sites, keeping Ras in an active state, as well as CPg/PAG thereby preventing recruitment of Csk to Src (reviewed in [81]). The SH2 domain of Grb2 was reported to interact with phosphorylated Shp2 in response to PDGF, and suggested to connect the receptor to MAPK signaling in this way as well [139]. Recent data however, suggest that the phosphatase activity of Shp2 rather than its ability to recruit Grb2 is involved in its activation of Erk (for review, see [81]).

Germline activating mutations of *PTPN11* are found in approximately 50% of cases with Noonan syndrome, a disease characterized by heart and developmental defects with an increased risk of leukemia. Somatic mutations have been identified in cases of ALL,

AML and JMML [140] as well as other types of tumors (reviewed in [138]). Given the crucial role in transformation of cells and the relatively high incidence of gain-of-function mutations in leukemias, Shp2 is an obvious target for cancer therapy.

Adaptor and scaffolding proteins

Adaptor proteins are defined as proteins lacking enzymatic activity with the capacity of interacting with several proteins at the same time. Upon activation, they create docking sites for other signaling intermediates, and act to bring these together. Adaptor (or scaffolding) proteins are important for the regulation and amplification of downstream signals and provide a basis for fine-tuned signaling in the cell. For example, many proteins can interact and be activated both in a direct manner and via adaptor proteins depending on the signal strength and intensity and also the cell and tissue type.

One of the best known adaptor proteins is the Growth factor receptor binding protein-2 (Grb2), which couples RTKs to the MAPK signaling pathway. Activated RTKs bind to the SH2 domain of Grb2 via the consensus sequence YXN, causing the association with the Ras guanine nucleotide exchange factor Sos that in turn activates Ras. c-Kit binds to Grb2 via tyrosines 703 and 936 [84] and Flt3 has been shown to interact with Grb2 via tyrosines 768, 955 and 969 [85].

The PH domain-containing docking protein Gab2 (Grb2-associating binding protein 2) was initially identified as a major binding protein for Shp2 in IL-3 dependent hematopoietic cells, and homologous to Gab1 [141]. The PH domain enables protein-protein complexes to localize to phospholipids and Gab2 has indeed been reported to be involved in processes like cell adhesion and motility via integrin signaling [142]. Gab2 associates with SH2 domain-containing proteins like Shp2 and PI3K [143] as well as adaptor proteins like Grb2. SCF induces phosphorylation of Gab2 [143] and also Flt3 has been shown to associate with Gab2 via Grb2 ([100] and BJH ref). As human Flt3 does not have a direct binding site (YXXM) for the p85 subunit of PI3K, the receptor is believed to instead form a complex with proteins such as Grb2, Gab2, Shc and SHIP to indirectly act on the downstream PI3K as well as Ras signaling pathways. Gab2 contributes to PI3K signaling by directly interacting with the p85 subunit which in turn activates the p110 catalytic subunit of PI3K and by translocating PI3K to the plasma membrane where it can access its phospholipid substrates. Gab2 was shown to be required for the Bcr-Abl induced transformation of murine myeloid cells [144], and mice transplanted with Gab2^{-/-} bone marrow cells displayed defects in multilineage repopulation in response to cytokines in early-phase hematopoiesis [145].

ShcA also interacts with Grb2, which couples RTKs to MAPK signaling and may be the mediator of this pathway for RTKs lacking direct Grb2 binding sites. Moreover, Grb10 and Grb7 interact with RTKs and, Grb7 was shown to bind to Y936 of c-Kit [84]. The Adaptor protein containing PH and SH2 domain (APS) is phosphorylated in response to SCF and found to bind to Y568 and Y936 [146]. APS and the two very closely related family members Lnk and SH2B, are involved in recruiting the E3 ubiquitin ligase Cbl to RTKs, thus mediating their downregulation by degradation. In

addition to APS, Lnk was recently found to interact with Y568 of c-Kit [147]. CrkL interacts with RTKs via the p85 subunit of PI3K and was also shown to recruit Cbl to c-Kit [148].

