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Review article

The role of SRC family kinases in FLT3 signaling

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ABSTRACT

The receptor tyrosine kinase FLT3 is expressed almost exclusively in the hematopoietic compartment. Binding of its ligand, FLT3 ligand (FL), induces dimerization and activation of its intrinsic tyrosine kinase activity. This leads to autophosphorylation of FLT3 on several tyrosine residues which constitute high affinity binding sites for signal transduction molecules. Recruitment of these signal transduction molecules to FLT3 leads to the activation of several signal transduction pathways that regulate cell survival, cell proliferation and differentiation. Oncogenic, constitutively active mutants of FLT3 are known to be expressed in acute myeloid leukemia and to correlate with poor prognosis. Activation of the receptor mediates cell survival, cell proliferation and differentiation of cells. Several of the signal transduction pathways downstream of FLT3 have been shown to include various members of the SRC family of kinases (SFKs). They are involved in regulating the activity of RAS/ERK pathways through the scaffolding protein GAB2 and the adaptor protein SHC. They are also involved in negative regulation of signaling through phosphorylation of the ubiquitin E3 ligase CBL. Initially studied as the SFKs, as if they were a homogenous group of kinases, recent data suggest that each SFK has its own specific signaling capabilities where some are involved in positive signaling, while others are involved in negative signaling. This review discusses some recent insights into how SFKs are involved in FLT3 signaling.

1. Introduction

The mammalian genome contains 56 receptor tyrosine kinases (RTKs) which can be subdivided into 20 different families (Blume-Jensen and Hunter, 2001; Kabir and Kazi, 2011; Kazi et al., 2008; Kreitman et al., 2018). The type III family of RTKs include the platelet-derived growth factor receptors (PDGFRA and PDGFRB), the stem cell factor receptor (SCFR or KIT), the colony stimulating factor 1 receptor (CSF1R) and FMS-like tyrosine kinase 3 (FLT3). FLT3 is predominantly expressed in hematopoietic progenitor cells of both myeloid and lymphoid lineage. Mutations in FLT3 are very common in AML and are found in 25–35 % of patients with acute myeloid leukemia (AML).

Under normal conditions, receptor tyrosine kinase activation is mediated through dimerization of receptors (Lemmon and Schlessinger, 2010). In most cases, dimerization is initiated through binding of the specific ligand to the receptor. Although homo-dimerization is the most common form of dimerization, hetero-dimerization also occurs in some receptor systems (Weiss and Schlessinger, 1998). The activation mechanism of the type III receptor tyrosine kinases has been extensively studied (Lennartsson and Rönnstrand, 2012). Although ligand-induced dimerization is generally viewed as the mechanism of activation of RTKs, there are some reports of pre-formed dimers that become activated upon ligand binding, where the activation occurs through ligand-induced rotation of the pre-existing receptor dimers (Moriki et al., 2001). This also seems to be the case of the insulin receptor where the receptor exists as a preformed heterotetramer and ligand binding induces conformational changes other than dimerization that causes activation of the receptor (Lee et al., 2014).

After translation FLT3 is transported to the plasma membrane. Monomeric receptors are inactive until the extracellular domain interacts with the ligand. The inactive state is maintained by the interactions between the juxtamembrane (JM) domain and the kinase domain. For this reason, mutations that lead to deletion or interference with the JM domain result in constitutive activation of the receptor (Kiyoi et al., 2002). Structural data show that the interaction between the JM domain and the kinase domain blocks the ATP binding site (Griffith et al., 2004). This mechanism of activation is quite distinct form the mechanisms of many other RTKs. In many cases, the activation of RTKs involves the phosphorylation of specific residues in the activation loop, which leads to conformational changes and activation of the kinase domain. However, in the type III RTKs, the mechanism of activation is different. Mutation of the activation loop tyrosine in FLT3 does not lead

