Translational studies on acute myeloid leukemia focus on the receptor tyrosine kinase FLT3

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UNIVERSITY

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Abstract

Among adults, acute myeloid leukemia (AML) is the second most frequent type of leukemia. In spite of recent improvements in the treatment of this disease, a majority of patients develop drug resistance, leading to poor overall survival. One of the factors behind this is mutations in the tyrosine kinase receptor (FLT3). It is one of the most commonly mutated genes and is present in almost 40% of the patients with AML. In order to deal with the resistance and improve current treatments, a better understanding of FLT3 signalling and functioning is required.

In our first study, we focused on the tyrosine residue at the 842 position of FLT3. We demonstrated that Y842 is not involved in FLT3 activation or ubiquitination, but is important in regulating downstream signalling via RAS/ERK pathway, as well as controlling receptor stability.

The following study's purpose was to understand if it was the location or the nature of the FLT3 mutation that played the larger role in leukemogenesis. Therefore, we looked at how ITD mutations in the juxtamembrane domain (JMD) compared with those in the tyrosine kinase domain (TKD), as well as with the point mutation in the tyrosine kinase domain, D835Y. The cells with TKD-ITD were able to activate the STAT5 pathway and had a higher cell proliferation and survival than cells expressing D835Y. Overall, TKD-ITD and JMD-ITD showed similar oncogenic potential, but still had a higher oncogenic potential than the D835Y point mutation.

In the third and fourth paper we focused on the associating proteins, since tyrosine kinase receptors signal with the help of those in order to activate downstream signalling. First, with the help of an SH2 domain array, we identified ABL2 as a potent interacting partner of FLT3. It turned out that ABL2 expression did not change the kinase activity of FLT3, its stability or ubiquitination. However, ABL2 acted as a negative regulator of downstream signalling of FLT3 via partial inhibition of AKT pathway. Another interacting partner of FLT3 was identified to be LCK, and its role was studied in paper four. In Ba/F3 cells the expression of LCK enhanced the FLT3-ITD STAT5 phosphorylation. We also observed that those cells exhibited a higher capacity to form colonies. Furthermore*, in vivo* these cells developed tumours faster than the control. In conclusion, our data show that LCK works together with the oncogenic FLT3-ITD in cellular transformation.

Key words: AML, signalling, FLT3 receptor, cancer

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The tree of signalling.

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MADE IN SWEDEN

To my beloved

Table of Contents

Abstract

Among adults, acute myeloid leukemia (AML) is the second most frequent type of leukemia. In spite of recent improvements in the treatment of this disease, a majority of patients develop drug resistance, leading to poor overall survival. One of the factors behind this is mutations in the tyrosine kinase receptor (FLT3). It is one of the most commonly mutated genes and is present in almost 40% of the patients with AML. In order to deal with the resistance and improve current treatments, a better understanding of FLT3 signalling and functioning is required.
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List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. *Tyrosine 842 in the activation loop is required for full transformation by the oncogenic mutant FLT3-ITD*
Julhash U. Kazi, Rohit A. Chougule, Tianfeng Li, Xianwei Su, Sausan A. Moharram, Kaja Rupar, **Alissa Marhäll**, Mohiuddin Gazi, Jianmin Sun, Hui Zhao, Lars Rönnstrand
Cell Mol Life Sci. 2017; 74(14): 2679–2688.

II. *Internal tandem duplication mutations in the tyrosine kinase domain of FLT3 display a higher oncogenic potential than the activation loop D835Y mutation*
Alissa Marhäll, Heidel F, Fischer T, Lars Rönnstrand
Annals of hematology. 2018; Epub DOI: 10.1007/s00277-018-3245-5.

III. *ABL2 suppresses FLT3-ITD-induced cell proliferation through negative regulation of AKT signaling*
Julhash U. Kazi, Kaja Rupar, **Alissa Marhäll**, Sausan A. Moharram, Fatima Khanum, Kinjal Shah, Mohiuddin Gazi, Sachin Raj M. Nagaraj, Jianmin Sun, Rohit A. Chougule, Lars Rönnstrand
Oncotarget. 2017; 8(7): 12194–12202.

IV. *The Src family kinase LCK cooperates with oncogenic FLT3/ITD in cellular transformation*
Alissa Marhäll, Julhash U. Kazi, Lars Rönnstrand
Scientific reports. 2017; 7(1): 13734.

List of papers not included in thesis

1. *The dual specificity PI3K/mTOR inhibitor PKI-587 displays efficacy against T-cell acute lymphoblastic leukemia (T-ALL)*
   Mohiuddin Gazi,Sausan A. Moharram, **Alissa Marhäll**, Julhash U. Kazi
   Cancer Letters. 2017; 392: 9-16

2. *Efficacy of the CDK inhibitor dinaciclib in vitro and in vivo in T-cell acute lymphoblastic leukemia*
   Sausan A. Moharram, Kinjal Shah, Fatima Khanum, **Alissa Marhäll**, Mohiuddin Gazi, Julhash U. Kazi
   Cancer letters 2017; 405: 73-78

List of Abbreviations

ABL2	Abelson tyrosine-protein kinase 2
AML	Acute Myeloid Leukemia
ANG1	Angiopoietin 1
BCR	Breakpoint Cluster Region Protein
CEBPA	CCAAT/Enhancer Binding Protein Alpha
CXCL12	C-X-C motif chemokine 12
DNMT3A	DNA Methyltransferase 3 Alpha
ERK	Extracellular Signal-regulated Kinase
FL	FLT3 ligand
FLT3	Fms-like Tyrosine Kinase 3
HSC	Hematopoietic Stem Cell
ITD	Internal Tandem Duplication
JMD	Juxtamembrane Domain
KIT	KIT Proto-Oncogene Receptor Tyrosine Kinase
LCK	Lymphocyte Cell-Specific Protein-Tyrosine Kinase
NRAS	Neuroblastoma RAS viral oncogene homolog
PI3K	Phosphoinositide 3 Kinase
RTK	Receptor Tyrosine Kinase
RUNX1	Runt Related Transcription Factor 1
SCF	Stem Cell Factor
SFK	Src Family Kinase
SH2	Src Homology 2
SHP2	Src Homology 2 containing Phosphatase 2

STAT5	Signal Transducer and Activator of Transcription 5
TET2	Tet Methylcytosine Dioxygenase 2
TGF-β	Transforming growth factor beta
TKD	Tyrosine Kinase Domain
TP53	Tumour Protein P53
TPO	Thrombopoietin
WT	Wild Type

Introduction

Hematopoiesis

Hematopoiesis is a process of blood formation that occurs during embryonic development and throughout the life. It is required in order to produce and replenish blood cells. In the healthy adult we have approximately 5 litres of blood. Each day the body produces 2.5 billion red blood cells, 2.5 billion platelets and 10 billion granulocytes per kilogram of body weight [1]. Since mature blood cells are predominantly short-lived, it requires hematopoietic stem cells (HSCs) to replenish the blood cells of different lineages. In contrast to continuous production of blood cells every day, HSCs only divide once every 40 weeks and during other times stay in a quiescent state [2, 3]. HSCs reside as rare cells in the bone marrow, featuring self-renewal ability and giving rise to progenitors that become restricted to several or single lineages [4]. In turn these progenitors give rise to the precursors devoted to differentiation and production of mature cells of different lineages: the erythroid (red blood cells), the lymphoid (B-, T- and natural killer cells), the myeloid (granulocytes, megakaryocytes and monocytes/macrophages) and dendritic cells [2, 4]. The support for HSCs is provided by the bone marrow microenvironment, which is composed of fibroblasts, mesenchymal stem cells, osteoblasts, adipocytes, endothelial cells, macrophages and glial cells. The cells of the microenvironment prevent stem cells from depletion and therefore protect it from excessive proliferation [5]. The homeostasis of HSCs depends on the molecular crosstalk between the cells of the microenvironment and HSCs. The osteoblasts secrete stem cell factor (SCF), C-X-C motif chemokine 12 (CXCL12), thrombopoietin (TPO) and angiopoietin 1 (ANG1) [6, 7]. SCF is a growth factor that binds the receptor tyrosine kinase KIT. HSCs, at all stages of development, express KIT [8]. Most CD34+ cells in the bone marrow express KIT. More committed progenitors including myeloid, erythroid, megakaryocytic, natural killer and dendritic cells, pro-B and T cells, as well as mature mast cells also express KIT [9]. Ligand binding to KIT leads to homodimerization of the receptor, activation of its intrinsic kinase activity and autophosphorylation of several tyrosine residues. This activation triggers multiple signalling cascades, including RAS/ERK, PI3K/AKT and JAK/STAT pathways [10]. Mice lacking expression of functional SCF or KIT die in the perinatal periods due to severe macrocytic

anaemia [11]. The chemokine CXCL12 is involved in regulating migration of the hematopoietic precursors. TPO with the combination of IL3 or SCF increases proliferation and survival of CD34+ hematopoietic progenitor cells *in vitro* [12]. It also plays a role in maintenance of HSCs, as inherited forms of amegakaryocytic thrombocytopenia often develop as a result of thrombopoietin receptor mutations leading to a reduced pool of HSCs [13]. ANG1 regulates quiescence of the HSCs via interaction with Tie-2 [14]. The nonmyelinating Schwann cells, a specific type of glial cells, is a major source of active transforming growth factor β (TGF-β) in the bone marrow [15]. TGF-β plays an important role as a regulator by inducing HSC quiescence *in vitro* and maintains HSC hibernation by regulating activation of latent TGF-β [15, 16]. The latent form of TGF-β is produced by other cells of the microenvironment.

The self-renewal of HSCs is regulated by STAT5 depending on its level of expression [17]. However, constitutive activation of STAT5 has been associated with lymphoma and leukemia [18]. Intermediate levels of STAT5 induce proliferation of HSC and CD34+ cells, while high levels of STAT5 block myelopoiesis and promote differentiation toward erythroid cells [19]. STAT5 also regulates the transcription factor CCAAT enhancer binding protein-α (C-EBPα), which in immature HSCs is expressed at low levels and is upregulated upon differentiation to more committed common myeloid progenitors and granulocyte monocyte progenitors [20]. Activated STAT5 in CD34+ cells has been shown to downregulate C-EBPα, and the effects of constitutively active STAT5 on self-renewal can be impaired by the re-expression of C-EBPα [21]. Therefore, it is expected to observe disrupted C-EBPα in acute myeloid leukemia (AML) [22]. Other pathways like NOTCH are also important in the regulation of hematopoiesis. It plays a role in cell fate determination, and constitutive activation of NOTCH1 enhances the self-renewal of HSCs. The levels of NOTCH are subsided upon differentiation of cells [23].

As mentioned above there are various cell types that contribute to the formation and maintenance of the HSC microenvironment, nevertheless the exact contribution of each of these cells has not yet been fully elucidated.

The bone marrow consists of the mixture of cells at several stages of differentiation with HSCs being a rare type. Different cell surface markers are used to isolate HSCs and progenitor cell populations. The classical surface marker is CD34+ [24]. Based on the research of other groups the CD34- is an even more primitive precursor than CD34+ [25]. Nevertheless, other markers must be used to identify the true HSCs, because based only on CD34 expression results in a heterogeneous pool of cells. The common examples of markers include CD133+, CD90+, kinase insert domain receptor + (KDR+) and CD38- [26-28]. When it

comes to identification of more differentiated progenitor cells, such as multipotent progenitors, lymphoid primed multipotential progenitors, it relies on the presence or absence of several other surface markers. FLT3 is not expressed on HSCs, whereas FLT3 expression can be observed on the multipotent progenitors [29]. The expression of FLT3 is associated with the contribution to the cell fate of multipotent progenitors as depicted in Figure 1. FLT3 signalling promotes hematopoiesis toward the lymphoid commitment from a multipotent progenitor [30]. The common myeloid progenitors also express FLT3, later on, the expression will be lost on more differentiated cells.

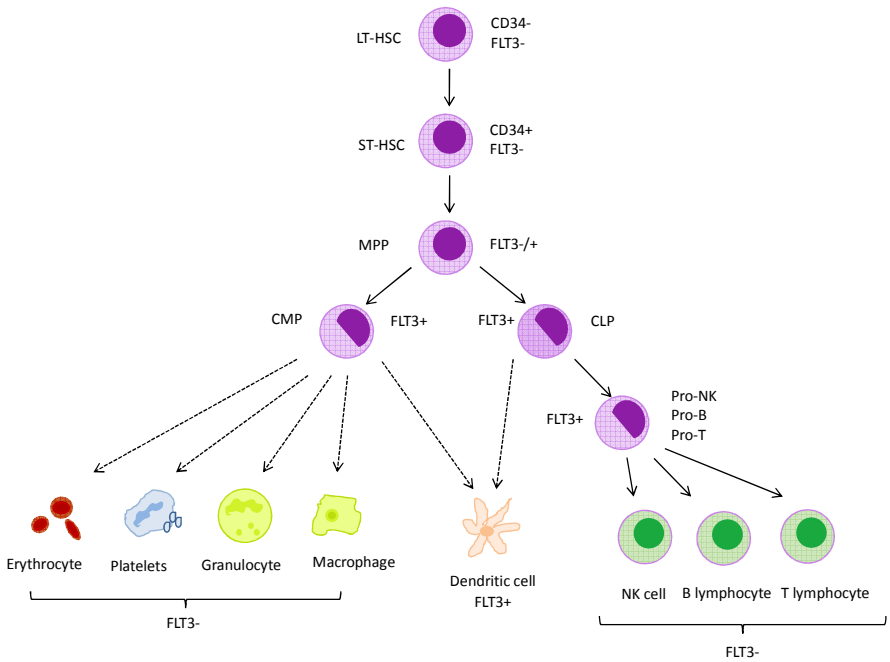

Figure 1

Schematic view of the hematopoiesis and FLT3 expression during blood cells maturation. Hematopoiesis is organized in a hierarchical manner, with rare long-term (LT) and short-term (ST) hematopoietic stem cells (HSCs), which have symmetrical division and lack FLT3 expression. They give rise to multipotent progenitor stem cells (MPPs). This cells further give rise to progenitors of two kind common lymphoid (CLP) or common myeloid (CMP), both types of progenitors express FLT3. The progenitor cells continue to proliferate extensively and finally generate mature blood cells at the bottom of the hierarchy. All mature blood cells, except dendritic cells, lose the expression of FLT3.

Due to the critical role of HSCs for development of all blood cell lineages, but also for maintenance of hematopoietic homeostasis, the dysfunction of HSCs, as well as progenitor cells, can lead to various blood disorders. The change from a normal cell to a cancer cell requires acquisition of multiple somatic mutations that collectively cause malignant transformation.

Acute Myeloid Leukemia

Every year approximately 700 new cases of leukemia are registered in Sweden. This disease can be divided into two groups: acute or chronic leukemia. It, in turn, can be subdivided into two more groups: myeloid and lymphoid depending on the origin of the affected cells.

Acute myeloid leukemia (AML) is the most common type of leukemia, with approximately 350 new cases each year in Sweden. The disease is characterized by an abnormal number of myeloid precursors with impaired differentiation and by reduced production of normal blood cells. At the beginning AML displays very diffuse symptoms; however, as the disease progresses the patient will commonly experience symptoms such as anaemia, increased bleeding and a higher risk of infections. To a large extent, AML is a disease of the elderly, mainly affecting people older than 65. Incidence increases with age [31]. Among potential risk factors for developing AML are exposure to radiation, benzene inhalation, alcohol use and pesticide exposure [32, 33]. In addition to regular risks, up to 10-15% of patients that have been exposed to cytotoxic chemotherapy as a treatment for solid tumours will develop AML [34]. If left untreated AML is fatal within months, due to the fast progression of the disease.

There has been considerable progress in the therapy of the disease over the past decades. A complete remission is achieved with the treatment in up to 70% of young patients [35-37]. Complete remission is defined as a bone marrow with < 5% blasts, a number of neutrophils > 1000 and platelets > 100000. Nevertheless, the relapse of AML patients with complete remission increases after three years, and only 40% of patients are alive after five years. In contrast to this, the survival rate for elderly patients is no more than 30% within the first year of treatment [38].

The high relapse rate of patients with AML suggests that the disease has a complex clonal diversity. Survival of some of the clones during chemotherapy, followed by their expansion, can result in relapse. In fact, AML is a highly heterogeneous disease with changes in expression of multiple genes, possible chromosome abnormalities and gene mutations. For many years, a two-hit model was an accepted hypothesis for development of AML. The model was based on the need of two different types of genetic mutations required for malignant transformation of a myeloid precursor [39]. Mutations leading to constitutive activity or impairment of downstream signalling molecules in genes such as FLT3, N-RAS, KIT, BCR-ABL were considered to be class I mutations, which promote uncontrolled proliferation and resistance to apoptosis [40]. These mutations were considered to be the later events in transformation, whereas it starts with mutations

in class II that affect transcription factors and impair genes targeting myeloid differentiation. Class II includes chromosomal abnormalities that cause fusion genes (PML-RAPA or RUNX1-RUNX1T1) and mutations in transcription factors (CEBPA or RUNX1) [39]. The support for the two-hit model was based on the observation that it is not enough with only one class mutation, therefore requiring mutations in both classes to develop AML in a mouse model [41]. Additionally, the frequency of patients with AML that have the mutations of both classes is high, thereby supporting the two hit model. Nevertheless, the two hit model is a simplistic way of looking at leukemogenesis and it does not include other types of mutations. A more complex network of other players involved in the malignant transformation is shown in Figure 2; the relative frequencies of the mutations are based on a study of 200 patients with AML [42].

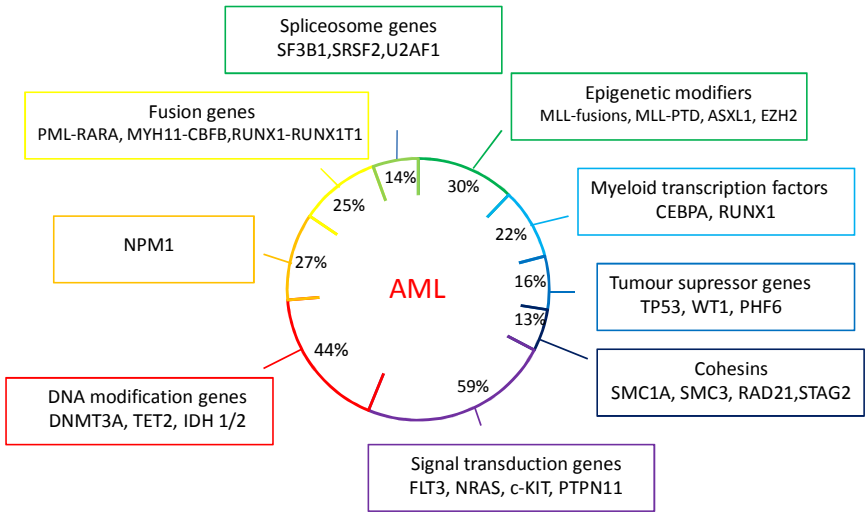

Figure 2

The chart represents the organization of different mutations. The relative frequencies of the mutations are shown based on the evaluation of 200 patients with AML. The mutations in FLT3, N-RAS, and KIT, are the most common among all the categories of mutations.

Based on this study, mutations in signal transduction genes, such as FLT3, N-RAS, and KIT, are the most common among all the categories mutations. However, in AML patients there is a large mutational heterogeneity.

Leukemogenesis therefore, is not a straightforward process, but rather a very complex network with many other driver mutations, which lead to transformation. Nevertheless, there are also studies which show FLT3 mutations alone can disrupt proliferation, differentiation and apoptosis [43-45].

AML therapy

Our understanding of AML biology has expanded in the field of genomic and molecular characterization. AML is a very heterogeneous disease; therefore it must be divided into different groups based on the biological diversity with an individualized treatment approach. The primary factors influencing the choice of treatment should be based on the molecular abnormalities, the driver mutations, pathogenic pathways, as well as the predicted response to therapy.

Prior to the choice of treatment, the diagnostic procedures take place to diagnose AML patients. Morphology tests are the first ones when a minimum of 200 leukocytes on blood smears and 500 nucleated cells on spiculated marrow smears are counted [46]. The requirement is the count of more than 20% of marrow or blood blasts. This is followed by the immunophenotyping test according to European Leukemia Network package, which examines the expression of cell surface and cytoplasmic markers [47]. Furthermore, a cytogenetic analysis that looks at the translocations, inversions and rearrangements is also included in general practice [48].

The use of molecular genetic testing that includes FLT3-ITD, CEBPA, NPM1, and KIT mutations is the current standard of care for determining prognostic subgroups. However, the prognostic significance of FLT3 TKD in AML has been debated. This controversy may be due to different frequencies of ITD and TKD, which range from 20% to 30% and from 5% to 10%, respectively.

After diagnosing AML, the treatment in younger (under 60 years old) patients is fairly standard and has not changed significantly in recent years. It involves the most common approach "3 + 7 regimen", which consists of 3 days of a short infusion of an anthracycline (idarubicin or daunorubicin) or anthracenedione (mitoxantrone), combined with 100-200 mg/m^2 of cytarabine (ara-C) as a continuous infusion for 7 days [49]. In many cases the therapy eventually fails due to relapse of the disease. In relapsed patients with AML, various chemotherapy regimens have been studied, but long-term survival has been demonstrated to be around 5 % [50]. After a successful induction therapy, in order to help prevent a relapse, young patients receive a high dose of chemo followed by either an

allogeneic or autologous stem cell transplant. There are serious complications associated with this alternative therapy; however the rate of relapse in such patients is dramatically reduced in comparison to standard chemo. A majority of older patients cannot tolerate the intensive chemotherapy and therefore represent a challenging group for finding a treatment. Currently, the available treatment is very limited and usually includes low-intensity chemotherapy and clinical trials.

Ideally, the management of each patient should completely depend on the nature of AML; it is therefore of high importance to optimally classify AML patients. The frequency of AML patients bearing FLT3-ITD mutation is high, and the mutation is associated with a higher risk of relapse and poor outcome. Therefore, in recent years there has been a great pursuit towards developing FLT3 inhibitors or compounds that structurally mimic the purine ring of adenosine and fit into the ATP-binding site of FLT3.

The first molecules tested to treat AML patients were lacking potency towards FLT3, mainly because they were general tyrosine kinase inhibitors developed to treat solid tumours. Semaxinib (SU5416), an inhibitor of KIT and VEGFR, was tested in phase II trials on AML patients, without screening the patients for FLT3 mutations [51, 52]. The results showed that the cytotoxicity caused by this drug was high and that the specificity towards FLT3 was low. Among the first generation inhibitors were drugs such as tandutinib, midostaurin (PKC412), sunitinib (SU11248), SU5614, sorafenib, and CT53518 [53-57]. These were the generation of multi-targeted receptor tyrosine kinase inhibitors leading to many additional adverse effects due to the targeting of not only FLT3, but also other members of the tyrosine kinase receptors family. Some of the drugs mentioned above were more successful than others; midostaurin in combination with chemotherapy in younger patients with FLT3 mutations did improve the overall survival [58]. In contrary sorafenib in combination with chemotherapy did not result in improvement of overall survival of the patients [59, 60]. Currently, the only FLT3 inhibitor that is approved by the FDA is midostaurin. In a phase I/II study, the combination of midostaurin and azacitidine (AZA) was tried on mostly elderly patients of which 74% harboured an FLT3 mutation, and 76% had had a relapse after previous therapy. The overall response rate was observed to be 26%, while patients carrying the FLT3 mutation had a response rate of 33% [61].