Oncogenic signaling from Flt3

Due to the distinct phenotypic differences caused by the Flt3 ITDs, much effort has been put into characterizing the downstream signaling of this receptor. The most striking deviation from wild-type Flt3 or TKD mutants lies in the ability to potently activate Stat5 [149-151]. Stat5 in turn activates several specific downstream targets such as Pim-1, Cdc25A and Bad, key mediators of cell cycle progression and anti-apoptotic signaling. Another transcriptional target of Stat5 in AML cells is Bcl-2, and a correlation between high Bcl-2 levels with a Flt3 ITD duplication of Y591 has been observed [152]. The enhanced anti-apoptotic signaling driven by Stat5 activity is one of the key features of Flt3 ITD expressing cells which might explain the worse outcome for these patients. In a recent report it was demonstrated that Flt3 ITD cells contain increased levels of reactive oxygen species (ROS) as compared to cells with wild-type Flt3, most likely via Stat5 activity. Phosphorylated Stat5 was shown to bind Rac1 in its GTP-bound state, a protein known for its function in activating the NADPH oxidase family to create superoxide, hence contributing to the elevated ROS levels [153]. This impacts the double-strand break (DSB) repair mechanism which also is part of the Flt3 ITD specific responses that result in a poor prognosis. Constitutively phosphorylated Stat5 has been verified in primary AML blasts carrying the Flt3 ITD mutation and the effects of Flt3 inhibition in AML blasts correlates with inhibition of Stat5 [154]. Flt3 ITD was also suggested to promote the inhibitory phosphorylation of Foxo Forkhead transcription factors and thereby repressing *p27* and *Bim* gene expression [155], similar to that observed in CML by Bcr-Abl [156]. Phosphorylation of Foxo3a in Flt3 ITD positive cells was further reported to be downstream of constitutively activated Akt [157].

Recently, using phosphospecific antibodies against potential tyrosine phosphorylation sites in Flt3, Razumovskaya and co-workers identified three novel phosphorylation sites in Flt3 (Y726, Y793, Y842; [158]). Furthermore, it was demonstrated that the Flt3 ITD, Flt3 D835Y and wild-type Flt3 showed different patterns of autophosphorylation and distinctively different patterns of kinetics of phosphorylation. Since the tyrosine phosphorylated residues are the initiator sites for the individual signal transduction pathways, this further suggests that the various mutants induce different signal transduction pathways. It is not yet known which proteins bind to the three novel phosphorylation sites.

Gene expression profiles of Flt3 ITD and TKD have revealed an upregulated expression of transforming growth factor- β stimulated clone-22 (TSC-22), a potential leukemia cell growth suppressor, exclusively by Flt3 TKD [159]. Microarray analysis identified Frizzled-4, one of the receptors in the Wnt signaling pathway, to be induced by Flt3 ITD in myeloid progenitor cells [160]. In addition, increased activation of Wnt

signaling was observed in six out of seven AML cases with Flt3 ITD, suggesting that the leukemogenic effects of Flt3 ITD might in part be due to the involvement of this pathway [161].

Hematopoiesis and hematopoietic stem cells

The term hematopoiesis refers to the tightly regulated and controlled process of blood formation where millions of cells are produced daily in a normal adult. Hematopoiesis is initiated by the development and self-renewal of hematopoietic stem cells (HSCs). HSCs were originally identified in 1961 [162] and first isolated in the Thy-1 low, Lineage specific antigen (Lin) negative, and Stem cell associated antigen (Sca-1) positive fraction [163], and were later characterized as cells lacking any expression of Lin but positive for Sca-1 and expressing c-Kit (so called LSK cells) [163, 164]. Adult HSCs reside within the bone marrow and represent only a very small fraction of the hematopoietic system in total. They are defined as clonogenic cells with the ability to self-renew and to differentiate into mature blood cells of all lineages, hence holding the capacity to regenerate the whole blood system of an organism [165].