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to defects in kinase activity, but instead affects downstream signaling (Kazi et al., 2017). Non-covalent ligand dimers bind a pair of receptors which is mediated through a single interaction site in the third Ig-like domain (D3) (Verstraete et al., 2011). The ligand binds with high affinity to FLT3, which in part is explained by the dimeric binding that further enhances the strength of interaction. The binding affinity (K_d) of FL for FLT3 has been estimated to between 200 to 500 pmol/L using human myeloid leukemia cells (Turner et al., 1996). The mode by which the ligand-receptor interaction occurs in FLT3 is different from all other RTKs, in that it lacks homotypic receptor interactions (Verstraete et al., 2011). Other type III receptor tyrosine kinases, such as CSF1R (Chen et al., 2008), PDGFR (Yang et al., 2008) and KIT (Yuzawa et al., 2007) use homotypic receptor interactions, which play important roles in receptor activation. While Ig-like domains D4 and D5 contribute to the homotypic interactions in other type III RTKs, FLT3 D4-D5 lacks the conserved set of residues required for homotypic interactions and no interactions between the FLT3 D4-D5 domains occur (Verstraete et al., 2011). Therefore, it is likely that FLT3 extracellular domain only plays a role in ligand binding and that the homo-dimerization is brought about through some other mechanisms.

Ligand binding leads to rapid activation of FLT3 that can be observed *in vitro* within a minute after ligand stimulation (Fig. 1). Ligand binding induces significant structural changes in the intracellular domain but it is not known whether it is the ligand-induced dimers that leads to activation or preformed dimers that undergo conformational changes upon ligand binding. The autoinhibitory juxtamembrane domain is released from the kinase domain, and thereby making it accessible to ATP. These changes initiate autophosphorylation of several tyrosine residues. In contrast to many other RTKs, phosphorylation of the activation loop tyrosine, Y842, is not required for kinase activity, but is rather involved in signaling events by recruiting the protein tyrosine phosphatase SHP2 (Kazi et al., 2017).

As described above, following ligand stimulation more than 10 tyrosine residues in the intracellular part of the receptor are phosphorylated. The kinetics of tyrosine phosphorylation has not been thoroughly studied. One study suggests a difference in the kinetics of phosphorylation of different tyrosine residues in wild-type and mutant receptors (Razumovskaya et al., 2009) However, we still don't know whether phosphorylation is an ordered event (as is the case for the closely related RTK KIT), or whether some phosphorylation sites are required to prime others for phosphorylation. In any case, we know that phosphorylation of several residues including Y589, Y591 and Y599 are critical for FLT3-mediated survival signaling (Vempati et al., 2008). The structural data and data from studies on KIT suggests the possibility that tyrosine residues in the juxtamembrane domain are phosphorylated first (DiNitto et al., 2010; Griffith et al., 2004; Mol et al., 2003). Phosphorylation of the juxtamembrane tyrosine residues in KIT leads to release of the juxtamembrane domain from the kinase domain, which releases the negative regulatory constraint it poses on the kinase activity (Mol et al., 2004, 2003). It is very likely that the scenario is similar in the case of FLT3, given their close similarity.

2. FLT3 signaling through SFKs

The SRC family of non-receptor tyrosine kinases (SFKs) include 11 protein kinases. While eight members, including BLK, FGR, FYN, HCK, LCK, LYN, SRC and YES, are considered as the core SFKs, the other three members, PTK6 (BRK), FRK and SRMS, are known as SFK-related kinases (Ingley, 2008). Expression of FYN, SRC and YES is ubiquitous but the other isoforms display a more restricted expression (Parsons and Parsons, 2004). All members carry a common domain structure: a unique domain (SH4 domain), a SRC-homology 3 (SH3) domain, a SRC-homology 2 (SH2) domain and a kinase domain (originally denoted SH1 domain) (Resh, 1993). Except SRMS, the SFK members have a C-terminal region involved in negative regulation of kinase activity where a conserved residue, corresponding to Y527 in chicken SRC, is present. This residue is phosphorylated C-terminal SRC kinase (CSK) and CSK-homologous kinase (CHK; reviewed in (Ia et al., 2010)). Phosphorylated Y527 binds to the SH2 domain in an intramolecular interaction which is strengthened by interaction between the SH2 domain and proline-rich