The second generation inhibitors include gilteritinib (ASP2215) towards general FLT3 mutants, and crenolanib, which is specific for both FLT3-ITD and FLT3-TKD mutants [62, 63]. Gilteritinib showed promising results in *in vivo* studies with FLT3 activity being decreased after administration of the drug with no shown toxicity effect. The regression of the tumour size and improved overall survival in xenograft mice was registered [62]. The use of crenolanib during a phase II study

on relapsed AML patients carrying FLT3 had an effect on the overall response rate, 38% in patients that had been previously treated with FLT3 inhibitors and 62% in patients that had not been treated, respectively [64].

A potent and selective inhibitor of FLT3, quizartinib (AC220), showed during phase II clinical trials a complete remission in 50 % of the patients [65]. In another phase I/II study, AC220 was used in combination with azacytidine or cytarabine for patients with relapsed AML. The overall response rate among patients carrying FLT3-ITD mutation was 73% [66]. However, the resistance to AC220 arises with time via reactivation of FLT3 and RAS/MAPK signalling, or due to secondary mutations including FLT3-TKD mutations [67].
The best way to improve the treatment of AML patients is likely a combination therapy, which can be developed through a better understanding of the mechanisms of relapse. Therefore, focus should be on both early and late steps of AML progression. The acquisition of secondary mutations that interfere with drug binding represents a problem in finding and developing drugs for treatment. Another faced challenge is that AML cell lines might show small variations in the molecular structure of the receptor, which will greatly affect the response to the inhibitor [68]. The group of patients that carry both wild type FLT3 and FLT3-ITD mutant alleles present an additional challenge because it means even worse survival and a higher risk of relapse in comparison to the patients who lost their wild type FLT3 and are left with only the FLT3-ITD mutant [69, 70].

The main focus here was FLT3 inhibitors; it is, however, worth mentioning that there are other novel therapies under investigation as a treatment for patients with AML, including new cytotoxic agents, epigenetic modulators, immunotherapy using antibodies, and mitochondrial inhibitors [71-73].

Receptor tyrosine kinases

A kinase is an enzyme that catalyses the transfer of phosphate groups from high-energy donor molecules to the target molecules. These enzymes play an essential role in most cellular processes, such as cell cycle progression, proliferation, differentiation, survival and apoptosis. All protein kinases are classified as either serine/threonine kinases or tyrosine. Serine/threonine protein kinases phosphorylate the OH group of serine or threonine, whereas tyrosine protein kinases phosphorylate tyrosine residues.

The human genome contains 90 tyrosine kinase genes, of which there are 58 receptor tyrosine kinases (RTK) and 32 non-receptor tyrosine kinases (NRTK) [74]. These 58 RTKs can be grouped into 20 subfamilies, based on kinase domain sequence, including, among others, insulin receptor, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [75]. The NRTK family includes, among others, the SRC family, Janus kinases (JAKs), and ABL.

RTKs consist of an extracellular domain that binds polypeptide ligands, a transmembrane helix, and a cytoplasmic domain that possesses tyrosine kinase catalytic activity and contains phosphorylation sites with tyrosine, serine, and threonine residues [76]. The extracellular domain contains, depending on the particular receptor, a variety of conserved elements including immunoglobulin (Ig)-like domains, fibronectin type III–like domains, cysteine-rich domains, and EGF-like domains [77]. The highest level of conservation can be observed in the organization of the cytoplasmic domain that consists of the juxtamembrane region, the catalytic tyrosine kinase and the carboxy-terminal region. Among different RTKs the length of the juxtamembrane and carboxy-terminal regions varies [77].

In the absence of ligand, a majority of RTKs are monomeric, existing as a single polypeptide chain. One of the exceptions is the insulin receptor and IGF1 receptor, which are expressed as disulfide linked $(\alpha\beta)_2$ heterotetramers on the cell surface [78]. Upon the binding of the specific ligand to the receptor, RTKs undergo dimerization or allosteric transition, resulting in the activation of the kinase activity of the receptor. Despite the similarities in the structure of RTKs, they have

different activating mechanisms of the intracellular tyrosine kinase domain. The structures of inactive tyrosine kinase domains differ substantially from receptor to receptor, whereas the active forms look very similar [79].

The autophosphorylation is triggered when the two cytoplasmic domains undergo dimerization. The phosphorylation of tyrosine residues in the activation loop causes it, in most cases, to undergo a conformational change which leads to activation of the kinase activity. The active receptor will then autophosphorylate on a number of tyrosine residues creating binding sites for SRC homology 2 (SH2) domain- and phosphotyrosine binding domain-containing proteins [80]. SH2 domains are highly conserved regions, yet different SH2 domains are associated with different binding motifs. Some of them act as adaptor proteins (GRB2, NCK, SHC, etc.) and help in activating downstream signal transduction pathways, while others contain an intrinsic enzymatic activity (PI3K, SHP2, PLC-γ, etc.).

One of the first signalling cascades triggered by RTKs to be studied is a mitogen associated protein kinase (MAPK) pathway, also called the RAS/ERK pathway. The activated RTKs cause RAS to bind GTP in place of GDP. The bound RAS-GTP activates the first serine-threonine kinase in the MAP kinase cascade, RAF, followed by the activation by phosphorylation of each of the remaining two kinases in the cascade, MEK and ERK. The last step in the pathway is the phosphorylation of the transcriptional regulators. RTKs use this and other signalling pathways to send information to the nucleus, among others the PI3K/AKT, mTOR and STAT pathways.

The protein tyrosine phosphatase SHP2 (encoded by PTPN11) contains SRC homology 2 (SH2) domains and is a positive regulator of the RAS/ERK signalling [81]. SHP2 is composed of two N-terminal SH2 domains (N- and C-SH2), PTP domain and a carboxy-terminal tail [82]. In the absence of stimuli, the SHP2 is found in closed autoinhibited conformation due to the interaction of PTP and N-SH2 domains. Upon the binding of SH2 domain to specific phosphotyrosine motifs, SHP2 is recruited directly, or via adaptor protein GRB2, to FLT3 [83]. When catalytically active SHP2dephosphorylates RAS p21 protein activator(RAS GTPase activating protein)docking sites, thereby increasing RAS activation; this results in the promotion of RAS/ERK signalling [81]. However, alternative mechanisms of SHP2-mediated activation of RAS/ERK signalling has also been proposed.

Dysregulation of tyrosine kinases has been implicated in a variety of human cancers, and therefore tyrosine kinases can be classed as proto-oncogenes. Due to this implication in different diseases, it is an attractive target for drug therapy. In terms of different targeted therapeutics, the extracellular regions of RTKs are

attractive in using monoclonal antibodies, whereas the cytoplasmic kinase domain is targeted by small molecule inhibitors.

FLT3 receptor

Feline McDonough Sarcoma (Fms)-like tyrosine kinase 3 (FLT3) belongs to the PDGFR subfamily of RTKs along with the following members: the stem cell factor receptor (KIT), the colony-stimulating factor-1 receptor (CSF1R), and the platelet-derived growth factor (PDGF) receptor α and β [84]. The FLT3 gene is located on chromosome 13 at band q12 [85]. It shares 30 % of homology with other members of the subfamily. FLT3 is composed of five immunoglobulin-like domains in the N-terminal extracellular region, a transmembrane region, a juxtamembrane domain, an intracellular kinase domain split in two by a specific hydrophilic insertion (the kinase insert), and a carboxyterminal tail (the structure is shown in Figure 3 [86]).

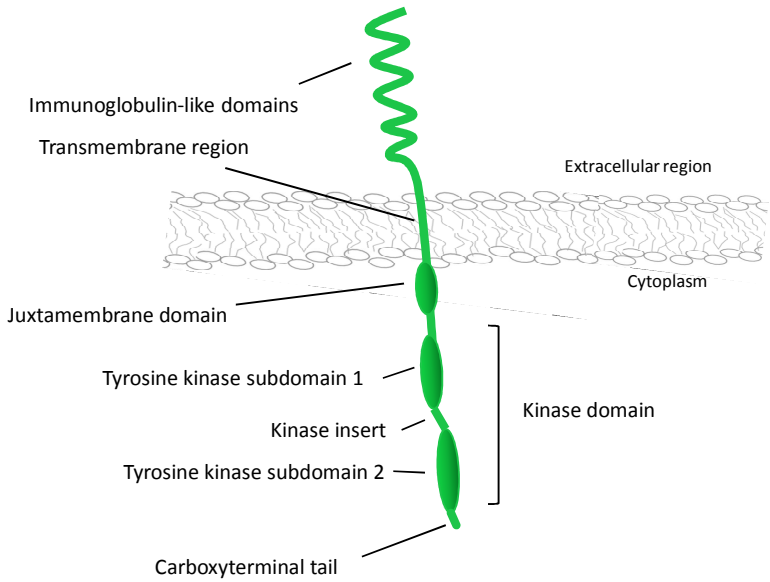

Figure 3

The schematic structure of FLT3.

The first three immunoglobulin-like domains are needed for the FLT3 ligand (FL) binding, while the two remaining immunoglobulin-like domains, located closest to

the plasma membrane, are involved in dimerization [87]. The kinase domain consists of the tyrosine kinase subdomains 1 and 2 (TKD1 and TKD2, respectively). The hematopoietic progenitor cells express FLT3 on the cell surface; however, upon the cell maturation its expression is lost. It plays a key role in controlling survival, proliferation and differentiation of hematopoietic cells.

The length of the human FLT3 receptor is 993 amino acids. Once it is synthesised in the ribosome, FLT3 goes through glycosylation in the endoplasmic reticulum to form an immature, partially glycosylated isoform with a molecular weight of 130–143 kDa, which straight away progresses to the Golgi apparatus to become fully glycosylated [88]. The highly glycosylated, mature form of FLT3, with a size of 155-160 kDa, appears on the cell surface with a high affinity binding domain for its ligand [89].

Stimulation of the FLT3 receptor is achieved through binding of the FLT3 ligand (FL). FL is a type I transmembrane protein and similar in structure to the other ligands in the subfamily. FL is comprised of a signalling peptide, an extracellular and a transmembrane domain, as well as a short cytoplasmic tail [9]. The ligand exists both as a membrane-bound form, as well as a soluble form that is generated by alternative splicing and proteolytic cleavage [90]. When FL binds FLT3, the receptor becomes homodimerized, leading to a conformational change which in turn results in phosphorylation of the tyrosine kinase domain.

In the insulin receptor when the tyrosines are unphosphorylated, the activation loop typically assumes the closed conformation by folding into the cleft between the N and C lobes, thereby preventing the ATP binding [91]. Despite the FLT3 and insulin receptor belong to the large family of the tyrosine kinase receptors the activation mechanisms are extremely different. The activation loop of the FLT3 has only one tyrosine residue at the 842 position, which is not involved in activation of the FLT3. Instead it is the phosphorylation of the tyrosine residues in juxtamembrane domain that plays an essential role in activation of the FLT3 [92].

This activation of FLT3 is a rapid process, followed by an equally rapid internalization and degradation of the homodimerized receptor [93]. Activation of FLT3 leads to association with the adaptor protein growth factor receptor bound protein-2 (GRB2). The GRB2 serves as the linker protein that can bind other proteins such as guanine nucleotide exchange factor (SOS). This results in dissociation of the GDP molecule, making the place vacant for GTP. The GTP binds RAS and thereby activates RAS/ERK pathway. FLT3 can interact through the association or phosphorylation with proteins such as GRB2, GAB2, SHIP, SHP2 and ultimately leading to activation of the PI3K/AKT pathway [94-96]. In

addition, human FLT3 also binds to the protein tyrosine phosphatase SHP2 and E3 ubiquitin ligase CBL, Figure 4 [96, 97].

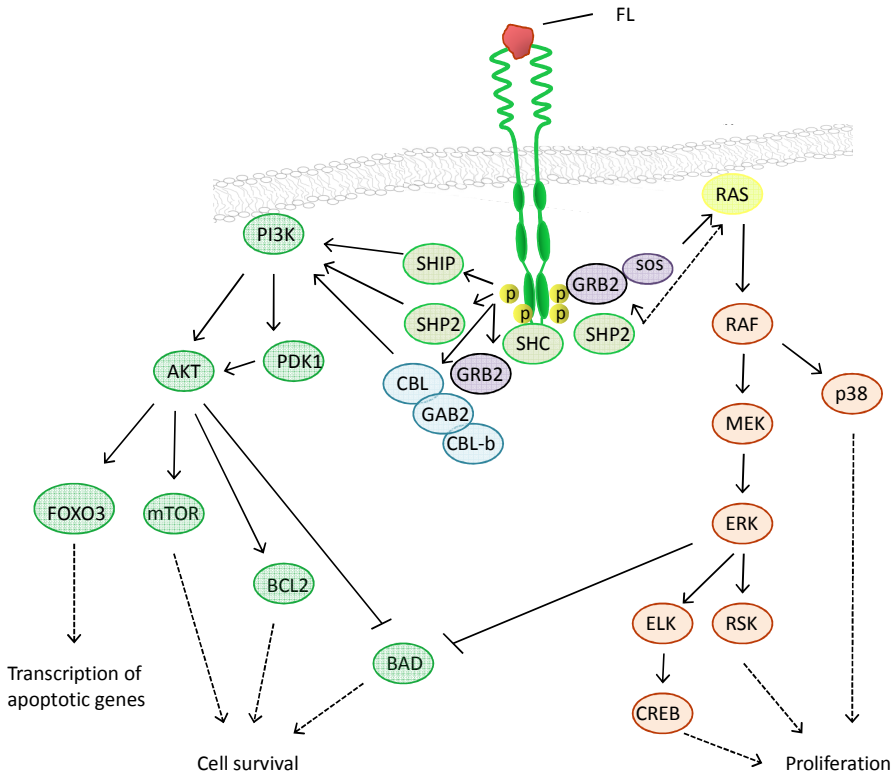

Figure 4

Signalling pathways activated by the wild type FLT3 upon binding FL.

Binding of FL to the FLT3 results in dimerization of the receptor and activation of downstream signalling.

During normal hematopoiesis the early hematopoietic progenitors express FLT3, and FL plays a key role in activating FLT3 and downstream signalling – thereby promoting the differentiation of a particular category of progenitor cells. Generation of a knock-out mouse model gave a better understanding of the role of FL. The survival of mice lacking the FL is not affected and they even appear healthy, nevertheless the amount of myeloid and B-lymphoid progenitors in the bone marrow, as well as dendritic cells and natural killer cells in the spleen and lymph nodes is reduced [98]. In comparison, FLT3 deficient mice are healthy and fertile. The amount of myeloid and lymphoid cell populations is normal in the spleen. However, in the bone marrow the percentage of B-lymphoid progenitors is

reduced [99]. Overall, the absence of the FL has mild effects, whereas the knockout of the FLT3 has an even milder effect on the bone marrow cellularity [100]. *In vitro* experiments FL in combination with other cytokines enhances the generation of B cells, but not erythroid or megakaryocyte lineages [101-103].

During the normal hematopoiesis, FLT3 requires its ligand for activation, in contrast to hematopoietic malignancies, when cells commonly have the FLT3 aberrantly expressed and/or mutated and will no longer depend on FL.

In 40% of the cases of AML, patients have an activating FLT3 mutation, thus making it one of the most frequently affected genes in AML. The most common mutation is FLT3 internal tandem duplications (FLT3-ITDs), present in 30% of the cases [104]. In second place there are point mutations within the activation loop of the TKD found in approximately 7-10% [105, 106]. Point mutations are rarely observed in the extracellular domain and the JMD, only accounting for around 2% of patients [107, 108].

First type of FLT3 mutation

Internal tandem duplication (ITD) mutation in the juxtamembrane domain (JMD) of FLT3 was the first activating mutation to be described. It remains one of the most common mutations in AML and the most studied. With the help of crystal structure, three distinct topological components were defined of the JMD: the JM binding motif (JM-B), the JM switch motif (JM-S), and the zipper or linker peptide segment (JM-Z) [109]. The ITD mutation consists of a head to tail replication of sequences coding for part of the juxtamembrane domain of FLT3, primarily found in the zipper peptide segment. The sequence length varies from patient to patient (from 3 to more than 400 base pairs), but it always occurs in multiples of three base pairs within the frame. In many cases, one can observe the addition of one or two amino acids before the repeat region [110]. Residues 590-600 of the receptor amino acid sequence are the most frequently affected. Nevertheless, the duplicated region can also be within exon 15 or in between exons 14 and 15. At present, there is no explanation as to how ITDs are generated, but one of the proposed theories is that it is due to a failure in mismatch repair during the replication of DNA [111].

It has been shown that in the cell lines COS-7, Ba/F3 and 32D cells, the outcome of the elongation is that the mutated receptor is constitutively active and autophosphorylated without requiring to bind FL, therefore resulting in a ligand-

independent, constitutive activation of FLT3 [44, 111, 112]. The autophosphorylation can also be caused by high expression levels of the receptor. Recent studies show that FL increases the activation of the FLT3-ITD [113] .
Among the downstream signalling of the active FLT3-ITD are RAS/ERK and PI3K/AKT pathways, but in addition, and in contrast to wild type FLT3, one can observe activation of the STAT5 pathway [114, 115]. C-Myc, cyclin D1 and p21 are the downstream targets of the STAT5 signalling and play a significant role in cell proliferation [116, 117]. Additionally, the proto-oncogene Pim-1 is upregulated through STAT5 signalling in leukemia cells with constitutively active FLT3 [118]. Due to the activation of these signalling pathways, the differentiation of hematopoietic progenitors is blocked and the cytokine independent proliferation is promoted.

The poor prognosis of AML patients with FLT3-ITD can also be a result of increased DNA double strand breaks (DSBs). FLT3-ITD mutations lead, via STAT5 signalling and activation of RAC1, to increased amounts of reactive oxygen species (ROS), which in turn contributes to increased DSBs and errors in repair [119].

Based on human trials, the presence of FLT3-ITD mutation has a negative effect on the long-term outcome [104, 120, 121]. The rate of complete remission among patients with FLT3-ITD is very high; however, with time they have a higher relapse rate, shorter disease free periods, and worse overall survival. Allogeneic transplant is often recommended as a treatment for FLT3-ITD AML patients with a normal karyotype. Nevertheless, a high risk of relapse remains. In addition to this poor prognosis, there is the fact that patients with AML frequently have mutations in more than one gene. The most common mutation in AML patients is in NPM1, which in combination with FLT3-ITD gives a worse prognosis. In contrast, patients with NPM1 mutation and wild type FLT3 mainly have a good outcome with long relapse free terms and improved overall survival [122].

Second type of FLT3 mutation

Point mutations in the activation loop of the tyrosine kinase domain (TKD) account for the second most common type of FLT3 mutations in AML. A majority of TKD mutations are an aspartate-to-tyrosine substitution at codon 835. Although other substitutions have also been identified they are not as frequent; among them substitutions from aspartate-835 to histidine, valine, glutamate and asparagine [105, 123]. Additionally, a small proportion of AML patients harbour a mutation

in exon 20 that is the result of an insertion of a glycine and serine residue between 840 and 841 amino acids [124].

These mutations cause a constitutively active receptor through a conformational change of the receptor and disruption of its autoinhibitory function. In contrast to FLT3-ITD mutation, which has an age association increase, the prevalence of point mutations affecting the receptor is constant across all ages [125]. This constitutive kinase activation results in the downstream signalling similar to FLT3-ITD via RAS/ERK and PI3K/AKT. For AML patients with TKD mutations, the prognosis and overall survival looks better than for patients with FLT3-ITD [126]. However, there can be a controversy of the prognostic significance of FLT3-TKD, since the frequency of such mutations is much lower than FLT3-ITD.

Third type of FLT3 mutation

The third type of FLT3 mutations are point mutations in the JMD. The occurrence of these mutations is extremely low. The cases with registered AML patients are mutations in F594L, Y591C, V579A, F590G, Y591D, etc. [108, 127]. Therefore, most studies have been carried out in the Ba/F3 cells harbouring one of these JM point mutations in FLT3. The growth of these cells is independent of interleukin 3 (IL-3) and has increased resistance to apoptosis via up-regulation of Bcl-x(L) and constitutive activation of the receptor and STAT5 [108]. When compared to other types of FLT3 mutations, the receptor shows lower autophosphorylation and therefore has a weaker transforming potential [127]. The crystal structure revealed that point mutations in JMD interfere with the stability of the autoinhibitory conformation of the juxtamembrane domain [108, 109].

SRC family kinases

The tyrosine kinases are a large multi-gene family that is implicated in many different signalling processes within the organism. They are responsible for regulating cell proliferation, migration, differentiation, survival and apoptosis, among other things. Out of 90 tyrosine kinase genes, there are 32 non-receptor tyrosine kinases that can be further divided into ten subfamilies based on kinase domain sequence [74]. In this chapter, the focus will be a subfamily of the SRC family kinases (SFKs). The SFKs family consists of eight members that are found in various cell types: SRC, YES, FYN, LYN, LCK, HCK, FGR, and BLK [128]. Most members of the SFKs are mainly expressed in cells of the hematopoietic system, except for SRC, FYN and YES that are found in other tissues like brain, fibroblasts and endothelial cells.

The size of proteins in the family varies from 52 to 62 kDa, which is due to similarities in the structure. The domain structure of SFKs represents a conserved sequence of an N-terminal region, a unique domain, SRC homology domain 3 (SH3), SRC homology domain 2 (SH2), linker, kinase domain and the C-terminal tail [129]. Each structure has its own unique role; the sequence of the N-terminal region allows for the attachment of the protein to the plasma membrane, via a covalently attached fatty acid, whereas the sequence of SH3 binds to proline-rich regions in proteins. The SH2 domain plays a role of signal transducer through interaction with phosphorylated tyrosine residues within a specific sequence of amino acids [130]. The kinase domain is composed of N-terminal and C-terminal lobes with the catalytic site in a cleft between them. The roles of the two lobes are orientation and anchoring of the ATP molecule, as well as binding the protein substrate by N- and C- lobes, respectively [131]. The activation loop lies in the cleft, where nucleotide binding and phosphor-transfer occurs [131]. The C-terminal tail serves as a negative regulator of the kinase. Upon its phosphorylation by CSK, another tyrosine kinase, a change in conformation occurs and through intramolecular interactions SH3 and SH2 domains turn inward and lock the kinase in an inactive state [131]. Therefore the activity of the SRC family members depends on both the dephosphorylation of the C-terminal tail and on the phosphorylation of the activation loop. SFKs also have the ability to autophosphorylate via a specific tyrosine residue found within the C-terminal lobe. This autophosphorylation triggers a conformational change from inactive to active

form [132]. Finally, the unique domain is what makes each member of the family recognizably different. It has also been proposed to play an important role in mediating interactions with receptors that are specific to each member. In the case of LCK, it has a specific sequence in the unique domain that enables its interaction with CD4 and CD8 on the surface of the T-cells [133, 134].