Within the LSK compartment, long term-HSCs lack expression of CD34 and Flt3 but short term-HSCs are LSK CD34+Flt3-. However, within this population there is a subgroup of Flt3+ cells, which have undergone a first step of lineage restriction through the loss of megakaryocyte and erythroid potential by downregulated expression of GATA-1, EpoR, SCL/Tal1 and TpoR [166].

The concept of the “stem cell niche” as the specific microenvironment in which the stem cells reside, was introduced in the late 1970s [167], and is today considered a key regulatory mechanism for the functions of stem cells. Interestingly, HSCs maintenance in their niche is dependent on membrane-bound SCF, and cleavage into the soluble ligand was shown to favor HSC differentiation [168]. This information, together with several studies on the requirement for HSCs to interact with integrins such as VLA-4 and VLA-5 for their function, supports the idea of HSC dependency on the physical association with their stem cell microenvironment [169].

c-Kit is expressed by most of the CD34+ cells in the bone marrow [170] and also by more committed progenitors such as myeloid, erythroid, megakaryocytic, natural killer and dendritic cell progenitors as well as pro-B and -T cells and mature mast cells [171]. Synergistic signaling between SCF and FL is exemplified in the growth of hematopoietic progenitor cells, where they show little response to either of the cytokines alone, but a strong proliferation when exposed to SCF and FL in combination. Given the importance of SCF and FL in hematopoiesis, these cytokines may be used in the expansion of BM stem cells for transplantation purposes, in immunotherapy by for

example expanding the subsets of DCs, and in cancer therapy in immune system mediated cases.

Acute myeloid leukemia

Acute myeloid leukemia (AML) accounts for approximately 30% of all adult leukemias, and is a disease characterized by an accumulation of myeloblasts in bone marrow and blood [172]. In most AML patients, many different clonal chromosomal abnormalities such as reciprocal translocations, insertions, deletions and unbalanced translocations are found, in contrast to CML where the cytogenetic picture is less complex. The heterogeneity of AML is a result of both cytogenetic alterations and molecular mutations and subclassifications are based on these factors as well as the morphology and differentiation status of the AML cells. Chromosomal abnormalities include gains or losses of parts of, or whole chromosomes, or translocations such as the *t(8;21)/AML1-ETO (RUNX1/RUNX1T1)*, *inv(16)/CBFB-MYH11* (smooth muscle heavy chain gene) or *t(15;17)/PML-RAR α* , occurring in about 40% of AML cases and considered strong prognostic factors.

The development of AML is described as a multi-step process where the presence of at least two complementing mutations, typically impairing differentiation and promoting proliferation, is a requirement. Through constitutive activation of signaling pathways promoting survival and proliferation, clones of affected hematopoietic progenitor cells are expanded and can further be exposed to alterations blocking myeloid differentiation. Neither of these types of mutations is enough to cause AML alone. The first category, referred to as class I mutations, are those activating signal transduction pathways such as in c-Kit and Flt3 and resulting in reduced apoptosis and enhanced stem cell self-renewal. The second category (class II mutations) includes mutations which result in transcriptional repression of differentiation. Examples of class II mutations in AML are gene fusions such as *CBF-MYH11*, *AML1-ETO* and *PML-RAR α* . Chromosomal rearrangements of the mixed lineage leukemia (*MLL*) gene also

fall under this category. The most common class I mutations are within the Flt3 receptor, either as ITD or TKD mutations. ITD is associated with an unfavorable prognosis [60, 173], but the impact of TKD mutations are not as clear [174]. In a study of leukemic blasts from AML patients, the incidence of Flt3 ITD mutations was found to be 27% [175], and several other studies have given similar results [176, 177]. Mutations in c-Kit occur in approximately 2% of AML cases and are more frequently found in CBF leukemias [178].