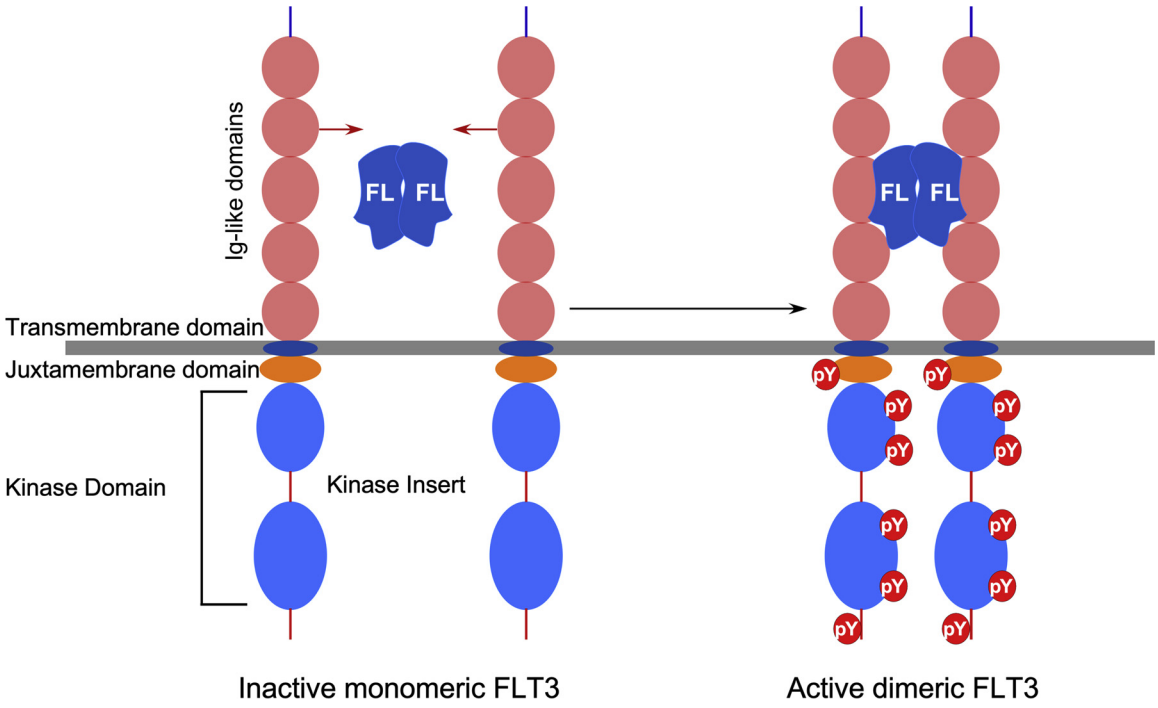

Fig. 1. FLT3 consists of five extracellular Ig-like domains, a transmembrane domain, the juxtamembrane domain followed by a bipartite kinase domain (interrupted by the so-called kinase insert) followed by the carboxyterminal tail. Binding of FL to FLT3 induces dimerization of receptors, a conformational change of the kinase domain which leads to its activation. This is followed by phosphorylation of FLT3 at numerous tyrosine residues that constitute high affinity binding sites for signal transduction molecules.