The involvement of members of the SFKs in the regulation of many biological activities has negative consequences when equilibrium is broken. The overexpression of SFKs has been associated with various cancers including breast, colon, lung, liver, gastric and leukemia [135-137]. The elevated levels can hardly be explained with mutations since mutations in SFKs genes are very uncommon in cancer [138]. Yet, there are sometimes mutations observed in the SH3 and SH2 domains that result in an active kinase. These intermolecular interactions otherwise serve as a blockade of the active site of the kinase [129]. However, the most possible cause of the increased expression of SFKs lies in the deregulated upstream regulators [139, 140]. The extensive studies of the members of SRC family provide evidence that they play an important role in the tumour progression, rather than initiation [141, 142]. Due to the implication in tumourigenesis, SFKs represent an interesting target for therapy.

LCK

The SRC family kinase lymphocyte-specific protein tyrosine kinase (LCK) is predominantly expressed in T cells, but also in B cells [143, 144]. This protein of 56 kDa size is abundant mostly at the plasma membrane. It is required for the normal development and also activation of mature T cells; it is therefore expressed throughout the entire life of T cells [143]. The receptors CD4 and CD8 on T cells are constitutively associated with LCK. During thymopoiesis LCK drives progression of thymocytes to CD4+/CD+8, and later on, it associates with CD4 or CD8 to promote even further progression of the T cells. At last, LCK is responsible for the antigen induced T cell activation via association with the co-receptor [145].

The members of the SRC family have been implicated in tumourigenesis, and LCK is not an exception. The abnormal expression of LCK has been detected in both liquid and solid tumours, including leukemia, breast, brain and colon cancers [146-151].

LCK is implicated in breast cancer progression and higher expression of the protein is associated with invasive breast cancer [147, 152]. In human glioma cells, LCK plays an important role in the fractionated radiation-induced expansion of glioma stem-like cell populations and the acquisition of resistance to chemotherapeutic agents [148]. The metastases of colon cancer are observed to have a higher expression of LCK [149].

The present Investigation

Paper I. Tyrosine 842 in the activation loop is required for full transformation by the oncogenic mutant FLT3-ITD

Aim

In this study, the aim was to study the role of Y842 residue in FLT3 signalling *in vitro* and *in vivo*.

Summary

Phosphorylation on several tyrosine residues is critical for the catalytic activity of FLT3. Y842 is a tyrosine residue located in the activation loop of the receptor. To study the role of Y842, we generated Y-to-F mutants of FLT3-WT and FLT3-ITD and overexpressed them in the murine myeloid cell line 32D. We compared the cell viability, apoptosis and colony formation ability between cells expressing FLT3-ITD and FLT3-ITD-Y842F mutant. A mouse xenograft model was used to verify data *in vivo*, and 32D cells were injected subcutaneously to follow the tumour formation. We also studied signalling downstream of FLT3 with western blotting and gene expression analysis using microarray in order to understand the mechanisms.

32D cells expressing FLT3-ITD/Y842F displayed reduced viability and higher levels of apoptosis. The colony forming capacity *in vitro* was dramatically reduced in cells after introducing the Y842 mutation in FLT3-ITD, suggesting that the Y842F mutation reduces the transformation potential of FLT3-ITD. *In vivo* verification showed that the average tumour weight was reduced by 70% in mice injected with cells expressing FLT3-ITD/Y842F compared to cells expressing FLT3-ITD. All observations led us into hypothesising that this mutation influences FLT3-ITD-induced gene expression. Thus, the microarray analysis comparing gene expression regulated by FLT3-ITD versus FLT3-ITD/Y842F was performed and demonstrated that mutation of Y842 leads to suppression of anti-apoptotic

genes. Furthermore, we showed that cells expressing FLT3-ITD/Y842F display impaired activity of the RAS/ERK pathway due to reduced interaction between FLT3 and the protein tyrosine phosphatase SHP2. This leads to reduced SHP2 activation. SHP2 has previously been implicated as a positive regulator of the RAS/ERK pathway in several receptor systems.

Discussion

Over the past decade, the molecular characterization has improved for patients with AML, as has the number of specific FLT3 inhibitors. There are several specific FLT3 inhibitors that are currently undergoing clinical trials either as a single agent or in combination with chemotherapy. The majority of the FLT3 inhibitors work via competitive inhibition of ATP binding sites that are located in the kinase domain of the FLT3 receptor [153, 154]. In theory, this is a good approach for treating AML patients with internal tandem duplications (ITD) in the juxtamembrane domain of FLT3, which accounts for 30% of the AML cases. Nevertheless, a significant proportion of the patients develop over time resistance towards the targeted therapy due to a novel mutation. One of the most common secondary mutations is a point mutation found in the kinase domain [67, 155, 156]. The contribution to the resistance of the secondary mutation is due to different mechanisms either by altering the conformation state of the receptor and binding ability of the inhibitor or by inducing other pro-survival pathways, or even changes in the expression of the receptor.

There are several different tyrosine residues that are essential for FLT3 signalling. The mutation in residue Y842 is not very frequent in AML patients, however, Y842C or Y842H mutations in combination with FLT3-ITD does lead to resistance to FLT3 inhibitors [157-159]. Since individual FLT3 kinase inhibitors are selective towards different activating TKD mutations, therefore it is of great importance to study and to be able to diagnose mutations at different sites [160, 161].

The Y842 residue in FLT3 corresponds to Y823 in KIT, and both play important roles in their receptors signalling as well as in transformation. Although the mechanism of action is slightly different between the Y823F mutation in KIT and the Y842F mutation in FLT3, one suppresses AKT, ERK1/2 and p38 pathways, whereas the other one only reduces ERK1/2 signalling [162]. In this paper, we could show that suppression of ERK1/2 signalling is due to impaired activation of the protein tyrosine phosphatase SHP2.

Despite belonging to the same family of tyrosine kinase receptors the tailoring of each drug should be unique to the specific receptor or interfere with the activity of the specific binding partners, in this particular case SHP2.

Paper II. Internal tandem duplication mutations in the tyrosine kinase domain of FLT3 display a higher oncogenic potential than the activation loop D835Y mutation

Aim

Previously, a group from Germany amplified and sub-cloned seven ITD-mutations from primary patient material to assess the functional properties of differentially located FLT3-ITD mutations [163]. In collaboration with them we received Ba/F3 cells infected with each of the five JMD-ITD constructs and each of the two TKD1-ITD constructs. Our aim was to compare the TKD-ITD domain mutations with a point mutation D835Y in the TKD.

Summary

Patients with ITD mutations in the TKD have a worse survival prognosis than those with point mutations in the TKD domain, the reason of which is unknown. Thus, a better understanding of FLT3 signalling as well as its downstream mediators could provide new insights into the molecular mechanism(s) and alternative drug targets for AML with different FLT3 mutations.

Our observations show that transfection of cytokine-dependent Ba/F3 cells with TKD-ITD is sufficient to induce formation of colonies in semi-solid medium in the absence of cytokines. The number and size of colonies were comparable to that of JMD-ITD, while D835Y-TKD transfected cells failed to form colonies. This suggests that the TKD-ITD mutations have stronger transforming potential than other TKD mutations. Similar to colony formation assays proliferation and cell survival was significantly higher in TKD-ITD transfected cells compared to cells transfected with D835Y-TKD. TKD-ITD selectively enhanced STAT5 phosphorylation while AKT, ERK1/2 and p38 phosphorylation remained unchanged.

Discussion

Patients with acute myeloid leukemia with internal tandem duplication (ITD) mutation represent 30% of the cases and generally have a poor prognosis. With the help of intensive chemotherapy the disease can be successfully treated into remission, but eventually, it routinely relapses [164, 165]. Previously it was thought that ITDs can only occur in the juxtamembrane region, but recent studies show that they can also be found in the kinase domain. Interestingly, if the relapse rates are compared between ITDs and point mutations, the relapse of patients with D835 that is located in the kinase domain is relatively low [166].

The newly identified ITDs within the kinase domain made us wonder whether it is the nature of the mutation or the location of the mutation that plays the main role in leukemogenesis. In this paper, we compared two recently characterized ITDs within the TKD and the most common point mutation, D835Y, within the TKD. While the paper is a good start, there are many limitations that should be considered. For example, we chose to use the D835Y mutation, because it is the most frequent point mutation, but there are other point mutations that it would be interesting to compare with. Especially the point mutations that are located in the first part of the tyrosine kinase domain would be of interest, since we used the cells with ITDs located in the same part. Another example, in this paper two sequences of ITD were used, even though, the sequence and the length of ITDs varies from patient to patient. It has been previously reported the length of ITDs does matter, and the longer sequence is associated with worse prognosis and survival [167]. Therefore, comparison of different ITDs would provide information on why the length is important. Additionally, it would be interesting to compare the ITDs of the same length, but with a different sequence. Thus one would gain a better understanding whether the length or the sequence of ITDs play a bigger role in leukemogenesis.

In the study that we performed, our data suggests that the two TKD-ITDs show oncogenic potential on a similar level to the JMD-ITD while the oncogenic potential is higher than for the D835Y point mutation. In this myeloid transformation the aberrant activation of STAT5 pathway played an essential step.

Paper III. ABL2 suppresses FLT3-ITD-induced cell proliferation through negative regulation of AKT signaling

Aim

To investigate the role of ABL2 in FLT3 signalling.

Summary

ABL2 belongs to ABL family of non-receptor tyrosine kinases and is involved in regulation various biological processes, including cell survival, apoptosis, cell motility and morphogenesis, as well as response to genotoxic stress [168]. In leukemia, the implication of ABL family is best known through an oncogenic BCR-ABL gene fusion[169]. However, the new studies show it is overexpressed as a single protein in various cancers [170, 171]. The involvement of the ABL family kinases with other tyrosine kinase receptors made us hypothesise that ABL2 can be implicated in FLT3 signalling as well.

As a proof of principle, with the SH2 domain array we could identify ABL2 as an FLT3 binding protein. ABL2 showed high affinity towards different phosphorylated residues (pY726, pY793 and pY842) in FLT3. To explore the role of ABL2 further, we generated the Ba/F3 cells expressing FLT3/ITD with ABL2 or empty vector. By performing different cell assays, we could observe that cells expressing ABL2 displayed reduced viability without any effects on apoptosis and reduced ability to form colonies. The ABL2 expression did not affect FLT3 activation or stability, but in turn, it partially blocks downstream signalling via the PI3K/AKT pathway.

Discussion

The regulation of downstream signalling is a well-known process that depends on phosphotyrosine residues that serve as docking sites for the SH2 domain containing proteins. The homeostasis of a cell largely depends on the activity of the receptor, which is in turn controlled by various associating proteins. Some associating proteins enhance the downstream signalling, whereas others block it. The fascinating fact is that ABL2 plays a double role depending on the tissue. In non-small cell lung carcinoma cell lines the depletion of ABL2 leads to decreased cell growth, whereas in an *in vivo* model of breast cancer, the knock-out of ABL2

accelerated tumour formation by increasing the rate of proliferation [172, 173]. In our scenario ABL2 appears to be a negative regulator of FLT3 signalling and transformation. Since we do not know the mechanism behind the regulation, further investigation is needed. Nevertheless, the ABL2 certainly represents a difficult case for the clinical use due to such a diverse function of the protein.

Paper IV. The Src family kinase LCK cooperates with oncogenic FLT3/ITD in cellular transformation

Aim

The aim of this paper was to investigate whether LCK is involved in FLT3 signalling and transformation.

Summary

LCK is a non-receptor tyrosine kinase that belongs to the SRC oncogene family. Overexpression of LCK had been previously described to induce STAT5 hyper-phosphorylation in Ba/F3 cells [174]. FLT3-ITD, but not FLT3 wild-type, strongly activates STAT5. These two factors made us hypothesize that LCK has a role in FLT3 transformation. Therefore, the purpose of our study was to determine the role of LCK in FLT3 signalling. We generated Ba/F3 cells expressing FLT3-WT or FLT3-ITD along with LCK or an empty control vector. FLT3 induced transformation is being studied both *in vitro* by using colony formation assay, proliferation and apoptosis assay, and *in vivo* with the help of a xenograft mouse model. We generated Ba/F3 cells which expressed FLT3-ITD LCK or the empty control vector, which were then injected subcutaneously into mice and 25 days later tumours were collected for staining.

LCK expression slightly potentiated FLT3-ITD-induced cell proliferation with no effect on apoptosis. However, in cells expressing wild-type FLT3, LCK seemed to partially rescue the Ba/F3 cells from apoptosis in comparison to the FLT3-WTempty vector. We also performed a colony formation assay in a semi-solid medium for FLT3/ITD. LCK expression dramatically increased the number of colonies. The same pattern was seen *in vivo* experiments the average tumour size is increased in mice injected with FLT3-ITD/LCK cells. Staining of the tissues from mouse xenografts showed higher Ki-67 staining in cells expressing LCK.

Discussion

A diverse spectrum of biological processes is controlled by the family of tyrosine kinase receptors. The signalling cascade is usually triggered by the ligand, which activates the receptor. The assistance in downstream signalling very often comes from additional tyrosine kinases that are being recruited by the tyrosine kinase receptors. Among them is the family of SRC non receptor tyrosine kinases that

assists in the tyrosine kinase receptors signalling [175]. The member of SFK family LCK has been implicated in various cancers [146-150]. When studying other members of SFK family, we observed that LCK is being upregulated in AML patients [176]. Yet, the role of LCK has not been studied in FLT3 transformation. These findings served as a background to this study.

For this study, we used the cells that lack endogenous LCK expression and could show that it is not involved in the FLT3 activation process. Yet, LCK is more likely to be involved in the FLT3 distinct downstream signalling pathway. To this conclusion we came since in FLT3/ITD - LCK did not affect the cell viability *in vitro*, but did however enhance the capacity of colony formation. Even more support came after looking at the downstream signalling, since STAT5 phosphorylation was enhanced. Additionally, *in vivo* studies show that tumours develop faster in mice that were injected with cells expressing LCK and FLT3-ITD than cells lacking LCK expression. This leads us to the conclusion that FLT3-ITD transformation potential is enhanced with LCK, making LCK a very promising target for therapy.

There are several SRC inhibitors in phase II/III clinical trials in patients with solid tumours, some of them show promising results [177-182]. Despite the high structural similarity between members of SFK, the individual members would require a specific inhibitor. Therefore, it is of great importance to understand the full biology of the LCK signalling and its involvement in AML in order to design a suitable inhibitor. One of the critical issues the potential LCK inhibitors might face is the lack of biomarkers to identify the patients that would respond and benefit.

Nevertheless, the future lies in identifying the AML patients that would potentially benefit from combination therapy of FLT3 and LCK inhibitor.

Overall conclusions and Future Perspectives

The low success in the outcome of AML patients with standard chemotherapeutic agents triggered the interest in exploring other therapeutic alternatives. Over the past decade the molecular targeting has shown great promise, yet there are many challenges with the wide-spreading of targeted therapies in clinical practice.

AML is a heterogeneous disease with many different types of mutations. Only a small fraction of these mutations are used in the clinic in defining the prognostic and predictive value. One of the reasons could be the cost of scanning all possible mutations in every patient. Another reason is that we still do not fully understand the biological impact of all downstream signalling pathways. The patients with multiple mutations also represent a challenge, since one needs to be able to distinguish the driver mutation(s) from the passenger mutation(s) and tailor the drugs accordingly.

FLT3 is one of the most commonly mutated oncogenes in patients with AML. Therefore, the development of specific FLT3 inhibitors has been a primary focus in research. Nevertheless, a common limiting factor to sustained efficacy of FLT3 inhibitors is the development of resistance in a significant amount of the patients. The emergence of resistance demonstrates the plasticity of cancer cells that allows them to adapt to therapeutic pressure with the formation of resistant clones. One of the common mechanisms of resistance connected toFLT3 receptor is a secondary mutation(s) in the target kinase that alters the conformational state of the receptor and therefore the binding ability of the inhibitor is reduced. Some mutations also cause the activation of additional pro-survival pathways causing off target resistance. In this thesis, we focused on different mutations within the FLT3 receptor, as well as associating proteins ABL2 and LCK and their role in FLT3 signalling. With the studies we obtained a better understanding of the importance of FLT3 in AML. In the future, this will hopefully aid in improving the prognostic values of AML patients, as well as in designing clinical trials involving the use of FLT3 inhibitors.

Currently, there are still some unresolved questions concerning the exact mechanism behind ABL2 and LCK interaction with the FLT3 receptor, therefore

further investigation is required. This is also true for the paper II, where only a limited number of mutations were included in the study; to have a comprehensive picture would require more mutations included in future studies.

Lastly, establishment of appropriate mouse models for AML is essential. Currently, the *in vivo* model that was used in these studies is not ideal and does not represent the physiological nature of AML. In the future, the relevant preclinical mouse models should be considered as crucial for studying the signalling, as well as testing new therapeutic agents.

Popular scientific summary

During a lifetime the human body will continuously produce new blood, consisting of red and white blood cells, and platelets, in the bone marrow. The white blood cells are the part of the immune system which defends the body against infections. When something goes wrong in the production of normal white blood cells, it can lead to the development of blood cancer, called leukemia.

With leukemia, the bone marrow will produce a lot of abnormal white blood cells, which despite their number are not effective in protecting the body from infections. Eventually the sheer amount of white blood cells produced starts decreasing the percentage of red blood cells (that are responsible for delivering oxygen to the organs) and platelets (responsible for clotting of blood). There are several types of leukemia and they can be divided into different groups depending on how quickly they progress (acute or chronic) and what cells (myeloid or lymphoid) they affect.

Every year in Sweden around 350 people are diagnosed with the form called acute myeloid leukemia (AML). The cause of AML in a majority of the cases remains unknown; however there are some risk factors that can contribute to the development of the disease, including age, smoking, exposure to certain chemicals, chemotherapy and radiation, and certain blood disorders. As a consequence it can be enough for even one cell to lose control and acquire mutations in its DNA. With the help of certain mutations the cell will grow and divide more rapidly and essentially continue living forever. There are many different types of mutations that can occur, however in 40% of patients with AML a gene called FLT3 is mutated. FLT3 is a receptor, a protein molecule that receives chemical signals from outside a cell and helps sending the information inside a cell. This receptor is found on the immature white blood cells and plays an important role in helping the cell to become a normal mature one over time. A normal FLT3 receptor requires a specific molecule, called FLT3 ligand (or FL for short), to become active and pass the signal into the cell. In acute myeloid leukemia there are different mutations within the FLT3 receptor. The most common is ITD (Internal Tandem Duplication), where a section of amino acids – the building blocks of all proteins, including the FLT3 receptor – is being duplicated. This results in an FLT3 receptor which no longer needs the FLT3 ligand, effectively becoming continuously active. Not only does it send signals all

the time, it also sends additional signals to the cell, causing the cell to become "immortal" and grow uncontrollably. The second type of mutation in FLT3 receptor is a point mutation (referred to TKD), where one amino acid is substituted with another one. This too results in a continuously active receptor and superfluous communication.

Currently, AML patients can be treated with chemotherapy, radiation therapy, stem cell transplants and targeted therapy (killing cancer cells with specific mutations without harming normal cells). Nevertheless, a significant portion of patients will relapse with time. This happens, because cancer cells can acquire new additional mutations and become resistant to the initial drug. A very common scenario is when patients with leukemia have an ITD mutation at first and after the treatment, get a second TKD mutation. Yet another scenario is when both types of mutations are present from the beginning. Acute myeloid leukemia is a very heterogeneous disease, where population of cells with different mutations can be present. In case of targeted therapy the drug can kill one population of cells towards which the drug is designed, whereas another population of cells with another mutation will continue growing and form resistance towards the drug.

This thesis focuses on different mutations of FLT3 in AML. **In Paper I**, we have focused on a specific amino acid (Y) that is located at position 842 of FLT3 receptor and its role in signalling. The mutation at this location is not a very frequent among patients with acute myeloid leukemia, nevertheless there are reports showing that this mutation along with ITD mutation contributes to the resistance to FLT3 drugs. Yet there were no studies on how this specific mutation affects FLT3 communication to the cell (downstream signalling). We used mouse cells that are grown in the lab on the plates (*in vitro*) and introduced to those cells a generated mutant of Y842F (Y842F – Y amino acid is substituted by F amino acid at the position 842) either with normal FLT3 receptor or FLT3 receptor with ITD mutation. Afterwards we looked how this mutation affects the cell behaviour by using different experiments, such as studying the ability of a single cell to grow into a colony, viability of the cells, as well as the death rate of the cells. The mutant Y842F showed reduced ability to grow colonies, as well as reduced viability and higher levels of death rate. In order to understand the mechanism we studied the downstream signalling; to see which specific communication line (specific signalling pathway) is being affected. Results showed that the specific signalling pathway called RAS/ERK had been impaired; i.e. the rate of cell division is reduced.

Studying the cell behaviour on a plate gives only part of the story, whereas studying the cells in the mouse model (*in vivo*) provides a more comprehensive image. By injecting the cells into mice we observed that the formed tumours were smaller for the mice injected with the mutant Y842F and FLT3-ITD than in the

case of just having FLT3-ITD mutation. The conclusion of the paper is that Y842 plays an important role in FLT3 signalling via RAS/ERK pathway and contributes to malignant transformation; a process by which cells acquire the properties of cancer.

In paper II, we compared the D835Y point mutation with ITDs that are located at different parts of the FLT3 receptor. For very long it was thought that ITD mutations can occur only in one specific region of the FLT3 receptor, called juxtamembrane domain (JMD). However, recently it has been identified that ITD mutations can also be located in the tyrosine kinase domain (TKD) of the FLT3 receptor. The D835Y (D amino acid is substituted by Y amino acid at the position 835) mutation is located in the TKD of the FLT3 receptor. There is a difference in relapse rates; patients with ITDs that are located in the JMD usually have a poor prognosis and high relapse rate, whereas patients with D835Y rarely relapse. We were interested in understanding which plays a major role in the development of leukemia: the location or the nature of the mutation. We therefore compared cells with introduced D835Y point mutation at TKD, with ITD mutations at different locations; both JMD and TKD. By performing different cell experiments and studying downstream signalling, we could conclude that ITD irrespective of location has a larger potential to cause cancer, than the D835Y point mutation.

In paper IV, we studied the role of an assisting protein (LCK) in FLT3 signalling. Quite commonly the FLT3 receptor requires help from assisting proteins to pass the signal into the cell. One type of assisting proteins is tyrosine kinases. They are being recruited by the FLT3 receptor to assist in downstream signalling. A protein called LCK is a type of tyrosine kinase, which has been involved in the development of different cancers. Yet, the role of LCK in FLT3 receptor signalling has never been studied before. The study was carried out *in vitro* and *in vivo* showing that LCK increases the ability of a single cell to grow into a colony, as well as mice growing tumours faster than the ones lacking LCK. Studying the downstream signalling revealed that a specific signalling pathway (STAT5 pathway) is involved. The conclusion of the paper is that LCK, together with the mutation FLT3-ITD drives the cells towards malignancy.

The regulation of the receptor activity is a very complicated process; therefore not all assisting proteins play a role in enhancing the downstream signalling. Some assisting proteins will do the opposite and block the downstream signalling. The protein ABL2 is also a tyrosine kinase, the same as LCK, however belonging to a different family. **In paper III**, we studied the role of ABL2 in FLT3 signalling. At first we showed that ABL2 can actually bind the FLT3 receptor. The importance of this experiment is to show that the interaction between these two proteins is possible. Using similar cell models as in previous studies and by performing different cell experiments we could see that cells with ABL2 have a

decreased ability to form colonies and decreased viability without an effect on the death rate. By studying the downstream signalling of the FLT3, we found out that ABL2 partially blocks one of the signalling pathways called PI3K/AKT pathway. The conclusion of the paper is that ABL2 partially blocks the malignant effects of FLT3-ITD mutation in cells.

