To my parents and teachers ...

From the Department of Medicine III,
University of Munich Hospital Grosshadern
Ludwig-Maximilians-University, Munich and the
HelmholtzZentrum München
German Research Center for Environmental Health
Clinical Cooperative Group ‘’Leukemia’’

Chair: Prof. Dr. med. Wolfgang Hiddemann

First Description and Analysis of the Novel SHIP1/ABL1 Fusion Gene in Acute Lymphoblastic Leukemia

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Purvi Mansukhbhai Kakadiya

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vorgelegt von

Purvi Mansukhbhai Kakadiya

Aus
Mumbai, Indien

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With Permission of the Faculty of Medicine
University of Munich

Supervisor/Examiner:	Prof. Dr. med. Stefan K. Bohlander
Co-Examiners:	Prof. Dr. Joachim-Ulrich Walther
	Prof. Dr. Elke Holinski-Feder
Dean:	Prof. Dr. Dr.h.c. Maximilian Reiser, FACR, FRCR
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der
Universität München

Berichterstatter:	Prof. Dr. med. Stefan K. Bohlander
Mitberichterstatter:	Prof. Dr. Joachim-Ulrich Walther
	Prof. Dr. Elke Holinski-Feder
Dekan:	Prof. Dr. Dr.h.c. Maximilian Reiser, FACR, FRCR

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1 Introduction

1.1 LEUKEMIA

Leukemia arises from the malignant transformation of hematopoietic cells, most probably through the transformation of a hematopoietic stem or early progenitor cell. Depending on the clinical course of the disease, leukemias can be broadly divided in to (i) Acute leukemias, which, if left untreated, would lead to death within days or weeks, and which are characterised by a block in cellular differentiation, leading to an imbalance between differentiation and proliferation and hence predominance of immature cells in the bone marrow and peripheral blood and (ii) Chronic leukemias, which are characterised by an expanded pool of proliferating cells which retain their capacity to differentiate into mature cells. Chronic leukemias have a protracted clinical course and would lead to the death of the patient within several months to years. The leukemias are further divided into myeloid and lymphoid types depending on the phenotype of the malignant cells. Though there are many different types of leukemia, the four most common types of leukemia can be introduced briefly as follows:

1.1.1 Acute myeloid Leukaemia (AML)

Acute myeloid leukemia is characterized by a massive proliferation of myeloid (the granulocytes; bacteria-destroying cells, or monocytes; macrophage-forming cells) precursor cells and entry of immature cells into the blood stream. The total incidence of AML is 2.5/100,000 per year and it is more common in adults; particularly in older adults above the age of 55-60 years (Sverre Heim, 1995b). The two most commonly used classification schemes for AML are The French-American-British (FAB) sytem and the World Health Organization (WHO) system.

1.1.1.1 The FAB classification of AML

The FAB classification system was first proposed in 1976 and was subsequently expanded, modified and clarified. The FAB classification

devides the acute myeloid leukemias into the subtypes M0 through M7, based on the type of cell from which the leukemia developed and the degree of maturation displayed by the leukemic cells (Bennett et al., 1976). The FAB classification is based mainly on morphology, i.e. how the leukemia cells look under the microscope after routine staining *(Table 1.1)*.

French-American-British (FAB) classification of AML	
M0	minimally differentiated
M1	myeloblastic leukemia without maturation
M2	myeloblastic leukemia with maturation
M3	hypergranular promyelocytic leukemia
M4	myelomonocytic leukemia
M4Eo	variant, increase in marrow eosinophils
M5	monocytic leukemia
M6	erythroleukemia (DiGuglielmo's disease)
M7	megakaryoblastic leukemia

Table 1.1: AML subtypes defined by the French-American-British (FAB) classification

1.1.1.2 The WHO classification of AML

The WHO classification system of AML is based on clinical data (previous clinical history, age) and cellular characteristics like, morphology, expression of certain proteins (cytochemistry, immunophenotype), and genetic lesion, which are assayed by cytogenetics and/or molecular biology techniques *(Table 1.2)* (Jaffe ES, 2001). This classification system allows the separation of more homogeneous classes to distinguish prognostic parameters and to identify groups of patients sensitive to specific drugs or to specific treatment regimens. Thus the WHO classification is superior to the older FAB classification for diagnostic, prognostic and therapeutic purposes.