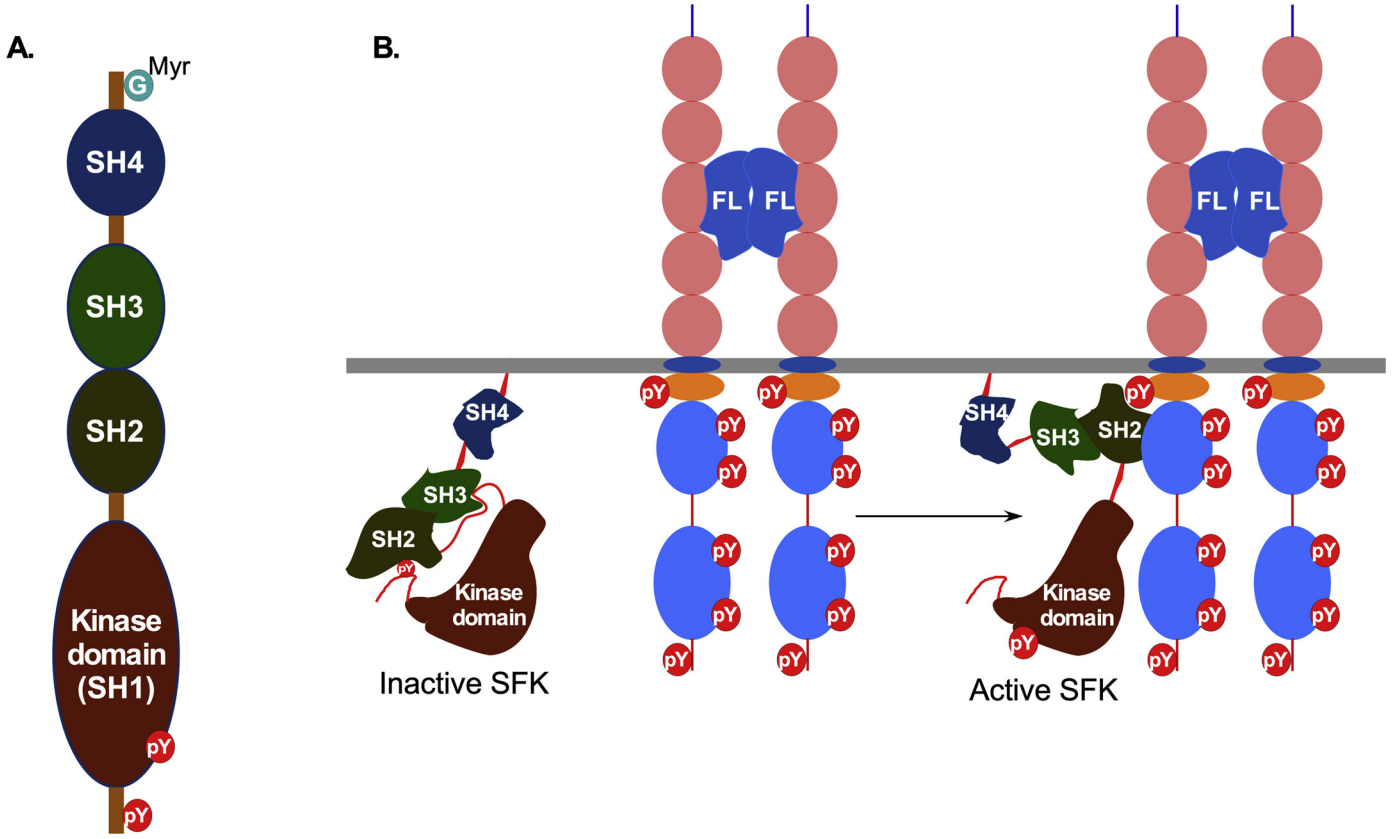

Fig. 2. A: SRC family kinases have a common structure in that they at their aminoterminus carry a myristic acid moiety that anchors the kinase to the inside of the plasma membrane. The SH4 domain, also called the unique domain, followed by the SH3 domain (which binds to proline-rich regions I proteins), the SH3 domain that binds to phosphotyrosine residues, followed by the kinase domain. The kinase domain carries an autophosphorylation site, Y416, in the activation loop that is involved in regulation of kinase activity. In the carboxyterminus Y527 is located, which is phosphorylated by CSK and which is a causes negative regulation of SFKs. B: upon binding to e. g. a receptor tyrosine kinase, the SH2 domain is released from Y527, which leads to opening up of the kinase structure, and its phosphorylation on Y416 which leads to full activation of SFKs.