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Paper I

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Cellular and Molecular Life Sciences

ORIGINAL ARTICLE

Tyrosine 842 in the activation loop is required for full transformation by the oncogenic mutant FLT3-ITD

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Abstract The type III receptor tyrosine kinase FLT3 is frequently mutated in acute myeloid leukemia. Oncogenic FLT3 mutants display constitutive activity leading to aberrant cell proliferation and survival. Phosphorylation on several critical tyrosine residues is known to be essential for FLT3 signaling. Among these tyrosine residues, Y842 is located in the so-called activation loop. The position of this tyrosine residue is well conserved in all receptor tyrosine kinases. It has been reported that phosphorylation of the activation loop tyrosine is critical for catalytic activity for some but not all receptor tyrosine kinases. The role of Y842 residue in FLT3 signaling has not yet been studied. In this report, we show that Y842 is not important for FLT3 activation or ubiquitination but plays a critical role in regulating signaling downstream of the receptor as well as controlling receptor stability. We found that mutation of Y842 in the FLT3-ITD oncogenic mutant background reduced cell viability and increased apoptosis. Furthermore, the introduction of the Y842 mutation in the FLT3-ITD background led to a dramatic reduction in in vitro colony forming capacity. Additionally, mice injected with cells expressing FLT3-ITD/Y842F displayed a significant delay in tumor formation, compared to FLT3-ITD expressing cells. Microarray analysis comparing gene expression regulated by FLT3-ITD versus FLT3-ITD/Y842F demonstrated that mutation of Y842 causes suppression of anti-apoptotic genes. Furthermore, we showed that cells expressing FLT3-ITD/Y842F display impaired activity of the RAS/ERK pathway due to reduced interaction between FLT3 and SHP2 leading to reduced SHP2 activation. Thus, we suggest that Y842 is critical for FLT3-mediated RAS/ERK signaling and cellular transformation.

Keywords FLT3 · FLT3-ITD · Activation loop · Acute myeloid leukemia · SHP2 · Transformation · Survival · Microarray

Abbreviations

AML	Acute myeloid leukemia
FBS	Fetal bovine serum
FL	FLT3 ligand
FLT3	Fms-like tyrosine kinase
ITD	Internal tandem duplication
MET	Hepatocyte growth factor receptor
RTK	Receptor tyrosine kinase
SH2	Src homology 2

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematopoietic disorder. The molecular genetics of AML has been thoroughly investigated identifying loss-of-function mutations in transcription factors and gain-of-function mutations in receptor tyrosine kinases. The most commonly mutated genes include *NPM1, CEBPA, TET2, IDH, DNMT3A* and *FLT3*. Mutations in the receptor tyrosine kinase FLT3 occurs in more than 30% of AML patients [1]. The internal tandem duplication (ITD) is a repetition of sequence that encodes the juxtamembrane domain and is the most common oncogenic mutations in FLT3 that correlates with a poor prognosis [2]. Other oncogenic mutations include point mutations or ITD mutations in the kinase domain.

FLT3 belongs to the type III receptor tyrosine kinase (RTK) family that includes five members PDGFRA, PDGFRB, KIT, CSF1R and FLT3. The characteristic feature of this family is an extracellular domain consisting of five immunoglobulin-like domains and an intracellular kinase domain interrupted by a kinase insert. The extracellular domain mediates association of the dimeric ligand and thereby induces dimerization of the receptor. Dimerization of receptor initiates a tyrosine phosphorylation program resulting in phosphorylation of several tyrosine residues in the receptor which are essential both for receptor activation and signal propagation [3]. Activation of FLT3 subsequently activates downstream signaling cascades including PI3K-AKT and RAS-ERK signaling through various SH2 domain-containing proteins such as GAB2, SHC and SHP2 [3]. Although wild-type FLT3 requires its ligand FLT3 ligand (FL) for activation, oncogenic mutants are constitutively active. Oncogenic FLT3 binds essentially to the same docking molecules as wild-type FLT3, and therefore controls similar signaling pathways [4].

The *FLT3-ITD* mutations significantly increase the risk of relapse, and therefore limit disease-free and overall survival [2, 5]. Inhibition of FLT3 displayed promising results in clinical trials [6]. However, in most of the cases responses were not sufficient for treatment of AML with a single drug [6]. Inhibitors mostly reduce peripheral blood blasts transiently, and bone marrow responses are rare [7, 8]. Limited response to the inhibitors is mainly due to primary and secondary mutations in FLT3 that make the receptor resistant to the inhibitor [9]. The second-generation FLT3 inhibitor, AC220 (quizartinib), induced a composite complete remission rates of 44–54% which is much better than that observed with other FLT3 inhibitors. However, later studies indicate that treatment with this drug also suffers from problems of acquired secondary resistance [10]. A recent study suggests that the multi-kinase inhibitor midostaurin prolongs survival when used in combination with chemotherapy [11]. Thus, we still need a better understanding of the best way of targeting FLT3 for AML treatment.

Phosphorylation of the tyrosine residue in the activation loop is known to be the hallmark of activation of many tyrosine kinases. For example, phosphorylation of activation loop tyrosine residues of fibroblast growth factor receptor leads to a 500 to 1000-fold increase in substrate phosphorylation [12], and is also crucial for activation of the insulin receptor [13] and hepatocyte growth factor receptor (MET) [14]. However, in both KIT and the PDGFR activation of the receptors intrinsic kinase activity was independent of phosphorylation of the activation loop tyrosine residue [15–17]. In this report, we show that the activation loop tyrosine is critical for FLT3-induced downstream ERK1/2 signaling as well as for FLT3-ITD-mediated oncogenesis.

Materials and methods

Reagents, plasmids and antibodies

Human recombinant FLT3 ligand was from ORF genetics (Kópavogur, Iceland). The transfection reagent Lipofectamine 2000 was from Thermo Scientific and cycloheximide was from Sigma-Aldrich. pcDNA3-FLT3-WT, pMSCVpuro-FLT3-WT and pMSCVpuro-FLT3-ITD were described previously [18]. pMSCVpuro-FLT3-WT/Y842F and pMSCVpuro-FLT3-ITD/Y842F plasmids were generated by site-directed mutagenesis using QuikChange mutagenesis XL kit (Agilent Technologies). The anti-FLT3 antibody was a rabbit polyclonal antibody produced in-house. Mouse monoclonal anti-beta-actin, horseradish peroxidase-conjugated anti-FLAG antibody and mouse monoclonal anti-FLAG antibodies were from Sigma-Aldrich. Mouse anti-phosphotyrosine (4G10) antibody and mouse mono-ubiquitin antibody were from Millipore and Covance Research Products, respectively. Rabbit anti-ERK2, rabbit anti-phospho ERK1/2 (pThr202/pThr204), goat anti-AKT antibodies were from Santa Cruz Biotechnology. Rabbit anti-tubulin, rabbit anti-phospho-AKT (pSer473) rabbit anti-phospho GAB2 and rabbit anti-phospho-SHP2 were from Cell Signaling Technology.

Cell culture, transient and stable transfection

COS-1 and 32D cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellen (DSMZ). COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 units/ml penicillin. 32D cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 µg/ml

streptomycin and 100 units/ml penicillin. Transient transfection of COS-1 cells and stable transfection of 32D cells were described previously [19]. Transfected 32D cells were maintained in the IL3-containing medium as described earlier [20].

Immunoprecipitation and western blotting

COS-1 cells were washed with cold PBS after 100 ng/ml ligand stimulation and lysed with Triton-X 100-based lysis buffer. 32D cells were starved of cytokines and serum for 4 h before stimulation. After stimulation cells were washed with cold PBS before lysis. Each ml of cell lysates was immunoprecipitated with 1 µg antibody and then processed for SDS-PAGE and western blotting analysis using the standard protocol [21].

Cell viability, apoptosis and colony formation assays

32D cells were washed three times to remove cytokines and re-suspended in RPMI 1640 containing 10% FBS. PrestoBlue cell viability assay and apoptosis assay were described previously [22]. Colony formation assay was performed as described elsewhere [23].

Animal experiment

Five male BALB/c nude mice in each group were used for animal experiments following Hong Kong animal ethical regulations. Mice were injected subcutaneously with 1,000,000 cells in 100 µl (1:1) PBS and Matrigel mixture. Mice were monitored for weight change and tumor size.

Gene expression analysis using microarray

32D cells expressing FLT3-ITD or FLT3-ITD/Y842F were washed three times to remove cytokines and serum. Cells were starved in medium containing 0.5% serum overnight before extraction of total RNA using the RNeasy mini kit (Qiagen). Bio-analyzer was used to check the quality of RNA. Gene expression was analyzed using Affymetrix GeneChip Mouse Gene 2.0 ST arrays. Raw data were normalized using RMA normalization.

Statistical analysis

All in vitro experiments were performed at least three times. Student's *t* test and one-way ANOVA with Bonferroni's post-tests were used for statistical analysis using GraphPad prism 5.0. Data were expressed as the mean ± SE and two-way *t* test was used.

Results

Expression of the FLT3/Y842F mutant results in reduced cell proliferation and enhanced apoptosis in myeloid cells

We have recently demonstrated that the activation loop tyrosine is of importance for KIT-mediated mitogenic signaling and KIT/D816V-mediated oncogenic transformation [15, 16]. Since FLT3 belongs to the same family of receptor tyrosine kinases as KIT, we hypothesized that the analogous FLT3 mutant would display a similar phenotype. We transduced a myeloid cell line, 32D lacking endogenous FLT3 expression, with oncogenic FLT3-ITD as well as with an activation loop tyrosine-to-phenylalanine mutant in ITD background, FLT3-ITD/Y842F. Since 32D cells are cytokine-dependent, withdrawal of cytokines leads to complete growth inhibition as well as to cell death. Both transfected cell lines displayed same cell surface expression of FLT3 (Fig. 1a, S1A and S1B) as well as total expression of FLT3 (Fig. 1b). We observed that while FLT3-ITD could fully support the viability of cytokine-starved 32D cells, FLT3-ITD-Y842F expressing cells displayed reduced viability. This suggests that, similar to KIT, phosphorylation of the FLT3 activation loop tyrosine is required for maintenance of cell viability (Fig. 1c). We also observed a significant increase in apoptosis in cytokine-starved cells expressing the FLT3-ITD/Y842F mutant compared to FLT3-ITD expressing cells (Fig. 1d).

Cells expressing the Y842F mutant have an impaired capacity to form colonies in vitro and tumors in vivo

As we observed that the FLT3-ITD/Y842F mutant cells had reduced cell viability and higher levels of apoptosis, we checked for their ability of in vitro colony formation in a semi-solid medium. We observed that cells expressing FLT3-ITD/Y842F induced a significantly lower number of colonies (Fig. 2a) as well as a reduced colony size (Fig. 2b) suggesting that the Y842F mutation reduces the transformation potential of FLT3-ITD. To verify our in vitro data in an animal model, we generated a mouse xenograft model by injecting 32D cells subcutaneously. The FLT3-ITD/Y842F mutant displayed significantly delayed tumor formation in xenotransplanted mice (Fig. 2c). Average tumor weight was reduced by 70% in mice injected with cells expressing FLT3-ITD-Y842F (Fig. 2d, e) compared to mice injected with FLT3-ITD expressing cells, suggesting that the phosphorylation of the activation loop tyrosine is an important event in FLT3-ITD-mediated transformation.

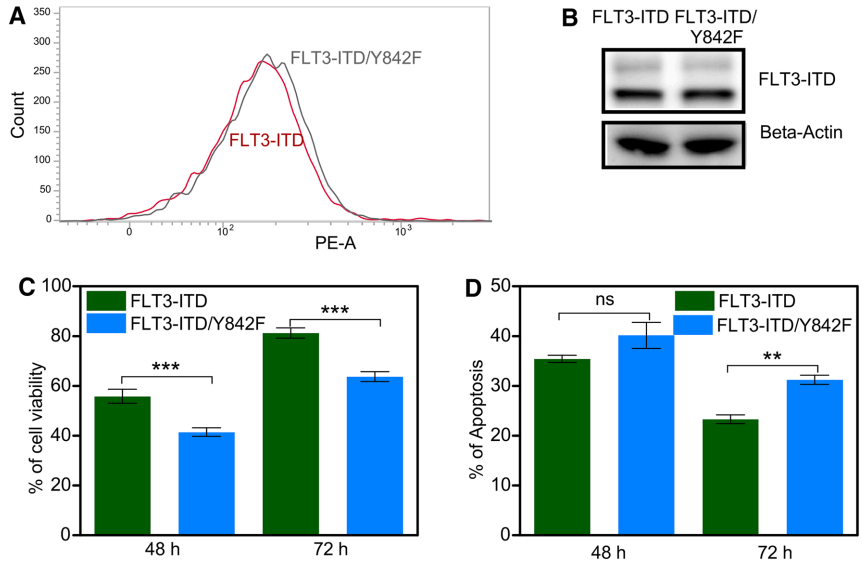

Fig. 1 Y842F mutation reduces cell viability and increases apoptosis. **a** Cell surface expressions of FLT3-ITD and FLT3-ITD/Y842F in stably transfected 32D cells were analyzed by flow cytometry using PE-conjugated anti-FLT3 antibody. **b** 32D cells expressing FLT3-ITD and FLT3-ITD/Y842F were lysed and lysates were analyzed using SDS-PAGE and western blotting. **c** Cell viability was measured upon cytokine depletion using PrestoBlue cell viability assay after 48 and 72 h. **d** Apoptosis was measured after 48 and 72 of cytokine depletion using Annexin V and 7AAD kit. ***$p<0.001$; **$p<0.01$; *ns* not significant; *error bar* represents SEM

Y842F mutation leads to downregulation of oncogenic signaling

As we observed that the Y842F mutation diminishes FLT3-ITD-mediated cell viability, colony formation and tumor formation, we hypothesized that this mutation might influence FLT3-ITD-induced gene expression. Thus, we checked global gene expression using Affymetrix Mouse Gene 2.0 ST arrays (EMBL-EBI arrayexpress accession: E-MTAB-5258). Cells expressing FLT3-ITD or FLT3-ITD/Y842F display difference in gene expression patterns (Fig. 3a) indicating that Y842F mutation influences FLT3-ITD-mediated gene expression. Expression of anti-apoptotic genes and oncogenes was suppressed in cells expressing the Y842F mutant (Fig. 3b). We then used the gene set which were downregulated in cells expressing Y842F mutant and analyzed for gene ontology using DAVID Functional Annotation Bioinformatics Microarray Analysis (https://david.ncifcrf.gov). Result showed an enrichment of GO:0070374 (positive regulation of ERK1 and ERK2 cascade, $p=3.405E{-}08$). Moreover, gene set enrichment analysis (GSEA) suggests that the deregulated genes are involved in several oncogenic pathways such as KRAS, SRC and loss of p53 (Fig. 3c). Since oncogenic signature genes were downregulated in Y842F expressing cells, it suggests that FLT3-ITD/Y842F has an impaired oncogenic capacity.

Y842F mutation selectively inhibited FL-induced ERK1/2 activation

To understand how mutation at Y842 affects FLT3-induced normal biological outcomes, we generated 32D cell lines expressing FLT3-WT and FLT3-WT/Y842F. Both cell lines expressed equal levels of FLT3 on the cell surface (Fig. 4a, S1C and S1D) as well as total FLT3 (Fig. 4b). Although expression of FLT3-ITD can partially support the growth of 32D cells upon cytokine withdrawal, cells expressing wild-type FLT3 cannot support the cell survival even when supplemented with FL. We analyzed FLT3 downstream signaling following stimulation with FLT3 ligand using phospho-specific antibodies. We observed that ligand stimulation of cells expressing the Y842F mutant activated phosphorylation of AKT to an equal extent as wild-type FLT3 (Fig. 4c). In contrast, mutation of Y842F led to a dramatic reduction in ERK1/2 phosphorylation (Fig. 4d). Similarly, FLT3-ITD-mediated constitutive activation of ERK1/2, but not AKT, was partially blocked by the Y842F

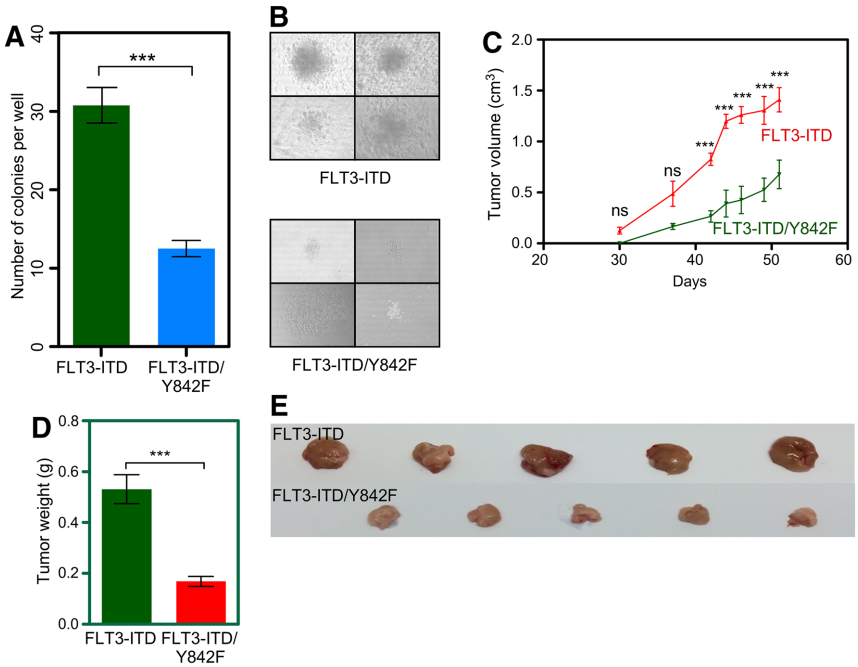

Fig. 2 Y842F mutant has impaired colony formation and tumor formation capacity. **a, b** Cells were washed to remove cytokine and serum and seeded in methylcellulose medium. Colonies were counted 7 days after seeding. **c–e** Immunocompromised mice were injected subcutaneously with 32D cells expressing FLT3-ITD or FLT3-ITD-Y842F. Tumor volume was measured twice a week and tumor weight was measured after killing mice. ***$p<0.001$; *ns* not significant; *error bar* represents SEM

mutation. However, we have not seen any reduction of STAT5 phosphorylation in cells expressing Y842F mutant (Fig. S2).

FLT3-Y842F mutant displays impaired SHP2 activation

FLT3 induces ERK1/2 activation through multiple signaling cascades. The two key signaling molecules, GAB2 and SHP2, are involved in this pathway [3]. Therefore, we examined the levels GAB2 and SHP2 phosphorylation following FL stimulation. We observed that while the presence or absence of the Y842F mutation did not affect GAB2 phosphorylation, the phosphorylation of SHP2 was strongly decreased in cells expressing the Y842F mutant (Fig. 5a). This observation suggests that somehow phosphorylation of Y842 in the activation loop is required for FLT3-mediated SHP2 phosphorylation which in turn is involved in activation of ERK1/2 signaling. We have previously demonstrated that SHP2 associated with phosphotyrosine Y599 in FLT3 [24]. Since we did not see a difference in phosphorylation of GAB2 (which in other systems has been demonstrated to associate with SHP2) in the Y842F mutant, it is likely that Y842 is an important SHP2 association site in FLT3. We observed that while wild-type FLT3 was able to associate with SHP2, the ligand-activated Y842F mutant displayed reduced SHP2 interaction (Fig. 5b) suggesting that Y842 is one of the binding sites for SHP2 in FLT3, either directly or indirectly. To determine whether SHP2 directly associates with pY842, we used a synthetic phospho-peptide corresponding to the FLT3-Y842 site. We did not observe any association between SHP2 and the FLT3-pY842 peptide suggesting that pY842 is not a direct binding site for SHP2 (Fig. 5c). However, we cannot exclude the possibility that an adapter protein bridges the binding of SHP2 to FLT3. Another possible explanation of the reduced binding capacity of the FLT3-Y842F mutant is that it could be due to a reduction in FLT3-Y599 phosphorylation. Therefore, we compared FLT3-Y599 phosphorylation between wild-type and Y842F mutant. We did not observe any reduction of FLT3-Y599

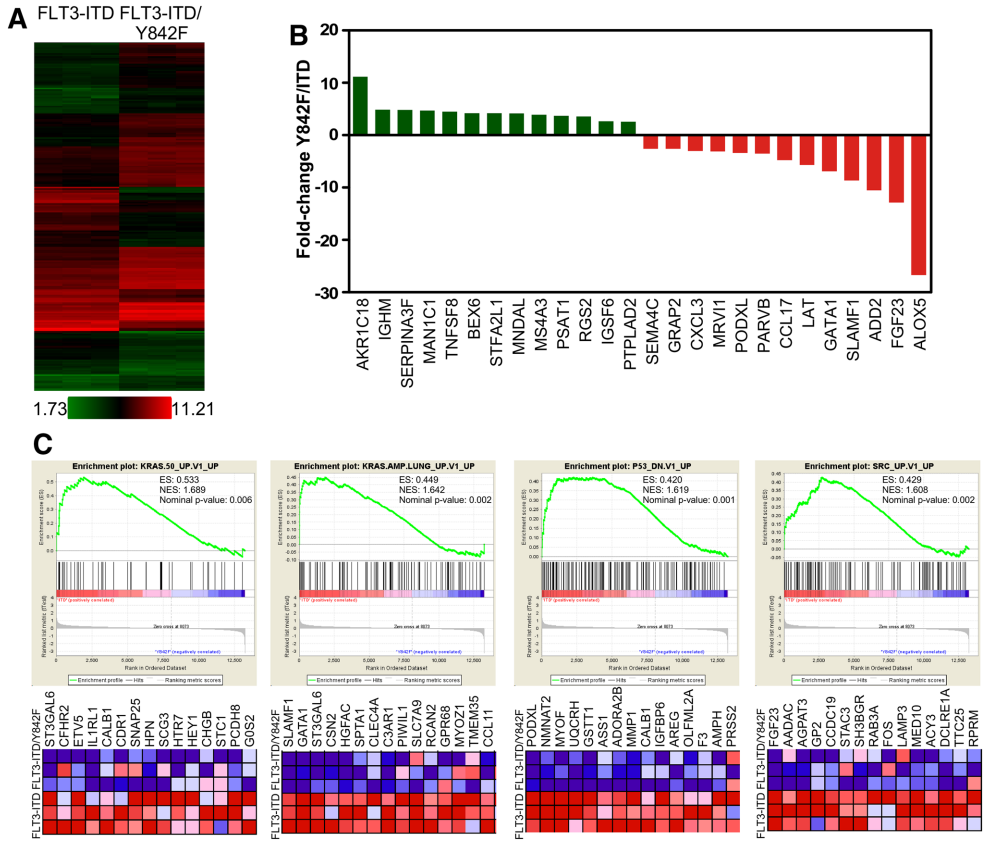

Fig. 3 Y842F mutation changes FLT3-ITD-induced gene expression. **a** Heat map shows the difference in gene expression in between FLT3-ITD and FLT3-ITD-Y842F. **b** Upregulated and downregulated genes in Y842F mutant. **c** Gene set enrichment analysis between FLT3-ITD and FLT3-ITD-Y842F transfected cells shows enrichment of different oncogenic signatures

site phosphorylation in Y842F expressing cells suggesting that the activation loop tyrosine has no role in regulating FLT3-Y599 phosphorylation (Fig. 5d).