The gene encoding for the nuclear protein nucleophosmin 1 (Npm1), involved in cell cycle regulation and apoptosis via its involvement in the p53 pathway, was recently found to be frequently mutated in AML cases, often along with Flt3 mutations [179]. Npm1 mutations commonly precede Flt3 ITD mutations and are therefore thought to be a primary event in leukemogenesis [180]. The hypothesis of cooperation is supported by the fact that Flt3 ITDs often are found in combination with Npm1 mutations, and also in about 30% of all *MLL*-PTD positive cases and in 40% of PML-RAR α AML. This is also seen in the case of c-Kit, where mutations are found in 40% of *AML1-ETO* and *CBFB/MYH11* cases but only about 2% in overall AML.

In leukemogenesis, the self-renewing stem cells are activated and may be more favored in accumulating mutations than the more mature and rapidly dying cell types. Thus, leukemia is believed to be sustained by a subpopulation of leukemic stem cells (LSCs, also referred to as leukemia-initiating cells) with self-renewing potential, and CD34⁺/CD38⁻ cells have been identified as the LSC fraction in AML (reviewed in [181]). The unique properties of stem cells in for example a reduced mitosis rate and decreased expression of the apoptosis-associated Fas/Fas-L, may contribute to their ability to escape therapy [182]. Thus, as the present AML treatment is aimed at cancer cells in general and not the cancer stem cells specifically, the response is disappointing. This is also reflected in the poor overall survival of AML patients with larger amounts of stem cells [183].

Some studies have shown that Flt3 ITD mutations are present in leukemic stem cells, and that treatment with the Flt3 inhibitor CEP-701 blocks the engraftment of Flt3 ITD expressing leukemic stem cells [184]. Contradictory to this, others have demonstrated that Flt3 ITD mutations in some cases are lost at relapse, indicating that the ITD occurred at subclones eradicated by the treatment [185-187]. Thus, for this smaller number of AML patients, the Flt3 ITD mutation appears not to be present in the leukemic

stem cell but for the majority of Flt3 ITD positive AML cases, the same mutation is present at equal or higher levels at relapse (reviewed in [188]).

AML therapy

The current treatments of AML patients are considered unsuccessful. Although a complete remission of adult AML is achieved by induction therapy using anthracyclins and cytarabine, the therapy eventually fails due to disease relapse. Moreover, due to the diversity of AML cytogenetics and molecular background, there is an increasing need for targeted therapy.

A panel of inhibitors against Flt3 as well as anti-Flt3 antibodies have been developed and are at present in different phases of clinical trials [189-192]. These are all small molecule heterocyclic compounds that competitively inhibits ATP binding to Flt3, but are not considered very specific. All reduce the cell proliferation rate by upregulating pro-apoptotic proteins but have had little effect in clinical studies. The therapeutic responses are usually modest with a transient reduction of peripheral blasts, and clinical trials are currently focused on combining Flt3 inhibitors with conventional chemotherapy. Most of the Flt3 inhibitors are rather unspecific like the majority of kinase inhibitors in general, and often recognize targets like PDGFR, c-Kit and VEGFR. This not only disables the evaluation of Flt3 inhibition, but may cause toxic responses due to the multiple targets when used in patients. Recently, a novel type of kinase inhibitor LS104, competing out substrate binding to the receptor instead of inhibiting the ATP binding site, was shown to target Flt3 ITD and its downstream signaling in primary AML blasts [193]. There have been reports of anti-Flt3 neutralizing antibodies in leukemic mouse models with positive responses [194], and this approach is more specific and less toxic. The antibodies mainly interfered with the cell surface expressed Flt3, and may for this reason not be the best option for the ITD mutant, predominantly expressed as the immature intracellular receptor.