sequences and keeps SFKs inactive. Activation of SFKs can be brought about in different ways. Binding of the SFK SH2 to other tyrosine phosphorylated proteins leads to release of the kinase domain and activation of SFKs (Fig. 2). Another way of activation is through dephosphorylation of Y527, which leads to release of the SFK SH2 domain and activation of the kinase. This leads to phosphorylation of SFKs at the Y416 position (chicken sequence). Several protein tyrosine phosphatases are known to be able to dephosphorylate Y527 including proline- enriched tyrosine phosphatase (PEP or PTPN22), T-cell protein tyrosine phosphatase (TCPTP or PTPN2) tandem SH2 domain-containing protein tyrosine phosphatase (SHP1 or PTPN6) and transmembrane receptor-like tyrosine phosphatase (PTPRC or CD45) (Reviewed in (Ingley, 2008)).

SFKs regulates several signaling pathways downstream of FLT3. Several SFKs, including FYN, HCK, LCK, LYN, FGR and SRC, have been shown to interact with and to be activated by FLT3. FGR, HCK, LYN and SRC interacts with FLT3 through Y589 and Y599 (Heiss et al., 2006). Thus, in contrast to the other type III RTKs, FLT3 does not only have one tyrosine residue involved in SFK binding, but two. The tyrosine residues corresponding to Y599 in FLT3 have not been demonstrated to be phosphorylated in either the PDGF receptors, KIT or the CSF1 receptor.

Mutation of Y589 F or Y599 F in FLT3 led to reduced tyrosine phosphorylation of CBL, a well-known SRC substrate (Heiss et al., 2006). These observations suggest that FLT3 activates SFKs which in turn activates downstream signaling cascades. Inhibition of SFKs by the inhibitors PP1 and PP2 induced growth inhibitory effects (Okamoto et al., 2007; Robinson et al., 2005) but did not block ERK activation (Heiss et al., 2006) suggesting that SFKs are not involved in FLT3-induced MAPK signaling. Conversely, PP1 and PP2 enhanced FLT3-induced ERK activation (Heiss et al., 2006). However, the interpretation of these data is complicated. Since FLT3-induced activation SFKs leads to tyrosine phosphorylation and activation of the ubiquitin E3-ligase activity of CBL, it will lead to ubiquitination of FLT3 and its subsequent degradation in the proteasomes. Therefore, PP1 and PP2-mediated inhibition of SFKs stabilizes FLT3 and probably enhances downstream MAPK activity (Heiss et al., 2006). Although SFKs have been demonstrated to contribute to MAPK signaling (Jurek et al., 2009), the role of SFKs might be more complex than initially assumed. SFKs have historically many times been studied as a group without distinguishing between the different family members. It has become increasingly clear that the different family members have distinct functions. Some are involved in positive signals, while others are more related to negative signaling. Overexpression of several SFKs such as FYN or LCK did not alter either wild-type or FLT3-ITD induced ERK1/2 and p38 activation (Chougule et al., 2016; Marhäll et al., 2017). Additionally, AKT activation was also unchanged in cells overexpressing either FYN or LCK but in both cases FLT3-ITD-induced STAT5 signaling was elevated (Chougule et al., 2016; Marhäll et al., 2017). Similarly, SRC also activates FLT3-ITD induced STAT5 signaling (Leischner et al., 2012), demonstrating the selectivity of SFKs to distinct FLT3 signaling cascade.