Mutation of the Y842 residue has no effect on ubiquitination or phosphorylation of FLT3 but leads to reduced stability of the protein

Ligand stimulation results in dimerization of FLT3 followed by autophosphorylation at several tyrosine residues, which in turn creates docking sites for, among other things, the ubiquitin E3 ligase CBL. In a previous report, we observed that ligand stimulation of wild-type KIT versus ligand stimulation of the activation loop tyrosine mutant of KIT (Y823F) leads to differential tyrosine phosphorylation as well as ubiquitination patterns [16]. In this study, we did not see any significant difference in ligand-induced ubiquitination or tyrosine phosphorylation between wild-type FLT3 and FLT3/Y842F (Fig. 6a). However, FLT3 degradation was increased by approximately 30% following ligand stimulation (Fig. 6b) in the Y824F mutant compared to wild-type FLT3.

Discussion

Oncogenic mutations in FLT3 lead to aberrant activation of survival and proliferation signaling. The most frequent

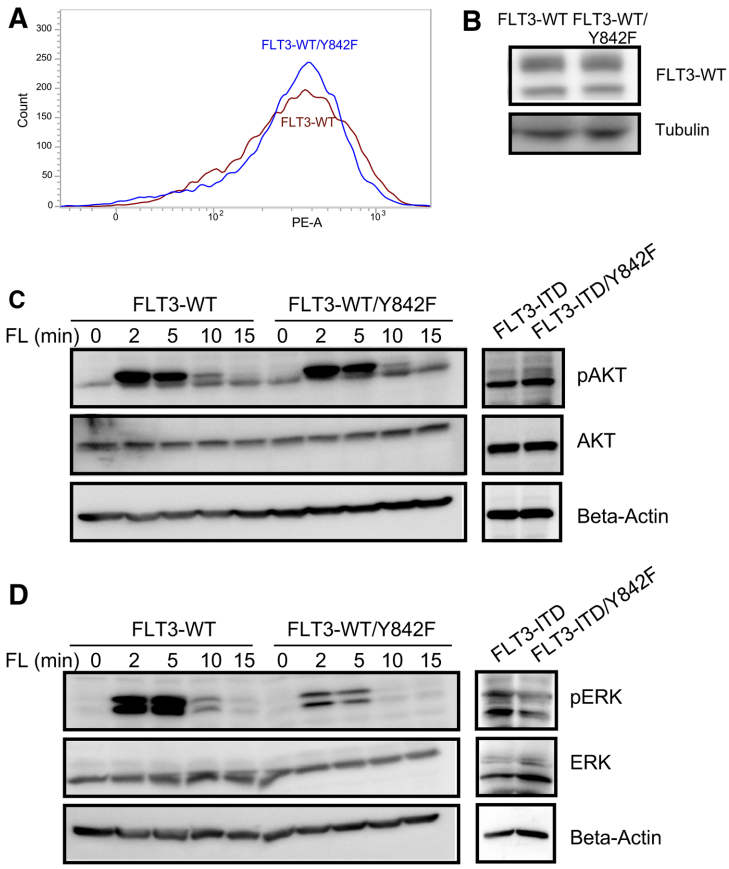

Fig. 4 Y842F mutation selectively reduces ERK phosphorylation. **a** Cell surface expressions of FLT3-WT and FLT3-WT/Y842F in stably transfected 32D cells were analyzed by flow cytometry using PE-conjugated anti-FLT3 antibody. **b** 32D cells expressing FLT3-WT and FLT3-WT/Y842F were lysed and lysates were analyzed using SDS-PAGE and western blotting. **c, d** Cells were serum and cytokine starved for 4 h before stimulating with 100 ng/ml FL for different time points. Cells were then lysed and lysates were used for SDS-PAGE and western blotting analysis using anti-phospho-AKT (**c**) and anti-phospho-ERK1/2 (**d**) antibodies

gain-of-function mutation, FLT3-ITD, is a potent and constitutive activator of downstream signaling. Other gain-of-function mutations include point mutations in the tyrosine kinase domain such as D835Y. Mutation in the tyrosine kinase domain also occurs in combination with a FLT3-ITD mutation leading to resistance to several FLT3 kinase inhibitors. Mutations of the activation loop tyrosine of FLT3 (Y842) is a less frequent event in leukemia. One report suggested that Y842C mutation results in constitutive activation of the receptor [25]. Y842C or Y842H mutations in combination with FLT3-ITD led to resistance to FLT3 kinase inhibitors [26, 27]. In the crystal structure of autoinhibited FLT3, Y842 makes extensive interactions with neighboring amino acid residues and thereby stabilizes the DFG-out inactive form of FLT3 [10, 17]. Mutation of Y842 to either H or C would likely result in loss of these interaction and lead to destabilization of the inactive form of FLT3. In contrast, mutation to F does most likely not interfere with the hydrophobic interactions and thus does not activate FLT3. Interestingly, in the closely related RTK KIT activating mutations have also been described at the homologous tyrosine residue, Y823. Different substitution mutations of this residue have been found but while Y823D is a constitutively active mutant [28], Y823A is kinase inactive [29] and Y823F does not affect kinase activity [16, 29].

To understand the functional role of Y842 we generated an Y842F mutant which is identical to wild-type FLT3 with the exception of the hydroxyl group missing in this position, thus preventing it from being tyrosine phosphorylated. We observed that Y842 is of great importance for FLT3-induced downstream signaling despite the fact that it does not affect the kinase activity of FLT3.

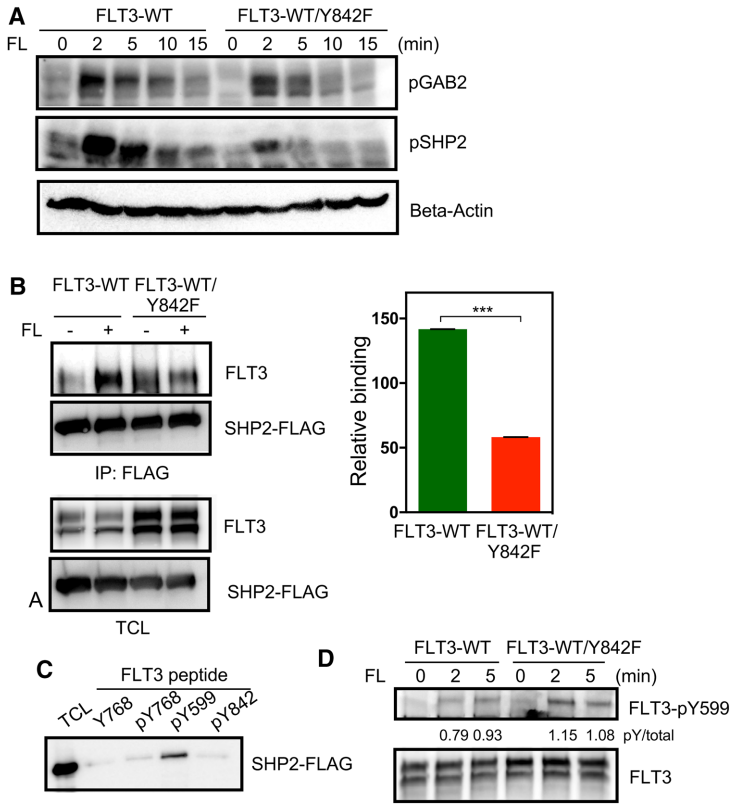

Fig. 5 Y842F mutation reduces SHP2 phosphorylation. **a** Cells were serum- and cytokine-starved for 4 h before stimulating with 100 ng/ml FL for different time points. Cells were then lysed and lysates were used for SDS-PAGE and western blotting analysis using anti-phospho-GAB2 and anti-phospho-SHP2 antibodies. **b** COS-1 cells were transfected with FLAG-tag SHP2 and FLT3-WT or FLT3-WT/Y842F. One day after transfection cells were stimulated with 100 ng/ml FL for 5 min before lysis. Lysates were immunoprecipitated using 1 µg anti-FLAG antibody. ***$p < 0.001$; *error bar* represents SEM. **c** Phospho-peptides corresponding to the different FLT3 phosphorylation sites were coupled to Ultralink beads. Slurry of immobilized peptides was incubated with cell lysates from SHP2 expressing cells. **d** Cells were serum-starved for 4 h before stimulation followed by lysis. Lysates were used for immunoprecipitation followed by western blotting analysis

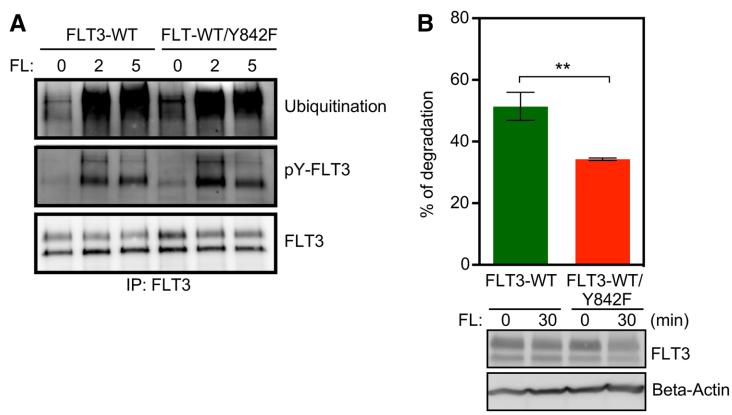

Fig. 6 Y842F mutation increases FLT3 degradation. **a** Cells were serum- and cytokine-starved for 4 h before stimulating with 100 ng/ml FL for different time points. Cells were then lysed and lysates were immunoprecipitated using 1 µg anti-FLT3 antibody. **b** Cells were treated with cycloheximide for 30 min followed by 30 min of ligand stimulation. Cells were then lysed and lysates were used to measure degradation. **$p < 0.01$; *error bar* represents SEM

Although phosphorylation of the activation loop tyrosine residues is critical for the kinase activity of several receptor tyrosine kinases such as the fibroblast growth factor receptor, the insulin receptor and MET [12–14], data suggest that it is dispensable for activation of type III RTKs [15–17]. However, Y842 in FLT3 and the corresponding residue in KIT (Y823) play important roles in receptor signaling as well as in transformation mediated by oncogenic mutants of either RTK. We observed that the Y842F mutation significantly reduced FLT3-ITD-induced cell viability and induced apoptosis in transfected 32D cells suggesting that Y842 plays a role in survival signaling mediated by oncogenic FLT3. Furthermore, the Y842F mutant cells exhibited impaired transforming capacity displayed as a reduced capacity to form colonies, which also were smaller, as well as reduced capacity to form tumors in xenografted mice. Thus, Y842F mutation displays in the FLT3-ITD background a similar phenotype to the Y823F mutation in the KIT/D816V background [15], in that it severely limits the oncogenic capacity of the transforming mutant.

Although the Y823F mutation suppresses several signaling pathways downstream of KIT, including AKT, ERK1/2 and p38 [16], the Y842F mutant of FLT3 selectively reduced only FLT3-mediated ERK1/2 signaling, while AKT signaling was intact. This further supports the notion that the receptor activation per se is not affected but selective downstream signaling events. The suppression of ERK1/2 signaling that we observed in mutant cells is likely to be due to the impaired activation of SHP2 signaling. SHP2 is a potent binding partner of FLT3, phosphorylated by FLT3 [30] and required for FLT3-mediated downstream signaling [24]. Activation of SHP2 occurs through binding of its two SH2 domains to phosphotyrosine residues, that leads to a conformational change and activation of its intrinsic protein tyrosine phosphatase activity [29]. Despite being a phosphatase, SHP2 is linked to positive signaling and activation of the RAS/ERK pathway through several mechanisms (for a review, see [10]). The observation that mutation of Y842 significantly reduced SHP2 interaction with FLT3 indicates that the impaired activation of ERK1/2 signaling in Y842F mutant cells is due to the reduced binding of SHP2 to FLT3 and reduced activation. The Y842F mutation did not completely eliminate the binding of SHP2, which can be explained by Y599 being an additional SHP2 binding site [24]. Taken together, our data suggests a unique function of the activation loop tyrosine residue in FLT3. Given the importance of both the activation loop and SHP2 in FLT3-ITD-mediated transformation, the development of drugs that interfere with binding of SHP2 to Y842 or with the activity of SHP2 could be useful drugs for the treatment of patients with acute leukemia.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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Paper II

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ORIGINAL ARTICLE

Internal tandem duplication mutations in the tyrosine kinase domain of FLT3 display a higher oncogenic potential than the activation loop D835Y mutation

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Abstract
Acute myeloid leukemia (AML) remains the most common form of acute leukemia among adults and accounts for a large number of leukemia-related deaths. Mutations in FMS-like tyrosine kinase 3 (FLT3) is one of the most prevalent findings in this heterogeneous disease. The major types of mutations in FLT3 can be categorized as internal tandem duplications (ITD) and point mutations. Recent studies suggest that ITDs not only occur in the juxtamembrane region as originally described, but also in the kinase domain. Although the juxtamembrane ITDs have been well characterized, the tyrosine kinase domain ITDs have not yet been thoroughly studied due to their recent discovery. For this reason, we compared ITD mutations in the juxtamembrane domain with those in the tyrosine kinase domain, as well as with the most common activating point mutation in the tyrosine kinase domain, D835Y. The purpose of this study was to understand whether it is the nature of the mutation or the location of the mutation that plays the main role in leukemogenesis. The various FLT3 mutants were expressed in the murine pro-B cell line Ba/F3 and examined for their capacity to form colonies in semisolid medium. The size and number of colonies formed by Ba/F3 cells expressing either the internal tandem duplication within juxtamembrane domain of the receptor (JMD-ITD) or the tyrosine kinase domain (TKD)-ITD were indistinguishable, while Ba/F3 cells expressing D835Y/FLT3 failed to form colonies. Cell proliferation and cell survival was also significantly higher in TKD-ITD expressing cells, compared to cells expressing D835Y/FLT3. Furthermore, TKD-ITD is capable of inducing phosphorylation of STAT5, while D835Y/FLT3 fails to induce tyrosine phosphorylation of STAT5. Other signal transduction pathways such as the RAS/ERK and the PI3K/AKT pathways were activated to the same level in TKD-ITD cells as compared to D835Y/FLT3 expressing cells. Taken together, our data suggest that TKD-ITD displays similar oncogenic potential to the JMD-ITD but a higher oncogenic potential than the D835Y point mutation.

Keywords FLT3 · Internal tandem duplication · ITD · Receptor tyrosine kinase · Acute myeloid leukemia

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Introduction

FMS-like tyrosine kinase 3 (FLT3) is a member of the class III family of receptor tyrosine kinases. FLT3 receptor is composed of an extracellular ligand-binding domain consisting of five immunoglobulin-like domains, a transmembrane domain, a juxtamembrane domain, and an intracellular kinase domain split into two parts by the so-called kinase insert. Upon binding of its ligand, FLT3 ligand (FL), receptors dimerize and become activated. The activated receptor promotes proliferation, survival, and differentiation of early myeloid and lymphoid precursors [1, 2]. Acute myeloid leukemia (AML) is characterized by clonal expansion of myeloid progenitor cells. Up to 30% of patients with AML harbor a

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mutation in FLT3 thus making it the most frequently mutated gene [3, 4].

The first mutation in FLT3 identified by Nakao and co-workers in 1996 was the so-called internal tandem duplication within juxtamembrane domain of the receptor (JMD-ITD) and for many years, this was thought to be the exclusive location of ITD mutations [4]. Thirteen years later, it was revealed that around 30% of ITDs are located within the tyrosine kinase domain (TKD) region [5]. A few years after the discovery of JMD-ITD, a second type of mutation was discovered in the activation loop of the FLT3 kinase domain, a point mutation at aspartate 835, which is present in around 10% of the AML patients [6]. AML patients with FLT3-ITD are characterized by early relapse and decreased survival in comparison to those expressing wild-type FLT3. Patients harboring ITD mutations within the TKD (TKD-ITD) have a worse survival prognosis in comparison to those with JMD-ITD, the reason for which is still unknown [7]. The length of ITD can be as short as 3 base pairs and up to 400 base pairs and always occurs in frame. Recently, using different JMD-ITD and TKD-ITD mutants, it has been shown that location of the ITD in FLT3 influences its sensitivity to tyrosine kinase inhibitors as well as disease progression in mice [8]. However, the mechanisms by which JMD-ITD and TKD-ITD differ in their oncogenic potentials remain unknown.

Despite the use of new generation kinase inhibitors, certain FLT3 mutations are still associated with high risk of relapse and poor survival. A better understanding of how individual FLT3 mutations contribute to higher risk of relapse and poor survival will help in the design of more effective treatments. Although the JMD-ITD has been well characterized, the TKD-ITDs have due to their recent discovery not yet been thoroughly studied. In this study, we compare ITD mutations in the JMD and TKD, as well as the point mutation located in the TKD, D835Y. The purpose of this study was to investigate whether it is the nature of the mutation or its location that plays a driving role in leukemogenesis. We observed that TKD-ITD mutations have stronger transforming potential that the D835Y mutation.

Material and methods

Cell culture and transfection

Ba/F3 cells expressing JMD-ITD and TKD-ITD constructs were provided by courtesy of T. Fischer and F. H. Heidel [8]. Ba/F3 cells expressing wild-type (WT) FLT3, EV, MIG, and D835Y were stably transfected using retroviral transfection system [9]. The cells were cultured in RPMI 1640 medium (Hyclone, Thermo Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA), 10 ng/ml recombinant murine interleukin 3 (IL3) and 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

Immunoprecipitation and Western blotting

Prior to stimulation with FLT3 ligand (100 ng/ml, 5 min) Ba/F3 cells were starved for 4 h in absence of cytokines and serum. After stimulation, cells were washed with cold PBS before lysis. Cell lysates were prepared by extracting proteins with lysis buffer [40 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet-P40] supplemented with protease inhibitors. For immunoprecipitation, 1 μg of antibody per ml of cell lysate was used. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline and incubated with primary antibodies for overnight at 4 °C. Blots were developed with a peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence (ECL) procedures (Amersham, Arlington Heights, IL), using the manufacturer's protocol. Antibodies used in this study: FLT3 (rabbit polyclonal antibody produced in-house) [10], FLAG (Sigma Aldrich), 4G10 (Millipore), ERK2 (Santa Cruz Biotechnology), pERK1/2 (Santa Cruz Biotechnology), AKT (Santa Cruz Biotechnology), pAKT (Cell Signaling Technology), p38 (BD Transduction Laboratories), pp38 (BD Transduction Laboratories), STAT5 (Abcam), pSTAT5 (Abcam), and tubulin (cell signaling).

Cell viability

Cells were washed three times and resuspended in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. To measure cell viability, 10,000 cells were seeded per well in 96-well plates. For each cell line, cells were treated with either FLT3 ligand (100 ng/ml), 10 ng/ml IL3, or no ligand. Following incubation for 48 h, cell viability was evaluated using AlamarBlue (Molecular Probe) according to the manufacturer's protocol.

Apoptosis

Cells were washed three times and resuspended in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. One hundred thousand cells were seeded per well in a 12-well plate. Each cell line was treated with either FLT3 ligand (100 ng/ml), 10 ng/ml IL3, or no cytokine. After incubation for 48 h, apoptotic cells were measured by flow cytometry using annexin V and 7-aminoactinomycin D (7-AAD) apoptosis kit (BD Biosciences). Cells positive for annexin V and both annexin V/7-AAD were counted as apoptotic cells.

Colony formation

Cells washed three times and resuspended in 20% IMDM medium and 80% methylcellulose medium (Stemcell Technologies). Five hundred cells were seeded in a 24-well plate and cultured for 7 days before counting colonies.

FLT3 degradation assay

Cells were washed three times with PBS to remove cytokines, followed by incubation with 100 μg/ml cycloheximide for 30 min. Cells were then stimulated with FLT3 ligand (100 ng/ml) for 30 min in presence of cycloheximide, followed by lysis of the cells. Cell lysates were subjected to the SDS-PAGE and Western blotting analysis.

Statistical analysis

All statistical analyses were performed using the unpaired, two-tailed Student's t test with $p < 0.05$.

Results

Higher proliferation and survival of cells expressing TKD-ITD compared to cells expressing D835Y

The ability of the cell to evade growth suppression and to divide uncontrollably, as well as its resistance to apoptosis, are some of the hallmarks of cancer [11]. Therefore, we first addressed the question whether there are any differences in viability and apoptosis between the recently identified ITDs—E611V (32) and G613E (33) [8]—and the most common point mutation D835Y, also located within the TKD. Ba/F3 cells stably expressing WT FLT3, D835Y, JMD-ITD 598/599 (22), TKD-ITD E611V (32), and G613E (33) [8] were used to investigate cell viability and apoptosis. An equal number of Ba/F3 cells were plated in three different groups: in the presence or in the absence of IL-3, as well as with the addition of FLT3 ligand (FL). The cells were cultured for 48 h and analyzed by PrestoBlue cell viability assay. WT FLT3 cells, grown in the presence or absence of IL-3, were used as positive and negative controls, respectively. As expected, there was no difference in cell viability in the presence of IL-3 in the cell lines expressing either form of FLT3. We observed that while TKD-ITD, regardless of location, can fully support viability in the absence of cytokines, D835Y-expressing cells displayed reduced viability (Fig. 1a). We also observed a significant increase in apoptosis in cytokine-starved cells expressing the D835Y mutant compared to TKD-ITD-expressing cells (Fig. 1b). Taken together, these results indicate that ITD mutations, regardless of their location in the gene, can support ligand-independent cell viability, whereas the D835Y mutation can only partially support cell viability.

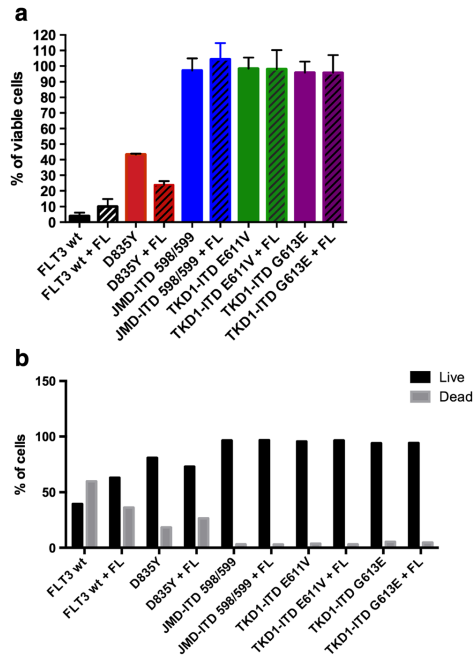

Fig. 1 Higher proliferation and cell survival in TKD-ITD mutants compared to D835Y. **a** Cells were cultured in the absence or presence of FL (100 ng/ml) and normalized against cell viability in the presence of IL-3. Forty-eight hours post-seeding, cell viability was assessed using the AlamarBlue cell viability assay. Each bar represents the mean ± SD of a representative triplicate experiment. **b** Apoptosis was measured using annexin V and 7-AAD kit after 48 h of cytokine depletion

Differences in downstream signaling between ITDs and the D835Y mutation

To investigate the underlying mechanisms that could explain the differences in phenotype between cells expressing ITDs and D835Y located within the tyrosine kinase domain, we compared several signaling pathways downstream of FLT3. For this purpose, Ba/F3 cells expressing either ITD-598/599 (at the JMD; 22), ITD-E611V (32) and ITD-G613E (33) (at the TKD), or D835Y were starved of IL-3 and serum for 4 h, followed by 5-min stimulation with FLT3 ligand (FL). As controls, Ba/F3 cells stably expressing WT FLT3 or empty vector (MIG EV) was used. Cell lysates were prepared and subjected to immunoblotting using specific antibodies against signal transduction intermediates. Previous studies have shown that ITDs at the JMD, but not the point mutation

D835Y in the TKD, can induce tyrosine phosphorylation of STAT5 [12, 13]. We observed a similar pattern between the point mutant and ITDs located within the TKD (Fig. 2). However, cells expressing D835Y showed very weak tyrosine phosphorylation of STAT5, whereas TKD-ITDs showed strongest STAT5 phosphorylation (Fig. 2). In a striking manner, constitutive activation of AKT in Ba/F3 cells expressing D835Y was stronger compared to the ITDs (Fig. 2). Meanwhile, the phosphorylation levels of ERK and p38 remained at the same level in cell lines expressing either D835Y or ITDs. The tyrosine phosphorylation of FLT3 did not show any difference between different FLT3 mutants, except for TKD-ITD E611V (32), where we could observe increased ligand-induced phosphorylation of the receptor (Fig. 2).