Activating mutations in c-Kit are found in AML cases with CBF abnormalities. Imatinib, best known for its function in inhibiting Bcr-Abl in CML, also inhibits c-Kit and may therefore represent a potential drug in CBF leukemias where c-Kit correlates to a worse prognosis [195]. However, in the case of CML, point mutations in the kinase domain of Bcr-Abl often result in imatinib resistance [196]. Moreover, since imatinib only inhibits the non-active receptor, the constitutively activated D816 mutations of c-Kit most often found in leukemias remain unaffected. Instead, the dual Src/Abl inhibitor dasatinib, at present in clinical trials, targets the activated receptor and may be used in hematological malignancies [197]. Dasatinib affects a wide array of targets [198], possibly causing the side effects like pleural effusions and suppression of the immune system observed in CML patients treated with this inhibitor [199]. In addition, nilotinib, another drug approved in CML treatment of patients with imatinib resistance, has shown

a more specific pattern of inhibition even though both nilotinib and imatinib recently proved to act on more targets than previously believed [198]. Because mutations in c-Kit and Flt3 are rather late events in leukemogenesis, conventional chemotherapy is still necessary but the response is likely to be enhanced when combined with a tyrosine kinase inhibitor.

In CML, oncogenic Bcr-Abl is associated with Stat5 activation leading to elevated levels of RAD51 [200]. Overexpression of RAD51 results in increased genomic instability associated with increased resistance (reviewed in [201]). It has been suggested that patients positive for Flt3 ITD have an increased risk of relapse (233), and considering the unique ability of the ITD receptor to activate Stat5, as similar mechanism as in CML might in part explain this. Indeed, levels of RAD51 have been positively correlated to Stat5 activation in Flt3 ITD expressing Ba/F3 cells, and the use of the Flt3 inhibitor PKC412 reduced the expression of the RAD51 transcript [202]. In a gene expression study of relapsed AML patients, the leukemic blasts showed an increased proliferation due to enhanced activation of Raf, Mek and Erk, most likely caused by upstream oncogenic events [203]. It has further been demonstrated that Flt3 ITD positive patients also lacking the Flt3 wild-type (wt) allele have a significantly shortened survival when compared to those with Flt3 ITD/wt or wt alone. These patients were found in about 35% of the cases with a Flt3 ITD allele [204]. One theory is that the presence of the wild-type Flt3 compensates for some of the oncogenic effects of the ITD receptor.

Conclusion

Detailed studies on the site-specific phosphorylation of RTKs like c-Kit and Flt3 are clinically relevant for diagnosis and potential treatment. The phosphorylation status of particularly important residues for the oncogenic signaling will provide information about which downstream proteins are activated and facilitate targeted therapy. The more details that can be worked out about these signaling networks, the more specific and efficient can the future cancer therapy potentially be.

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Figure legends

Table 1. Signaling molecules known to directly bind to the respective

phosphorylation sites of c-Kit. Question mark denotes unknown binding partner.

SFK: Src family kinase; p85 α : p85 α subunit of PI3-kinase; PLC- γ 2: phospholipase C- γ 2.

Table 2. Signaling molecules known to directly bind to the respective

phosphorylation sites of Flt3. Question mark denotes unknown binding partner. SFK:

Src family kinase.

Fig. 1 Signal transduction pathways downstream of c-Kit

SCF: stem cell factor; SFK: Src family kinase

Fig. 2. Signal transduction pathways downstream of Flt3

FL: Flt3 ligand; SFK: Src family kinase

Tyrosine- residue	568	570	703	721	730	823	900	936
Docking protein	SFKs, APS, SHP2, Cbl, Lnk, SOCS6 CHK	SFKs, SHP1	Grb2	p85α	Plcy-2 (?)	?	p85α (Crk)	Grb2, APS, Cbl, Grb7

Table 1

Tyrosine residue	572	589	591	599	726	768	793	842	955	969
Docking protein	?	SFKs (Sta5)	SFKs (Sta5)	SFKs SHP2	?	Grb2	?	?	Grb2	Grb2

Table 2