3. LYN

Expression of LYN is quite widespread but high levels of expression is predominantly found in the hematopoietic compartment. Its role in B

cell biology has been thoroughly investigated, where it has a role both in positive and negative signaling in a context-dependent manner (for review, see (Xu et al., 2005)). However, LYN is also activated by a number of RTKs, including the CSF1 receptor, KIT and FLT3 (reviewed in (Hibbs and Harder, 2006)). LYN is the predominantly expressed SFK in the murine myeloid cell 32D and it is also the predominantly expressed SFK in patient-derived AML cell lines (Roginskaya et al., 1999). Ligand-stimulation of FLT3 expressed in the myeloid cell line 32D or in the lymphocytic cell line Ba/F3 leads to induction of phosphorylation of LYN (Robinson et al., 2005; Heiss et al., 2006). LYN binds to Y589 in FLT3 (Heiss et al., 2006) and mutation of Y589 to phenylalanine leads to dramatically reduced ligand-induced activation of LYN. Furthermore, LYN is constitutively phosphorylated in the human AML cell line MV4-11, expressing FLT3-ITD (Robinson et al., 2005). It has also been shown that FLT3-ITD binds stronger to LYN that wild-type FLT3 (Okamoto et al., 2007). Although SFKs have been demonstrated to be linked to be linked to phosphorylation and activation of the ubiquitin E3 ligase CBL (Tanaka et al., 1996; Blake et al., 2000), there are conflicting data on whether inhibition of SFKs affects FLT3 stability. While Robinson and co-workers didn't see any effect of inhibition of SFKs with the small drug PP1 on FLT3 protein stability (Robinson et al., 2005), such an effect was described by Heiss and coworkers (Heiss et al., 2006). One possible explanation to the discrepancy is that two different hematopoietic cell lines were used in the studies, that have different patterns of expression of SFKs. In general, too few studies have been performed using selective inhibitors of SFKs, but rather used pan-SFK inhibitors such as PP1 and PP2. In a more thorough study, Okamoto et al. used shRNA to selectively silence the expression of LYN in 32D cells (Okamoto et al., 2007). They also demonstrated that knockdown of LYN partially reduced the phosphorylation of STAT5 and could demonstrate that treatment of mice with the SFK inhibitor PP2 blocked the onset of tumors and decreased the size of established tumors in a mouse transplant model.

4. FYN

Under normal physiological conditions, FYN is a SFK with important functions in the nervous system and in T cell biology (reviewed in (Elias and Ditzel, 2015)). It has also been described as an important kinase in various forms of cancer and in the development of resistance to targeted therapies. In chronic myeloid leukemia, resistance to various targeted inhibitors, including Gleevec, involves an upregulation of FYN expression (Grosso et al., 2009). Chougule and co-workers investigated the expression of SFKs in AML patient samples and correlated it to patient survival (Chougule et al., 2016). It was found that high expression of FYN correlated with poor survival, in particular the group of patients carrying the oncogenic mutation FLT3-ITD. FYN was found to associate with wild-type FLT3 in a ligand-dependent manner, while it was constitutively associated with FLT3-ITD. Overexpression of FYN in wild-type FLT3 expressing Ba/F3 cells did not influence FLT3 protein stability, and led to a minor increase in ERK, AKT and p38 phosphorylation. In contrast, in cells expressing FLT3-ITD, overexpression of FYN caused an increase in the phosphorylation of STAT5, an important mediator of FLT3-ITD transformation.

It could also be shown that AML cells that expressed high levels of FYN also showed an enrichment of the STAT5 signaling pathway (gene set enrichment analysis). FYN expression did not markedly influence cell survival but increased the capacity of FLT3-ITD expressing cells to form colonies in semi-solid medium. Taken together, these data point towards a positive role of FYN in FLT3-ITD-mediated transformation.