The TKD-ITDs and D835Y display different transforming potential

To further analyze the transforming potential of ITDs and D835Y, the colony-forming capacity in cytokine-free methylcellulose medium was evaluated. The cells were washed to remove IL-3 and cultured in methylcellulose medium for 7 days, and the number of colonies was counted. As expected, WT FLT3 cells deprived of IL-3 failed to form any colonies.

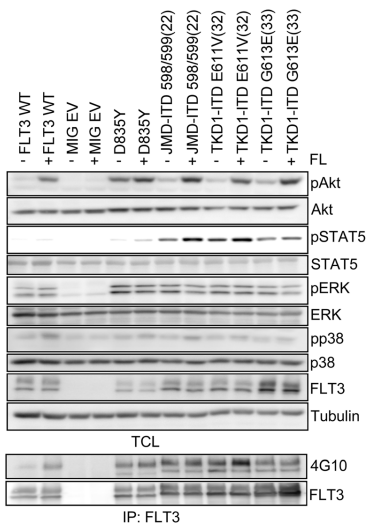

Fig. 2 Activation of downstream signal transduction pathways by the different oncogenic mutants of FLT3 Ba/F3 cells were starved in serum- and IL-3-free media for 4 h before stimulating with 100 ng/ml of FL for 5 min. Cells were then lysed and lysates were used for SDS-PAGE and Western blotting analysis. The membrane was probed for different downstream signaling proteins and their phosphorylated forms

Ba/F3 cells expressing ITD mutants showed similar number of colonies regardless of ITD location, in agreement with a previous report [8]. In contrast to TKD-ITD, the expression of D835Y led to a significantly decreased number of colonies (Fig. 3a, b). Along with a count of the number of colonies, the area of each colony was also measured. Despite a lower number of colonies formed by D835Y cells, the areas of the colonies were larger, but not as evenly shaped as those formed by ITDs (Fig. 3c). Inspecting the cells expressing TKD-ITD-E611V (32) and TKD-ITD-G613E (33), we observed no change in the number of colonies, although the size of the colonies differed (Fig. 3c). We can conclude that ITDs at the TKD are capable for growth factor-independent proliferation and clonal growth of single cells, whereas the D835Y point mutant is more similar in its phenotype to ligand-activated WT FLT3. Overall, these results show that the transforming capacity of the TKD-ITD mutations is stronger than that of the D835Y mutant.

Regardless of the nature of the activating FLT3 mutations, the stability of FLT3 remains unaltered

Finally, we analyzed whether the point mutation D835Y within TKD or ITDs within JMD or TKD have any effect on FLT3 stability. Ba/F3 cells were treated with cycloheximide for 30 min in order to stop protein synthesis, followed by immediate lysis of the cells or by stimulation with FL for additional 30 min in the presence of cycloheximide. We did not observe any differences in FLT3 degradation (Fig. 4a). In Fig. 4b, the quantification and normalization of three independent Western blots indicated no significant change. Therefore, the stability of the FLT3 receptor is not influenced by the nature of the activating mutations.

Discussion

Since the discovery of FLT3 mutations, they have been intensively studied both regarding their molecular biology and their clinical relevance. Over the past years, new generations of tyrosine kinase inhibitors were developed; nevertheless, the relapse of patients with AML remains an undeniable problem. The number of relapsed patients with D835 point mutations is relatively low, suggesting that FLT3 point mutations are not involved in the development of the relapse [14]. In contrast, patients with the ITD mutations are associated with higher rates of relapse and poor overall survival [15, 16]. The purpose of this study was to directly compare recently characterized ITDs within TKD and the well-known point mutation D835Y within TKD, to understand whether the mutation's nature or location plays the driving role in leukemogenesis. All three types of FLT3 mutations result in the constitutive ligand-

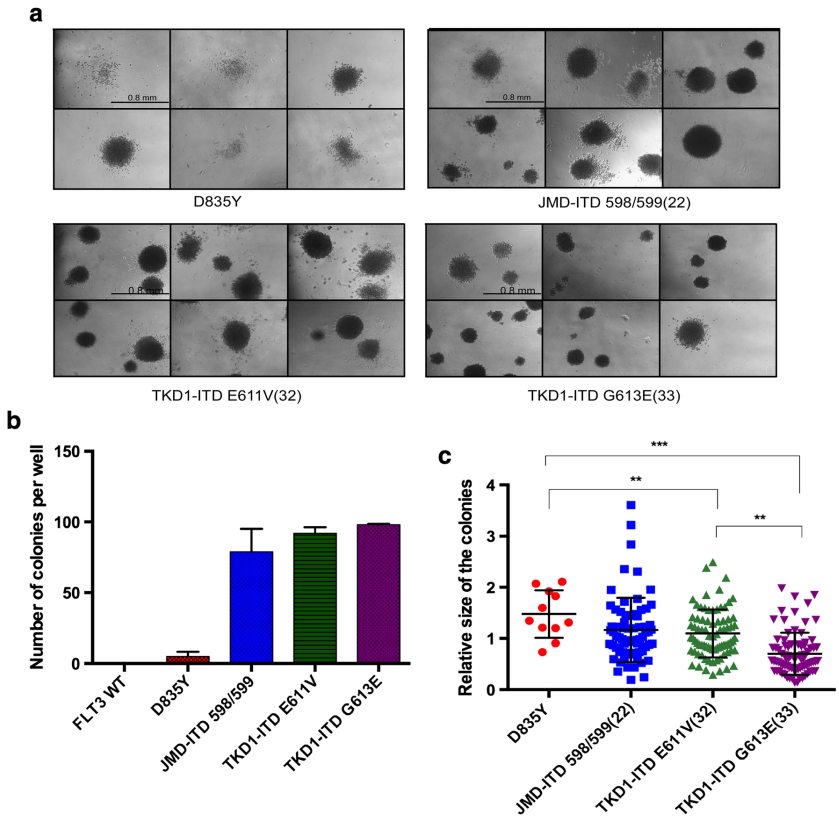

Fig. 3 Both JM-ITD and TKD-ITD induce clonogenic growth in semisolid media in the absence of IL-3, while D835Y fails to induce colonies. The Ba/F3 cells stably expressing the indicated FLT3 mutants were plated in triplicate at a concentration of 500 cells per well. The plates were cultured for 7 days. **a** Photographs of the representative areas of the wells demonstrating the number of colonies. WT FLT3-expressing Ba/F3 cells failed to induce colonies (data not shown) **b** Quantification of the number of colonies per plate. Each bar represents the mean ± SD of a representative triplicate experiment. **c** The total area of each colony was analyzed with help of ImageJ. The data presented indicate the relative size of the colonies (*p value = $p < 0.05$)

independent activation; nevertheless, the downstream players and transformation capabilities are different.

In spite of mutations being located within the tyrosine kinase domain, only ITDs are capable of activating STAT5. It is likely that the TKD-ITDs localizes in the same manner, as was previously reported for JMD-ITDs, to the endoplasmic reticulum (ER) where it aberrantly activates STAT5 [17]. It has been shown that STAT5 activation by ITDs located within JMD domain is mediated through the SRC family kinases [18]. It was also shown that phosphorylation of the SRC binding sites in FLT3, Y589 and Y591, were phosphorylated to a higher extent in FLT3-ITD compared to FLT3/D835Y, which could explain the stronger STAT5 phosphorylation in FLT3-ITD compared to FLT3/D835Y. It is not completely known which SRC family members are involved in phosphorylation. We have previously demonstrated that both FYN and LCK can positively contribute to FLT3-ITD-mediated STAT5 phosphorylation [19, 20]. Regardless of the mechanism, the aberrant activation of STAT5 is an essential step in myeloid transformation [21, 22]. Therefore, cells expressing ITDs independent of their location in the gene show a stronger phenotype in terms of activation of STAT5 and thus proliferation rate and anti-apoptotic activity in comparison to D835Y.

Some signaling pathways, such as the phosphorylation of ERK and p38, were very similar between the various FLT3 mutants. In contrast, cells expressing the D835Y mutant of

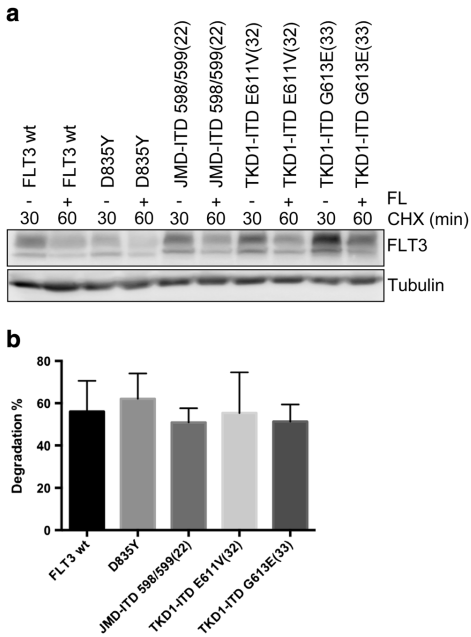

Fig. 4 Stability of the various FLT3 mutants. **a** Cells were treated with cycloheximide for 30 min and lysed or stimulated with FL (100 ng/ml) for additional 30 min. Western blotting analysis was used to measure the degree of degradation. **b** The graph presents a summary of quantified degradation of the FLT3 receptor. Each bar represents the mean ± SD of a representative triplicate experiment

FLT3 displayed constitutive AKT activation, whereas the ITD mutants displayed stronger ligand-induced AKT phosphorylation. The reason for this discrepancy is not completely clear. We know that the D835Y mutation not only leads to constitutive activity of FLT3, but that the cells expressing D835Y are independent of SRC kinase activity for transformation [18]. This might be explained by altered kinase specificity of the D835Y mutant compared to wild-type FLT3. In the closely related KIT, mutation of the analogous site D816 to valine leads not only to constitutive activity of the receptor, but also to an altered kinase specificity resembling the kinase activity of SRC family kinases [23]. The reason for ligand-induced AKT phosphorylation is not clear. It seems counter intuitive that a mutant that is trapped in the ER could have the ability to induce AKT activity in a ligand-dependent manner. However, even though a majority of FLT3-ITD remain trapped in the ER, there is also FLT3-ITD expression on the cell surface and some signaling pathways are activated from the cell surface, such as activation of KRAS [24]. Activation of PI3-kinase/AKT by FLT3 is known to occur through the scaffolding protein GAB2, which is phosphorylated by SRC family kinases and the subsequent recruitment of PI3-kinase to GAB2 [25, 26]. Thus, it is likely to be the mechanism behind the stronger ligand-induced AKT in the ITD mutants.

Degradation of receptor tyrosine kinases has been shown to be regulated by CBL-mediated ubiquitination, leading to internalization and degradation of the receptors. It is known that CBL-mediated ubiquitination in many cases is regulated by SRC-mediated tyrosine phosphorylation [27]. Thus, we hypothesized that there might be a difference in the stability of FLT3 depending on the nature of the oncogenic mutation. However, we found no difference in stability between the various oncogenic mutants of FLT3.

The presented results raise interesting questions about the role of the location and the nature of the mutation in the molecular pathogenesis of AML. In this study, we chose to only compare TKD-ITDs to FLT3/D835Y. However, it should be noted that, despite D835Y being the most frequent point mutation in AML patients, several other activating mutations have been reported in this region including other substitutions of D835 and small deletions or insertion mutations. Furthermore, the ITD mutations are located to the first part of the tyrosine kinase domain, whereas the FLT3/D835Y mutation is localized to the second part of the TKD, after the so-called kinase insert. One of the characterized FLT3-ITD mutations is located within the second part of TKD region (A627E), sharing a similar location to the point mutation D835Y [5].

In this study, we have used a specific FLT3-ITD sequence (N51; [28]); however, the sequence and the length of ITD mutations is extremely heterogeneous and varies from patient to patient [29]. In previous studies, it has been reported that in patients with FLT3-ITD mutations, the length of the ITD may change after therapy [30]. Additionally, a longer ITD size is associated with shorter overall and relapse-free survival and it has been suggested that FLT3-ITD size has a prognostic significance in AML [31]. Therefore, comparison of different ITD mutants will provide information on why the length of the ITD is important for patient survival. Taken together, our current study suggests that in the BaF3 cell model, the location of ITD mutations is of minor importance for transformation and that the D835Y mutation has a weaker transforming capacity than ITD in the TKD. In a previous study, it was found that FLT3-ITD location influences disease biology in vivo and leads to changes in global gene expression. ITD location altered proliferative capacity and sensitivity to FLT3-TKI treatment in vivo [8]. Therefore, it will be of major interest to study sensitivity of the FLT3-mutated clones and response rates to FLT3 inhibitor therapy in patients harboring JM-ITD versus TKD-ITD mutations.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Paper III

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Research Paper

ABL2 suppresses FLT3-ITD-induced cell proliferation through negative regulation of AKT signaling

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ABSTRACT

The type III receptor tyrosine kinase FLT3 is one of the most commonly mutated oncogenes in acute myeloid leukemia (AML). Inhibition of mutated FLT3 in combination with chemotherapy has displayed promising results in clinical trials. However, one of the major obstacles in targeting FLT3 is the development of resistant disease due to secondary mutations in FLT3 that lead to relapse. FLT3 and its oncogenic mutants signal through associating proteins that activate downstream signaling. Thus, targeting proteins that interact with FLT3 and their downstream signaling cascades can be an alternative approach to treat FLT3-dependent AML. We used an SH2 domain array screen to identify novel FLT3 interacting proteins and identified ABL2 as a potent interacting partner of FLT3. To understand the role of ABL2 in FLT3-mediated biological and cellular events, we used the murine pro-B cell line Ba/F3 as a model system. Overexpression of ABL2 in Ba/F3 cells expressing an oncogenic mutant of FLT3 (FLT3-ITD) resulted in partial inhibition of FLT3-ITD-dependent cell proliferation and colony formation. ABL2 expression did not alter the kinase activity of FLT3, its ubiquitination or its stability. However, it partially blocked FLT3-induced AKT phosphorylation without affecting ERK1/2 and p38 activation. Taken together our data suggest that ABL2 acts as negative regulator of signaling downstream of FLT3.

INTRODUCTION

The mammalian genome encodes more than 500 protein kinases that contribute to the regulation of almost all cellular events. Around 60 protein kinases are characterized as receptor tyrosine kinases which are regulated by extracellular stimuli including growth factors [1, 2]. The type III receptor tyrosine kinase family includes the receptors for platelet derived growth factors (PDGFRA and PDGFRB), the receptor for stem cell factor (SCFR or KIT), FMS-like tyrosine kinase 3 (FLT3, the receptor for FLT3 ligand, FL) and the colony-stimulating factor 1 receptor (CSF1R). Several members of this family are important regulators of the hematopoietic system and have been implicated in various hematological malignancies including acute myeloid leukemia (AML). AML originates from the myeloid lineage of hematopoietic cells [3] and more than 30% of AML patients carry an oncogenic mutation in the FLT3 gene [4]. FLT3 and other type III receptor tyrosine kinases share common domain arrangements such as an extracellular ligand binding domain, a transmembrane domain, a juxtamembrane domain and a kinase domain (split by a short kinase insert). Its ligand, FL, forms spontaneous dimers and binds to the extracellular domain of FLT3 and thereby induces dimerization of FLT3 which further promotes activation

of its intrinsic kinase activity and autophosphorylation on several tyrosine residues. Phosphotyrosine residues are well-known as docking sites for SH2 domain-containing signaling proteins that regulate, depending on the characteristic of the partner protein, either the activation or inhibition of signaling downstream of the receptor. For instance, ubiquitin E3 ligases such as CBL, SOCS2 and SOCS6 bind to FLT3 and negatively regulate downstream signaling. In contrast, the adaptor proteins GRB10 [5] and GADS [6], and the non-receptor tyrosine kinases SYK [7] and FYN [8], enhance downstream signaling.

The mammalian Abelson (ABL) family of non-receptor tyrosine kinase includes the two members ABL and ABL2 (also called ARG, ABL-related gene). ABL and ABL2 transduce signals from upstream receptors and regulate numerous biological processes such as cell survival, apoptosis, response to genotoxic stress, morphogenesis and cell motility [9]. ABL family kinases have been implicated in leukemia as the BCR-ABL fusion gene [10]. The BCR-ABL fusion gene is the major oncogene in chronic myelogenous leukemia (CML). The BCR-ABL fusion gene has also been reported less frequently in acute lymphoblastic leukemia (ALL) and rarely in AML [11]. Like ABL, ABL2 forms fusion genes with TEL transcription factors. However, this is a rare event in AML [12]. Recent studies suggest that, besides gene fusions, expression of ABL family kinases is upregulated in several cancers such as pancreatic cancer, anaplastic thyroid cancers, colorectal cancer, melanoma and non-small-cell lung cancers [13-16]. ABL family kinases regulate invasion, proliferation and survival mediated by the epidermal growth factor receptor (EGFR), the insulin-like growth factor receptor (IGFR), HER2 and SRC kinases [17-21]. However, the role of this family of kinases has not been studied with respect to signaling downstream of FLT3. Here we identify ABL2 as an FLT3 interacting protein and show that ABL2 plays a role in signaling downstream of FLT3.

RESULTS

Identification of ABL2 as a FLT3 binding protein

Receptor tyrosine kinases such as FLT3 signal through proteins that associate with the activated receptor. In order to identify novel FLT3-interacting proteins we used an SH2 domain array [22]. Seventy-four recombinant SH2 domains from 64 different proteins were used. Three different tyrosine phosphorylated peptides corresponding to residues Y726, Y793 and Y842 in FLT3 were used to determine the binding to the SH2 domains. We observed that the ABL2 SH2 domain displayed the highest affinity to the tyrosine phosphorylated FLT3 peptides (Figure 1A). Other associating proteins included several SRC family kinases, SOCS6, ABL, CRK, CRKL etc. Furthermore, we could show that association between ABL2 and FLT3 is FL-dependent and that oncogenic FLT3-ITD displays constitutive association with ABL2 (Figure 1B).

ABL2 expression negatively regulates FLT3-ITD-mediated cell viability and colony formation

To explore the role of ABL2 in oncogenic FLT3-ITD-mediated biological responses, we generated Ba/F3 cells overexpressing both FLT-ITD and ABL2 or empty control vector (EV). FLT3-ITD surface expression was analyzed by flow cytometry and the total expression was determined by western blotting. The results demonstrate an equal surface (Figure 2A) as well as total (Figure 2B) expression of FLT3-ITD in both ABL2 and EV expressing cells. We used these cell lines to determine the dependency of ABL2 for cell viability and colony formation. Cell viability was determined using the dye PrestoBlue. We observed that cells expressing ABL2 displayed significantly reduced viability when compared to cells expressing empty control vector (Figure 2C). However, ABL2 expression did not affect the apoptotic rate (Figure 2D). The potential to form colonies in semi-solid medium was also reduced in cells expressing ABL2 (Figure 2E).

ABL2 expression negatively regulates AKT phosphorylation

Wild-type FLT3 activates the PI3K/AKT, RAS/ERK and p38 pathways in response to FL-stimulation while FLT3-ITD constitutively activates those pathways [23-25]. Since signaling downstream of the wild-type receptor is controlled by FL stimulation, we generated Ba/F3 cell lines expressing wild-type FLT3 and ABL2 or EV in order to study how ABL2 regulates signaling downstream of FLT3. Flow cytometry and western blotting experiments verified equal surface (Figure 3A) as well as total (Figure 3B) expression of wild-type FLT3 in cells expressing ABL2 or empty vector. The expression of ABL2 partially blocked FLT3-mediated AKT phosphorylation (Figure 3C). However, ABL2 expression did not alter FL-induced ERK1/2 (Figure 3D) or p38 (Figure 3E) phosphorylation.

ABL2 expression has no effect on FLT3 activation, ubiquitination or degradation

A majority of the proteins that associate with receptor tyrosine kinases affect either receptor activation, ubiquitination or stability [26, 27]. We analyzed whether ABL2 has a role in regulating FLT3 protein stability. Ba/F3-FLT3-EV and Ba/F3-FLT3-ABL2 cells were treated with cycloheximide for 30 minutes, in order to block protein synthesis, and then stimulated with FL for 30 minutes in the presence of cycloheximide. We did not observe any difference in FLT3 degradation whether ABL2

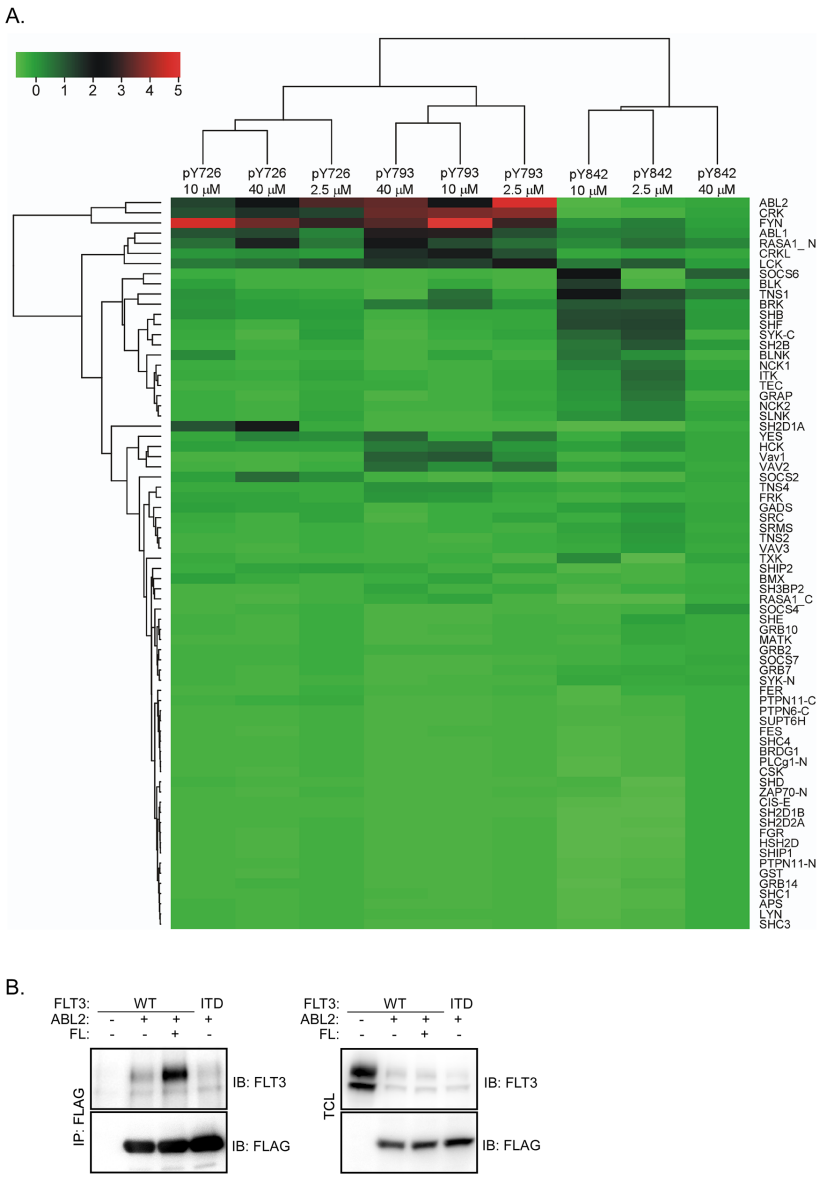

Figure 1: ABL2 binds to FLT3 in response to FL stimulation: A. An SH2 domain array screen identifies ABL2 as a novel FLT3-associating protein. Seventy-four SH2 domains from different proteins were used. **B.** COS-1 cells were transfected with plasmids expressing FLT3-WT and empty vector or FLAG-tagged ABL2. After 24 hours of transfection cells were stimulated for 5 minutes with 100 ng/ml FL followed by lysis. Lysates were immunoprecipitated using an anti-FLAG antibody.

was overexpressed or not (Figure 4A). ABL2 expression neither altered FL-induced tyrosine phosphorylation nor ubiquitination of FLT3 (Figure 4B).

DISCUSSION

Signaling downstream of receptor tyrosine kinases is tightly controlled by various associating proteins such as kinases, phosphatases, ubiquitin E3 ligases, adaptors and scaffolding proteins. In order to maintain a proper balance in signaling, many interacting proteins enhance receptor signaling, but some of them turn off the signal by dephosphorylating the receptor or directing the receptor for ubiquitin-mediated degradation. Thus, the level of signaling is maintained at a level that is appropriate for the cell. Too much RTK signaling can be deleterious and lead to induction of apoptosis [28]. Here we identify ABL2 as a novel FLT3-associating protein using an SH2 domain array screening. We show that ABL2 negatively regulates FLT3-ITD-mediated biological events through partial inhibition of AKT signaling.

The recombinant SH2 domain of ABL2 displayed a high affinity for multiple phosphopeptides corresponding to known FLT3 tyrosine phosphorylation sites. In addition to the ABL2 SH2 domain, the SH2 domains of CRK, CRKL, FYN, ABL, LCK, SOCS6, SYK etc. also displayed a considerable affinity for those phosphopeptides. Many of those proteins have already been verified by biochemical methods and identified as critical regulators of FLT3 signaling [7, 8, 29]. ABL2 associates with FLT3 only when FLT3 is activated, suggesting that FLT3 tyrosine phosphorylation is required for the interaction and that the interaction is mediated through pY-SH2 binding.

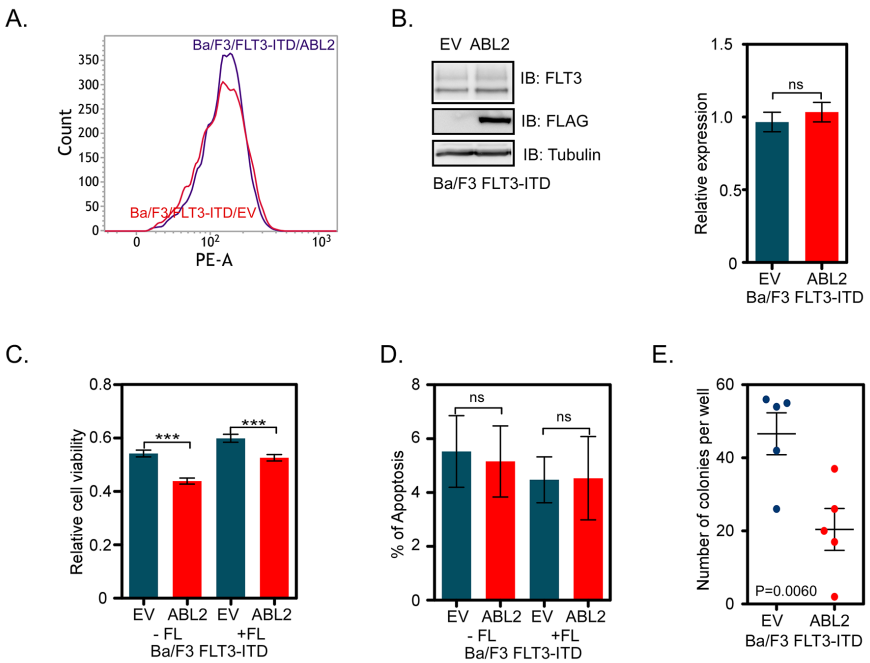

Figure 2: ABL2 expression reduces FLT3-ITD-mediated cell viability and colony formation: A. Ba/F3-FLT3-ITD cells expressing ABL2, or empty vector were labeled with phycoerythrin-conjugated anti-FLT3 antibody. Samples were then analyzed by flow cytometry. **B.** Ba/F3-FLT3-ITD cells expressing ABL2, or empty vector were lysed. Equal amount of proteins from total cell lysates were subjected to Western blot analysis. Blots from multiple experiments were quantified and analyzed using student's t-test. **C.** Ba/F3 cells expressing FLT3-ITD and ABL2 or FLT3-ITD and empty vector were used for the PrestoBlue cell viability assay. **D.** Cells positive for Annexin V and 7-aminoactinomycin D (7-AAD) or only for Annexin V were counted as apoptotic cells. **E.** Cells were washed to remove IL-3 and serum. Cells were mixed with 80% methylcellulose medium and seeded in a 24-well plate. ns, not significant; ***, $p<0.001$.

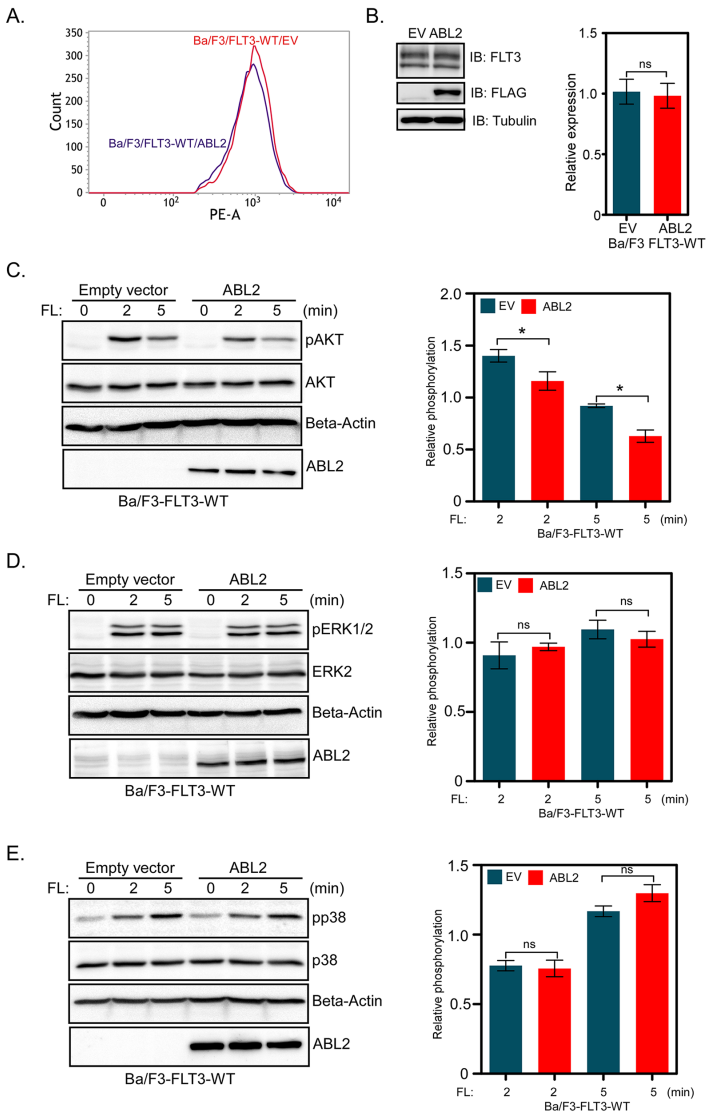

Figure 3: Ba/F3-FLT3 cells expressing ABL2 display reduced AKT phosphorylation: A. Ba/F3 cells expressing FLT3 and ABL2 or empty vector were labeled with phycoerythrin-conjugated anti-FLT3 antibody and then analyzed by flow cytometry. **B.** Ba/F3-FLT3-ABL2 and Ba/F3-FLT3-EV cells were lysed, and lysates were analyzed by Western blotting. Blots from multiple experiments were quantified and analyzed using student t-test. (C-E) Ba/F3-FLT3-WT cells expressing ABL2 or empty vector were washed to remove IL-3 and serum starved four hours before FL stimulation. Total cell lysates were used for Western blotting analysis using anti-phospho-AKT antibody **C.**, anti-phospho-ERK1/2 antibody **D.** and anti-phospho-p38 antibody **E.** Multiple blots were quantified and subjected to statistical analysis. ns, not significant; *, $p<0.05$.

FLT3 and its oncogenic counterpart, FLT3-ITD, exert survival, proliferative and transforming signals by activating mainly three different signaling cascades, i. e. the PI3K/AKT, RAS/ERK and p38 pathways [24, 25]. When ABL family proteins form fusion proteins with BCR or TEL they mediate signals that lead to cell proliferation, survival and transformation [12]. Depletion of ABL2 in non-small cell lung carcinoma cell lines led to decreased cell growth [30]. However, the effect of ABL2 expression seems to some extent to be context dependent, since in a breast cancer model, loss of ABL2 accelerated tumor growth by enhancing cell proliferation [21]. In line with these findings, we also observed that overexpression of ABL2 reduced FLT3-ITD-induced cell proliferation as well as colony formation. Although ABL2 is a tyrosine kinase, overexpression of ABL2 did not increase the total tyrosine phosphorylation of FLT3 suggesting that ABL2 is not involved in the FLT3 activation process. Furthermore, association of ABL2 did not affect FLT3 ubiquitination or degradation. Therefore, it is likely that ABL2 has no direct effect on FLT3 but that association of ABL2 with FLT3 disrupts selective downstream signaling pathways e. g. the AKT pathway. In contrast to its close relative ABL1, ABL2 seems to be a negative regulator of signaling and transformation. This observation may have different explanations: ABL2 might compete for binding to FLT3 with proteins essential for activation of the AKT pathway or ABL2 directly targets FLT3 downstream signaling proteins. The mechanism behind these signaling events will be further investigated in future studies.

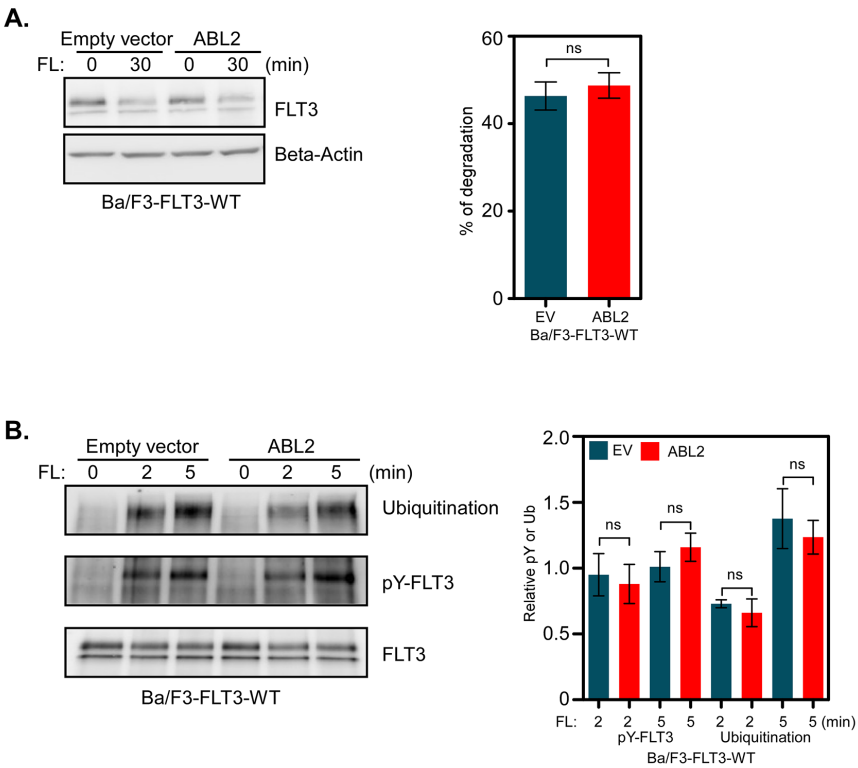

Figure 4: ABL2 expression does not alter FLT3 activation, ubiquitination or degradation: A. Cells were incubated with cycloheximide before stimulation with FL. Total cell lysates were used for Western blot analysis. Multiple blots were quantified for statistical analysis. **B.** Ba/F3-FLT3-WT cells expressing ABL2 or empty vector were washed to remove IL-3 and serum starved for four hours followed by FL stimulation, lysis and immunoprecipitation using an anti-FLT3 antibody. ns, not significant.

MATERIALS AND METHODS

Reagents and antibodies

Lipofectamine 2000 and horseradish peroxidase (HRP)-coupled secondary anti-mouse and anti-rabbit antibodies were from ThermoFisher Scientific (Waltham, MA). Cycloheximide and mouse anti-FLAG (M2) antibody were from Sigma-Aldrich (St Louis, MI). FLT3 ligand (FL) was from ORF Genetics (Reykjavik, Iceland). Rabbit anti-phospho-AKT (pSer473), mouse anti-phosphotyrosine (4G10), mouse anti-mono-ubiquitin antibodies were from Abcam (Cambridge, UK), Merck Millipore (Billerica, MA) and Covance Research Products (Princeton, NJ), respectively. Rabbit anti-phospho-ERK1/2 (pThr202/pThr204), goat anti-AKT, rabbit anti-ERK2 and anti-goat secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The phycoerythrin-labeled anti-FLT3, mouse anti-phospho-p38 and anti-p38 antibodies were from BD Transduction Laboratories (Franklin Lakes, NJ). Rabbit anti-FLT3 antibody was described previously [29].

Plasmids

Expression plasmids pcDNA3-FLT3-WT, pcDNA3-FLT3-ITD, pMSCVpuro-FLT3-WT and pMSCVpuro-FLT3-ITD plasmids were previously described [29]. Human full-length ABL2 plasmids in pCMV-Myc-FLAG vector was obtained from OriGene (Rockville, MD). Retroviral plasmid pMSCVneo-ABL2-WT-Myc-FLAG was generated by ligating ABL2-WT-Myc-FLAG fragment into the pMSCVneo vector.

Cell culture

COS-1 and Ba/F3 cells were purchased from DSMZ (Braunschweig, Germany). EcoPack cells were from Clontech (Mountain View, CA). Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100µg/ml streptomycin was used for maintaining COS-1 and EcoPack cells. RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 10 ng/ml recombinant murine interleukin-3 (IL-3), 100 units/ml penicillin and 100µg/ml streptomycin was used for Ba/F3 cells.

Transient transfection

Lipofectamine 2000 was used for transient transfection. COS-1 cells were transfected with pCMV-Myc-FLAG and pcDNA3 plasmids according to the manufacturer's protocol.

Stable transfection

Retroviral vector pMSCV was used to produce virus particles in EcoPack packaging cells. EcoPack cells were transfected with 10µg pMSCVpuro-FLT3-WT, pMSCVpuro-FLT3-ITD, pMSCVneo-ABL2-Myc-FLAG or pMSCVneo constructs using Lipofectamine 2000 followed by collection of virus-containing supernatants after 72 hours of transfection. Then Ba/F3 cells were infected with FLT3-WT or FLT3-ITD virus particles. Cells were selected using 1.2 µg/ml puromycin for two weeks and FLT3 expression was checked by flow cytometry and Western blotting. Ba/F3-FLT3-WT and Ba/F3-FLT3-ITD cells were further infected with ABL2 or empty vector virus particles and then further selected against 0.8 mg/ml G-418 for 2 weeks. ABL2 expression was verified using Western blotting. All stably transfected Ba/F3 cells were maintained in Ba/F3 medium as described above and recommended previously [31].

Immunoprecipitation and western blotting

Ba/F3 cells were washed three times and then starved of IL3 and serum for 4 hours in RPMI-1640 before stimulation with 100 ng/ml of FL. Cells were then washed with ice-cold PBS and lysed in 1% Triton X-100 lysis buffer. For immunoprecipitation of 1 ml cell lysates one µg primary antibody was used. The Western blotting and immunodetection procedures were described elsewhere [32, 33].

SH2 domain array screening

Screening of SH2 domain specificity of the various phosphopeptides was performed by Dr. Shawn Li's Laboratory, University of Ontario, as a service. Peptide synthesis, array constructions and assay conditions were essentially as described elsewhere [34].

Cell viability

Ba/F3-FLT3-ITD cells expressing ABL2 or empty vector were washed three times and resuspended in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin and 100µg/ml streptomycin. Cells were then seeded in a 96-well plate (10,000 cells per well). Two days after seeding, cell viability was counted by PrestoBlue (Molecular Probes, Eugene, OR) assay.

Apoptosis

Ba/F3-FLT3-ITD cells expressing ABL2 or empty vector were washed three times and resuspended in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin and 100µg/ml streptomycin. Cells were then seeded in a 12-well plate (100,000 cells per well). Two days after seeding, apoptosis was measured by flow cytometry using Annexin V and 7-aminoactinomycin D (7-AAD) apoptosis kit (BD Biosciences, Franklin Lakes, NJ).

Colony formation assay

Ba/F3-FLT3-ITD cells expressing ABL2 or empty vector were washed three times and 1,000 cells were mixed with 500 µl of 80% methylcellulose medium and seeded in a 24-well plate. Cells were incubated for 7 days and colonies were counted by two individual researchers.

FLT3 degradation assay

Ba/F3-FLT3-WT cells expressing ABL2 or empty vector were washed three times with PBS and treated with 100 µg/ml cycloheximide for 30 minutes. Cells were then stimulated with FL for 30 minutes in presence of cycloheximide followed by lysis in Triton X-100 lysis buffer. Cell lysates were subjected to the Western blotting analysis.

Statistical analysis

Blots were quantified using ImageJ software and statistical analysis was performed using GraphPad Prism 5.0. Data were presented as the mean ± SE. One-way ANOVA with Bonferroni's post-tests and student's t-test were used to check statistical significance.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interests.

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Paper IV

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The Src family kinase LCK cooperates with oncogenic FLT3/ITD in cellular transformation

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The non-receptor tyrosine kinase LCK belongs to the SRC family of kinases. SRC family kinases are proto-oncogenes that have long been known to play key roles in cell proliferation, motility, morphology and survival. Here we show that LCK regulates the function of the type III receptor tyrosine kinase FLT3 in murine pro-B cells. We observed that expression of LCK significantly enhances the colony forming capacity of the constitutively active FLT3 mutant FLT3-ITD (internal tandem duplication). Furthermore, cells expressing LCK developed tumor earlier compared to cells transfected with empty control vector. Staining of the tissues from mouse xenografts showed higher Ki67 staining in cells expressing LCK suggesting that expression of LCK enhances the FLT3-ITD-mediated proliferative capacity. LCK expression did not affect either FLT3-WT or FLT3-ITD -induced AKT, ERK1/2 or p38 phosphorylation. However, LCK expression significantly enhanced FLT3-ITD-mediated STAT5 phosphorylation. Taken together, our data suggest that LCK cooperates with oncogenic FLT3-ITD in cellular transformation.

Oncogenic mutations or overexpression of tyrosine kinases are very common in a wide range of cancers. Several members of type III receptor tyrosine kinases including FLT3, KIT and CSF1R have been implicated in hematopoietic malignancies[1,2]. FLT3 was found to be mutated in as high as 35% of acute myeloid leukemia (AML) and in a small portion of acute lymphoblastic leukemia (ALL)[3,4]. One of the most common FLT3 mutations includes the internal tandem duplication (ITD) in the juxtamembrane domain of the receptor. Although the wild-type receptor needs its ligand, FLT3 ligand (FL), to trigger downstream signaling, FLT3-ITD is constitutively active and can activate downstream signaling cascade in the absence of ligand stimulation. The downstream signaling is tightly controlled by associating proteins, which directly or indirectly interact with the activated receptor. Associating proteins include protein kinases, protein phosphatases, ubiquitin ligases and adaptor proteins[5–12]. Protein kinase, such as SYK[6] and FYN[13], cooperate with oncogenic FLT3-ITD, while CSK[14] and ABL2[15] partially block mitogenic signaling. The protein tyrosine phosphatase DEP1 negatively regulates FLT3-ITD-mediated colony formation[16] and loss of STS1/STS2 function results in hyperactivation of FLT3[11]. In contrast, association of another phosphatase, SHP2, seems to be essential for FLT3-ITD-mediated cellular transformation[17]. These findings suggest that the role of protein kinases or phosphatases cannot be simplified and specific kinase or phosphatase can act as negative or positive regulators of FLT3 signaling. Furthermore, although several E3 ubiquitin ligases such as SOCS2[18], SOCS6[19], SLAP[20] and SLAP2[9] accelerate ubiquitination-directed degradation of FLT3, signaling molecules play diverse roles in regulating mitogenic signaling. For instance, SLAP depletion partially blocked activation of FLT3 downstream signaling cascades[20] while depletion of SOCS6 accelerated mitogenesis[19]. Therefore, knowledge of individual FLT3 interacting proteins is required in order to understand how FLT3 downstream signaling is regulated. The lymphocyte-specific protein tyrosine kinase, LCK, is a member of the SRC family of kinases (SFKs). SFKs are a family of 11 non-receptor tyrosine kinases[21]. LCK has important functions in T cell development, homeostasis and activation[22]. LCK knockout mice display a strong decline in the CD4 and CD8 positive thymocyte population and carry only a few peripheral T cells[23]. Although LCK under normal physiological conditions primarily is expressed in T cells and in some subpopulations of B cells[24], it is highly expressed both in B and T cell leukemia[25,26] and contributes to the malignant phenotype. Loss of LCK expression in T-cell leukemia cells, or peripheral T lymphocytes, results in impaired T cell receptor activation[27,28]. In B-cell leukemia,

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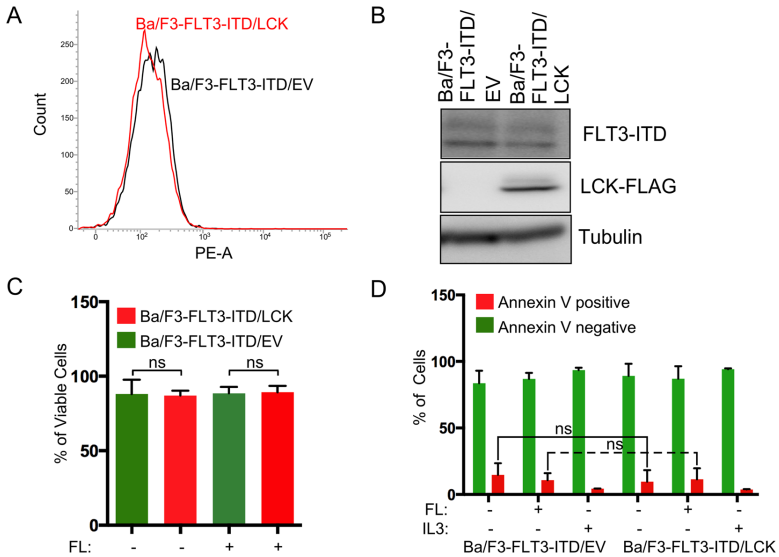

Figure 1. LCK expression does neither alter FLT3-ITD-mediated cell viability nor apoptosis. (**A**) Ba/F3-FLT3-ITD cells expressing LCK, or empty vector (EV), were labeled with phycoerythrin-conjugated anti-FLT3 for analysis with flow cytometry. (**B**) Ba/F3-FLT3-ITD transfected with LCK or empty vector were subjected to Western blot analysis for analysis of total expression of FLT3. Three independent experiments were quantified. The blots were cropped to focus upon the specific proteins indicated. (**C**) Cell viability was assessed by adding Prestoblue reagent 48 h post seeding. The graph represents relative cell viability with or without FL stimulation. (**D**) Cells were washed to remove IL3 and seeded in a 12-well plate in IL3 free medium. After 48 h cells were stained with phycoerythrin (PE)-labeled annexin V and 7-aminoactinomycin D (7-AAD). Cells were then analyzed using flow cytometry. Cells positive for annexin V and 7-aminoactinomycin D (7-AAD), or only for annexin V, were counted as apoptotic cells. Not significant, ns.

cells with hyperphosphorylated FLT3 also display high levels of LCK phosphorylation[29] suggesting a possible role of FLT3 in LCK activation or *vice versa*.