5. HCK

HCK is an SFK mainly expressed in the hematopoietic compartment. Knockout mice lacking HCK expression display a relatively mild phenotype, with reduced phagocytic ability of the macrophages. However, the combined knockout of HCK and FGR display impairment of myeloid cell degranulation and migration, which results in increased sensitivity to infections.

HCK associates with phosphorylated Y589 and Y591 in the juxtamembrane region of FLT3 (Heiss et al., 2006; Mitina et al., 2007). HCK, as well as LYN and to a less extent FYN, are capable of phosphorylating FLT3 on tyrosine residues in the juxtamembrane region and could thereby contribute to FLT3 signaling. However, one should keep in mind that those experiments were performed on HEK293 cells that strongly overexpress these kinases, and it remains to be shown whether it also takes place in cells expressing physiological levels of the kinases.

HCK was shown to be involved in FLT3-ITD-dependent upregulation of CDK6 in AML cells (Lopez et al., 2016). The upregulation of CDK6 by HCK is independent of STAT5 and essential for the transforming capacity of FLT3-ITD. Recently dual specificity inhibitors of FLT3 and HCK were developed (Koda et al., 2017). Given the importance of both HCK and FLT3 in leukemogenesis, it will be very interesting to see the effects of these inhibitors in patients suffering from FLT3-ITD positive AML.

6. LCK

Lymphocyte-specific protein tyrosine kinase, LCK, has important roles in T cell development, homeostasis and activation (reviewed in (Alarcon and van Santen, 2010)). Mice with a targeted deletion of LCK show a strong decline in the number of CD4 and CD8 positive thymocyte populations and have low numbers of peripheral T cells (Molina et al., 1992). Apart from the T cells, LCK is also expressed in certain populations of B cells (Majolini et al., 1998). LCK is highly expressed in B and T cell leukemias and contributes to transformation (Von Knethen et al., 1997). Despite being of non-lymphocytic origin, there are several reports about a role of LCK in AML. One study demonstrated higher expression of LCK in less differentiated cases of AML (Rouer et al., 1994). Another study showed a correlation between high expression of LCK and good response to a PI3K/mTOR-specific inhibitor (Casado et al., 2013). Overexpression of LCK in Ba/F3 cells stably transfected with FLT3-ITD did not influence viability of cells (Marhäll et al., 2017), but increases their capacity form colonies in semi-solid medium. In a xenograft model, cells overexpressing LCK formed larger tumors than control cells, which also stained stronger for Ki67, a marker for proliferation. Phosphorylation of STAT5 was increased in cells overexpressing LCK, while the phosphorylation or ERK, AKT and p38 was unchanged. Finally, the stability of FLT3 was not influenced by LCK.

7. FGR

Mice lacking expression of FGR show a very mild phenotype, just like mice lacking HCK. However, the combined deletion of both FGR and HCK leads to defects in neutrophil function (Lowell et al., 1996); and resistance to endotoxic shock (Lowell and Berton, 1998). FGR is highly expressed in human AML cells (Chougule et al., 2016). The exact role of FGR in relation to FLT3-ITD-driven AML is not yet known. Recently a FGR selective inhibitor was developed and evaluated in a clinical trial of AML patients (Weir et al., 2018). The best responding patients were all FLT3-ITD negative, which suggests that FGR might not be positively contributing to FLT3-ITD signaling.

8. SRC-like adapter proteins

The SRC-like adapter proteins (SLAP and SLAP2) share sequence similarities with SRC but lack catalytic activity (reviewed in (Kazi et al., 2015)). Like SRC, SLAP contains an SH3 domain followed by an SH2 domain, but there is no kinase domain. These adapter proteins are expressed in a variety of cell types. They are involved in signaling through the B cell receptor, the T cell receptor, cytokine receptors as well as receptor tyrosine kinases, including FLT3. Following receptor binding,

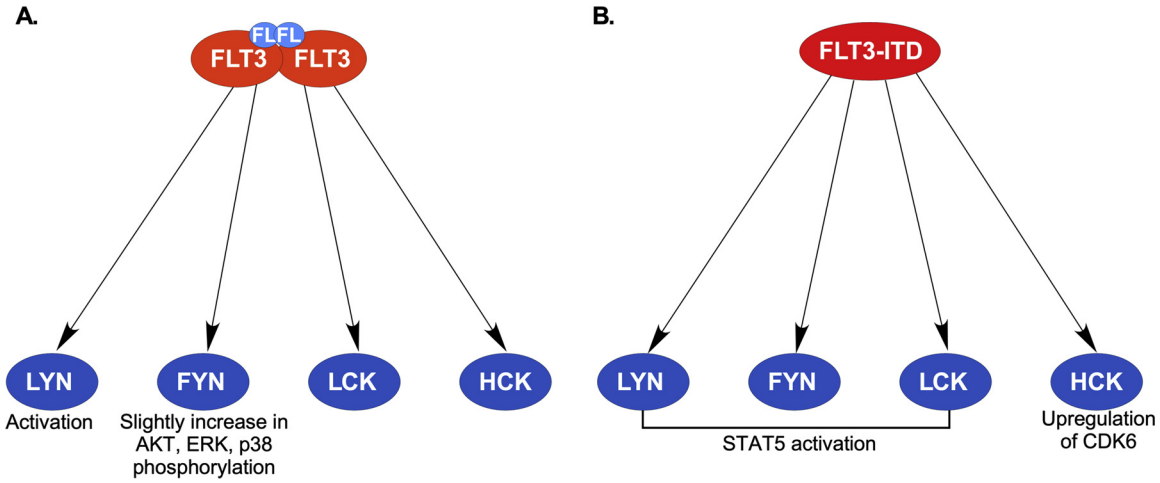

Fig. 3. wild-type FLT3 and the oncogenic mutant FLT3-ITD show specificity in which individual SFKs they activate, which leads to both isoform specific signaling outcomes, but also differences between wild-type FLT3 AND FLT3-ITD signaling.

SLAP is tyrosine phosphorylated. This creates a binding site for the ubiquitin E3 ligase CBL, which thereby is recruited and leads to ubiquitination-dependent degradation of the receptor. We could demonstrate that SLAP binds to FLT3 through its SH2 domains and influence receptor stability as well as downstream signaling (Kazi and Rönnstrand, 2012). shRNA-mediated depletion of SLAP in FLT3 expressing Ba/F3 cells led to weaker activation of FL-induced PI3-kinase/AKT signaling as well as weaker RAS/ERK signaling. Finally, the presence of FLT3-ITD mutation in AML patients was correlated with a strong increase in SLAP expression.

SLAP2 is closely related to SLAP that also binds to phosphorylated FLT3 (Moharram et al., 2016) and inhibits FLT3-ITD-mediated oncogenic transformation, both as judged by colony formation in vitro and tumor formation in vivo. Bioinformatics analysis revealed that a higher expression of SLAP2 in AML patients correlated with a better prognosis. SLAP2 is similar to SLAP involved in recruitment of the ubiquitin E3 ligase CBL to FLT3.

9. Conclusions

While the SRC family kinases share overall structure, their function is more diverse than originally anticipated. While many isoforms, such as LYN and FYN are mainly contributing positive signals (activation of the RAS/ERK pathway and the PI3-kinase/AKT pathway, others like FGR seem to signal through the ubiquitination machinery, thereby regulating the stability of proteins, e. g. FLT3. Furthermore, there are differences between how wild-type FLT3 signals through SFKs compared to how FLT3-ITD signals (Fig. 3). It should be noted that there are no data on whether LCK or HCK contribute to wild-type FLT3 signaling. There are several SFK inhibitors on the market for the treatment of patients with diverse cancers but given the both positive and negative roles of individual SFKs, more selective SFK inhibitors are likely to be more successful in treatment of leukemia patients.

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