Apart from cells of hematopoietic origin, LCK is also aberrantly expressed in a number of other cancer types, including breast cancer, colon cancer and small cell lung carcinoma[30–32] suggesting that it has general cancer promoting activities. Several studies have reported high levels of LCK expression in acute myeloid leukemia. Early studies indicated high expression of LCK in leukemic cells from patients with less differentiated AML, i. e. AML-0 and AML-1[33]. However, a role of LCK in FLT3-dependent AML has not yet been defined. In this report we show, using the proB cell line Ba/F3 that lacks endogenous LCK, that LCK expression is not essential for wild-type FLT3 signaling but plays an important role in oncogenic FLT3-ITD-mediated cellular transformation.

Results

LCK expression is dispensable for FLT3-ITD dependent cell viability and survival. It is long been known that SRC family kinases (SFKs) play important roles in mitogenic signaling. SFKs act as an intermediate mediator of various receptor tyrosine kinases. We have shown that SRC[34,35] and FYN[13] bind to FLT3 and cooperate with FLT3-ITD in cellular transformation. To understand the role of another SFK, LCK, we generated Ba/F3 cells stably expressing FLT3-ITD and either empty vector or LCK. FLT3 surface expression was analyzed by flow cytometry (Fig. 1A) and the total protein expression was measured by Western blotting (Fig. 1B). FLT3 surface and total expression appeared to be the same for both LCK and empty vector expressing cells, making it a suitable model for studying the impact of LCK on FLT3-ITD mediated biological events. To investigate whether LCK has any effect on cell growth, we examined the cell viability using PrestoBlue assay. Forty-eight hours post seeding of cells we observed, in comparison to the control, no alteration caused by LCK expression on the number of viable cells, regardless of FLT3 ligand stimulation (Fig. 1C). Furthermore, LCK expression neither increased nor decreased the fraction of apoptotic cells in an annexin V/7-AAD assay (Fig. 1D). Thus, we suggest that LCK expression is not essential for FLT3-ITD induced cell viability or survival *in vitro*.

LCK expression cooperates with FLT3-ITD in colony formation and tumor formation. Since we did not see any effect of LCK on FLT3-ITD-mediated *in vitro* cell survival, we asked whether it affects

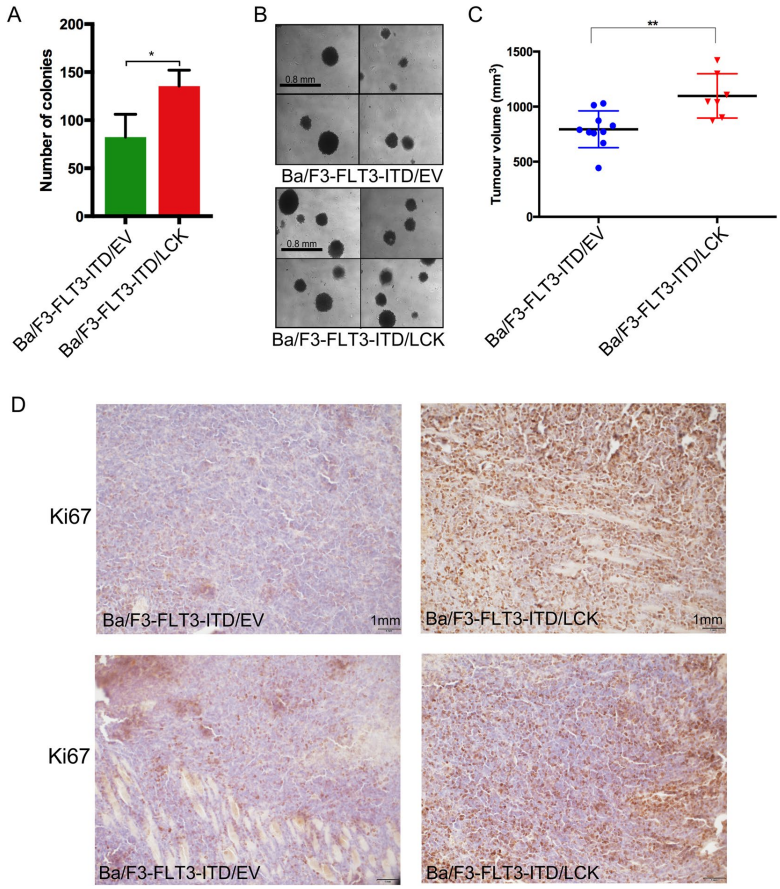

Figure 2. LCK expression promotes colony formation *in vitro* and tumor growth *in vivo*. (**A**) Cells were washed to remove IL-3 and serum. Cells were mixed with 80% methylcellulose medium and seeded in a 24-well plate. Quantified number of colonies formed *$p < 0.05$. (**B**) For quantification at least 10 pictures were taken per each well with colonies. (**C**) NOD/SCID mice were injected subcutaneously with 1×10^6 cells. The total volume size was measured after mice were sacrificed. (**D**) IHC of the tumor paraffin sections stained for Ki67, the photos are taken at 20X magnification. Empty vector, EV. *$p < 0.05$, **$p < 0.01$.

FLT3-ITD-induced *in vitro* colony formation. We observed that the potential to form colonies in the semi-solid medium was significantly increased in cells expressing LCK when compared to cells expressing empty vector control (Fig. 2A). However, the size of the colonies remained basically unchanged compared to controls (Fig. 2B). This suggests that LCK might play a role in FLT3-ITD-mediated cellular transformation. To further verify the *in vitro* findings, NOD/SCID mice were injected subcutaneously with Ba/F3-FLT3-ITD cells transfected with LCK or empty vector. After 25 days mice were sacrificed and the total volume of the tumors was measured. We could show that LCK expression significantly increased the tumor size in xenografted mice (Fig. 2C). To investigate whether the increased tumor size of LCK mice was due to an increase in proliferation, we stained tumor tissues for Ki67 and observed that tumors expressing LCK showed higher Ki67 staining, indicative of a higher proliferative potential (Fig. 2D). Therefore, we suggest that LCK accelerates the FLT3-ITD-mediated transformation potential *in vivo*.

LCK expression increases FLT3-ITD-mediated STAT5 phosphorylation. In contrast to the constitutively active oncogenic mutant FLT3-ITD, wild-type FLT3 is dependent on FL stimulation for activation of

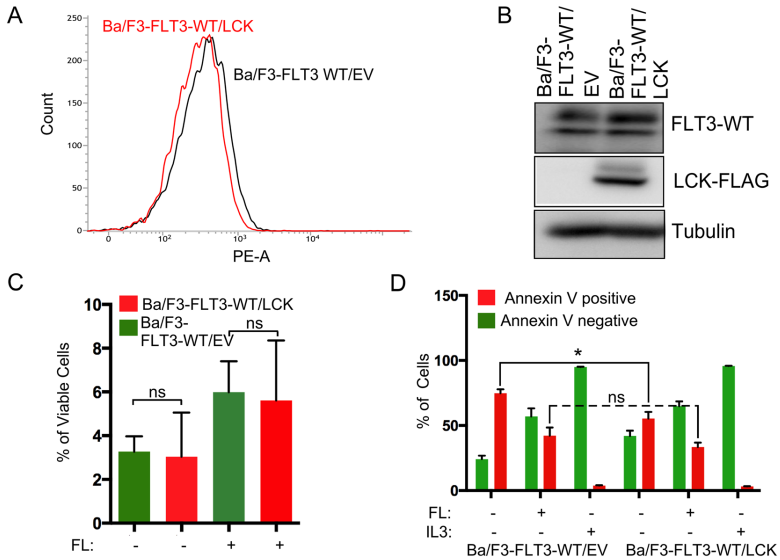

Figure 3. Expression of LCK does not affect wild-type FLT3 mediated cell viability or apoptosis. (**A**) Ba/F3 expressing FLT3 and LCK or empty vector (EV) were labeled with phycoerythrin-conjugated anti-FLT3 and then analyzed by flow cytometry. (**B**) The total amount of FLT3 expression was measured by Western blot. The blots were cropped to focus upon the specific proteins indicated. (**C**) Cells were washed to remove IL3 and seeded in a 96-well plate. PrestoBlue cell viability assay was used to measure viable cells. (**D**) Cells were washed to remove IL3 and seeded in a 12-well plate in IL3-free medium. After 48 h cells were stained with phycoerythrin (PE)-labeled annexin V and 7-aminoactinomycin D (7-AAD). Cells were then analyzed using flow cytometry. Cells positive for annexin V and 7-aminoactinomycin D (7-AAD), or only for annexin V, were counted as apoptotic cells. Not significant, ns; *$p < 0.05$.

the PI3K/AKT, RAS/ERK and p38 pathways[36]. In order to study how LCK regulates downstream signaling of FLT3, we therefore generated Ba/F3 cell lines expressing wild-type FLT3 with LCK or empty vector. Similar to the FLT3-ITD experiments, we used flow cytometry and Western blotting to verify equal surface expression (Fig. 3A) and total expression (Fig. 3B) of wild-type FLT3 in cells expressing LCK or empty vector. Ba/F3 cell line expressing wild-type FLT3 requires IL-3 for proliferation and survival, and LCK does not rescue that phenotype (Fig. 3C). LCK also does not influence the apoptotic rate of Ba/F3 cells in response to FL stimulation, while unstimulated cells expressing LCK displayed significant lower level of apoptotic cells (Fig. 3D). To gain insight into the mediators involved in downstream signaling, we looked at the known FLT3 downstream signaling pathways (Fig. 4A). We observed no change in phosphorylation of AKT (Fig. 4B), ERK (Fig. 4C) or p38 (Fig. 4D). However, STAT5 phosphorylation was significantly increased in Ba/F3 FLT3-ITD expressing LCK compared to the empty vector control (Fig. 4E), explaining the increased proliferation rate of the LCK tumors. However, we did not see any change in total FLT3 tyrosine phosphorylation in the absence or presence of LCK expression (Fig. 4F,G). Finally, we showed that FLT3-ITD was associated with LCK (Fig. 4H).

Discussion

Receptor tyrosine kinases transduce signals predominantly through interacting proteins. Therefore, understanding of the function of individual interacting partners of a specific receptor is important for understanding the regulation of downstream signaling cascades. Using an SH2 domain array we have recently shown that several SH2 domain-containing proteins such as ABL2, CRK, FYN, ABL1, RASA1, CRKL, LCK, SOCS6, BLK, TNS1, BRK etc. associate with several phosphotyrosine residues in FLT3[15]. We and others have characterized several of those proteins and demonstrated important roles in FLT3 signaling. The role of SRC family kinases, including SRC, LYN, FYN and HCK, has been outlined. Activation of FLT3 results in elevated tyrosine phosphorylation of LYN and SRC, and inhibitors targeting the SRC family kinases significantly reduced cell viability in FLT3-ITD-dependent AML cell lines, suggesting that the function of SRC family kinases is required for FLT3-induced cell survival[37,38]. While SRC and LYN displayed a role in cell survival, FYN appeared to be involved in FLT3-ITD-mediated cell transformation[13]. A recent report suggests that HCK is involved in FLT3-ITD mediated CDK6 expression and thereby supports cell survival and transformation[39]. Therefore, it is likely that, despite the high structural

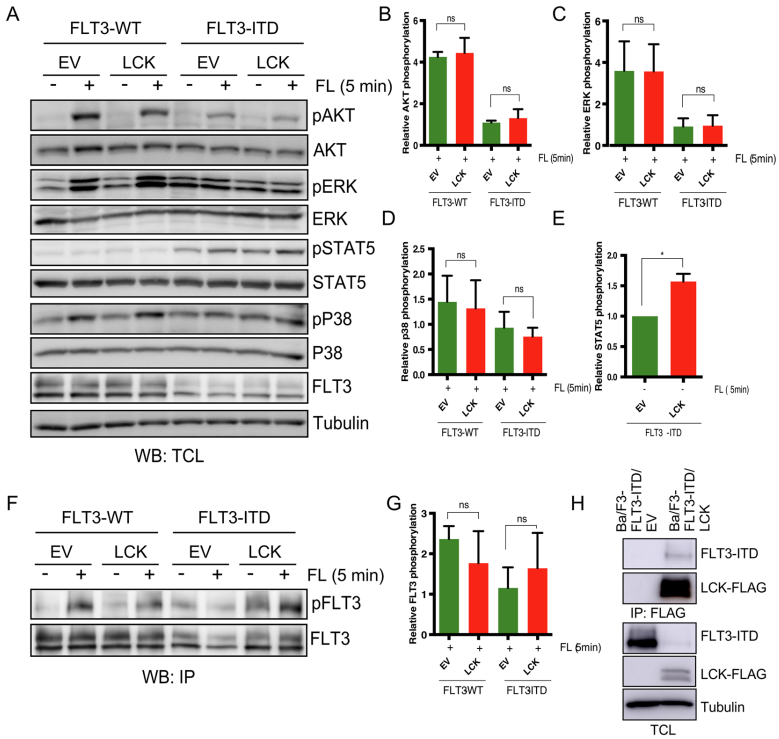

Figure 4. Ba/F3 FLT3-ITD cells expressing LCK display increased STAT5 phosphorylation. (**A**) Ba/F3 cells expressing either wild-type FLT3 or FLT3-ITD and either LCK or empty vector (EV) were washed to remove IL-3 and starved four hours before FL stimulation. Total cell lysates were subjected to Western blotting analysis using phosphospecific antibodies against AKT, ERK1/2, p38 and STAT5. (**B**–**E**) Blots from three independent experiments were quantified. Signals of phosphorylated proteins were normalized against total protein. (**F**) A fraction of the lysate was used for immunoprecipitation using an anti-FLT3 antibody. The blots were cropped to focus upon the specific proteins indicated. (**G**) Blots from three independent experiments from experiment F were quantified. Signals of phosphorylated FLT3 were normalized against total FLT3. (**H**) COS-1 cells were transfected with FLT3-ITD and LCK-FLAG or empty vector. Cells were lysed and lysates were subjected to anti-FLAG antibody immunoprecipitation. Not significant, ns; *$p < 0.05$.

similarity between SRC family kinases, individual members play distinct roles in FLT3 downstream signaling. In this report, we define the role of LCK in FLT3 signaling.

The function of LCK has mainly been studied in lymphocytes due to the abundant expression in the lymphoid lineage, in particular in T cells. It is highly expressed in several chronic lymphocytic leukemia's of both B cell and T cell lineages[28,29]. In addition, the myeloid cell line 32D and several non-lymphoid human tumor cell lines also show LCK expression[30,31,34]. Thus, LCK function may not only be restricted to the lymphoid lineage. Several studies have demonstrated a role of LCK in acute myeloid leukemia. Early studies[33] demonstrated higher expression of LCK in less differentiated cases of AML. In a recent proteomics study the role of individual kinases in AML was investigated and a correlation between high expression of LCK correlated with good response to a PI3K/mTOR-specific inhibitor[40]. Using a bioinformatics approach aiming at identifying relevant therapeutic targets in AML[41], several transcripts were identified that were differentially expressed between normal bone marrow samples and AML samples. Based on these data, they constructed a protein-protein interaction network and identified, among other proteins, LCK as one of the proteins of the hub nodes. Additionally, activation of FLT3 in AML samples resulted in abundant phosphorylation in the activation loop of LCK[29].

Taken together, these data collectively suggest that LCK might play a role in FLT3-ITD-mediated AML. Using Ba/F3 cells, lacking endogenous LCK expression, as a model system, we could show that LCK expression did not contribute to overall tyrosine phosphorylation of FLT3 suggesting that LCK does not have a role in the

FLT3 activation process. Furthermore, LCK did not contribute to FLT3-ITD-induced *in vitro* cell viability, but enhanced colony formation capacity, suggesting that LCK regulates distinct signaling pathway downstream of FLT3. This is also supported by the data that STAT5 phosphorylation, but not AKT, ERK1/2 and p38 phosphorylation, was enhanced in the presence of LCK. This is similar to what has been described for PCP-ALL cells, where a PAX5 fusion protein drives overexpression of LCK. In those cells, there is an LCK-dependent hyperphosphorylation of STAT5[42]. Similar to *in vitro* colony formation data, mice injected with cells expressing LCK and FLT3-ITD developed tumors quicker than cells lacking LCK expression. Collectively, our data suggest that LCK enhances the FLT3-ITD mediated transformation potential by cooperating with STAT5 pathway activation. Thus, LCK is a potential target for the development of selective SRC family kinase inhibitors that could potentially be used together with FLT3 inhibitors to treat patients with FLT3-ITD positive AML.

Materials and Methods

Cell culture and transfection. Murine pro-B cell line Ba/F3 (DSMZ, Braunschweig, Germany), was cultured in RPMI-1640 medium (Hyclone, Thermo Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA), 10 ng/ml recombinant murine interleukin 3 (IL3) and 100 units/ml penicillin, and 100 µg/ml streptomycin. Generation of Ba/F3-FLT3-ITD cells was described previously[1]. FLT3-ITD-transfected Ba/F3 cells were then further transfected with the pMSCV-FLAG-LCK or empty vector construct. Cells were selected with 0.8 mg/ml G-418 for 2 weeks. Transfected cells were maintained in Ba/F3 medium as previously described[43]. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

Immunoprecipitation and Western blotting. For signaling studies, before stimulation, Ba/F3 cells were starved for 4 hours in RPMI-1640 medium without serum or cytokines. Cells were stimulated with 100 ng/mL FL (ORF Genetics, Kópavogur, Iceland) for the indicated periods of time at 37 °C. Cells were washed once with cold PBS and lysed in lysis buffer [40 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet-P40] supplemented with protease inhibitors. Lysates were cleared by centrifugation at 14,000 × g for 10 min at 4 °C. For immunoprecipitation, 1 µg of antibody was used for 1 ml of cell lysate. Lysate and antibodies were mixed and kept on ice for 1 h before adding 20 µl of Dynabeads Protein G (ThermoFisher Scientific) followed by mixing end-over-end for 20 minutes. Beads were then washed three times with lysis buffer. Where the total cell lysates were used, equal amounts of proteins were electrophoretically separated on 8% SDS–PAGE gel and transferred to a PVDF membrane (Amersham, Arlington Heights, IL). Membranes were blocked with 5% non-fat dry milk in PBS-T, and probed with antibodies towards FLT3 (1 µg/ml, homemade, previously described[44,45]), 4G10 (1 µg/ml, Millipore), phospho-p38 (1 µg/ml, BD Biosciences) and p38 (1 µg/ml, BD Biosciences), phospho-ERK1/2, ERK2, phospho-STAT5, STAT5 and AKT (all at the dilution 1:200 Santa-Cruz Biotechnology), phospho-AKT (1:500, Epitomics), FLAG(1:2000, Sigma-Aldrich), LCK (1:200, Santa Cruz) and β-actin (1:5000, Sigma–Aldrich), followed by incubation with a horseradish peroxidase-labeled secondary antibody (1:5000). Immunodetection was performed by using ECL (Millipore Corporation, Billerica, MA) and a CCD camera (LAS-3000, Fujifilm, Tokyo, Japan). Signal intensity was quantified by MultiGauge software (Fujifilm).

Cell proliferation, apoptosis, and colony formation assay. Cells were washed three times to remove cytokine before all experiments. annexin V and 7-aminoactinomycin D (7-AAD) apoptosis kit (BD Biosciences) was used to measure apoptosis in cytokine-depleted cells. Cells positive for annexin V and both annexin V/7-AAD were counted as apoptotic cells. To measure cell proliferation, 10,000 cells were seeded into each well of a 96-well plate and incubated for 48 h. PrestoBlue (Thermo Fisher Scientific) was used to measure cell viability. Semi-solid methylcellulose medium (Stem Cell Technologies) was used for colony formation assay. Around 500 cells were seeded and cultured for seven days before counting colonies.

Animal work. NOD/SCID female mice were purchased from Charles River laboratories. 1×10^6 control or LCK expressing Ba/F3 FLT3-ITD cells were injected subcutaneously into 7 mice in each group. The tumor progression was monitored for 25 days. On the day of sacrifice, the tumors were measured and stored in 4% PFA for 24 h followed by the standard protocol of fixation. The experiment was performed under ethical permit from the Swedish Animal Welfare Authority following approved guideline.

Immunohistochemistry. After formalin fixation, tumors were embedded in paraffin. Tumor sections (4 µm thick) were deparaffinized using xylene, followed by graded ethanol series. Heat-mediated antigen retrieval was performed in retrieval buffer, PT module buffer pH 6 (TA-050-Pm1X), using pressure boiler. Ki67 (1:100, Abcam) antibody staining was performed in Autostainer 480 (Thermo Fisher Scientific Anatomical Pathology, Astmoor Runcorn, UK) for 30 min at room temperature, washed (x2) and incubated with secondary antibody for 30 min. Developed in Vulcan Fast Red chromogen kit (Biocare Medical).

Statistical analysis. Where required Western blots from three independent experiments were quantified. All statistical analyses were performed using the unpaired, two-tailed Student's t-test.

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Author Contributions

A.M. ran different experiments and analyzed data and prepared the manuscript. J.U.K. and L.R. designed experiments, analyzed data and prepared the manuscript.

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