1.1.2 Acute lymphoblastic leukemia (ALL)

Acute lymphoblastic leukemia (ALL) is characterised by the accumulation of malignant, immature lymphoid cells in the bone marrow and frequently also in the peripheral blood. ALL is much more common in children than in adults. The total incidence of ALL in children is 3/100,000 per year (Sverre Heim, 1995a).

World Health Organization (WHO) Classification of AML
AML with recurrent cytogenetic translocations
AML with t(8;21)(q22;q22); fusion gene: RUNX1RUNX1T1
Acute promyelocytic leukemia: AML with t(15;17)(q22;q12) and variants; fusion gene PML/RARA
AML with abnormal bone marrow eosinophils and with inv(16)(p13;q22) or t(16;16)(p13;q22) / fusion gene: CBFB/MYH11
AML with 11q23 / MLL abnormalities
AML with multilineage dysplasia
With prior MDS
Without prior MDS
AML with myelodysplastic syndrome, therapy related
AML not otherwise categorized

Table 1.2: **AML categories defined by the WHO classification**

The FAB classification *(Table 1.3)* is the most widely used classification scheme for ALL, in which both the characteristic morphology of individual cells and the degree of heterogeneity within the leukemic cell population are taken into account (Bennett et al., 1976). In contrast to the FAB classification of AML, ALL FAB subgroups L1, L2 and L3 do correlate to some extent with clinical ALL subcategories and with immunologic characteristics of leukemic cells *(Table 1.4)*. In addition, in 70-90% of ALL cases cytogenetic abnormalities can be found. Some of these cytogenetic abnormalities are

associated with both B- and T-lineage ALL (e.g., 6q-, 9p-), while others are specific to one lineage or to a specific immunophenotype in a lineage. For example, translocations involving immunoglobulin genes are generally B lineage specific and those involving the TCR (T-cell receptor) genes are specific to T-lineage ALL. Considering these facts, a classification system integrating morphology, immunophenotype and cytogenetics was proposed. This classification was named MIC (morphology, immunophenotype, cytogenetics) classification (MIC Cooperative Study Group, 1986; MIC Cooperative Study Group, 1988).

FAB category->	L1 ALL	L2 ALL	L3 ALL
Cell size	*Mainly small*	*Large, heterogeneous*	*Large, homogeneous*
Nuclear chromatin	*Fairly homogeneous*	*Heterogeneous*	*Finely stippled, homogeneous*
Nuclear shape	*Mainly regular*	*Irregular; clefting and indentation common*	*Regular; oval or round*
Nucleolus	*Not visible or small*	*Usually visible, often large*	*Usually prominent*
Amount of cytoplasm	*Scanty*	*Variable, often abundant*	*Moderately abundant*
cytoplasmic basophilia	*Slight to moderate*	*Variable*	*Strong*
Cytoplasmic vacuolation	*Variable*	*Variable*	*Often prominent*

Table 1.3: **Acute lymphoblastic leukaemia (ALL) subtypes defined by the FAB Classification** (Bennett et al., 1976).

FAB category	**L1 ALL**			**L2 ALL**			**L3 ALL**
% of total ALL	70-80%			20-25%			1-2%
Age group	Children			Mainly Adults			-
Cell lineage	**B lineage**	**T lineage**		**B lineage**	**T lineage**		**mainly mature B-cell**
Immunophenotype	CD10+/ CALLA+	T-cell Surface receptor negative	T-cell surface receptor positive	CALLA+	T-cell Surface receptor negative	T-cell surface receptor positive	Expression of cell surface Ig-light chain
Relevant Immunologic subgroup of ALL	**early preB-ALL, cALL, pre-B-ALL**	**early pre T-ALL**	**T-ALL**	**early preB-ALL, cALL, pre-B-ALL**	**early pre T-ALL**	**T-ALL**	**B-ALL**

Table 1.4: Clinical correlation of the FAB and immunologic subtypes of ALL

However, current protocols for diagnosing and treating ALL are based on the underlying genetic abnormality. This approach is also supported by newer data from microarray-based gene expression profiling. The data from the microarray expression analysis has shown that the aberrant expression of certain transcription factor oncogenes defines distinct biological, clinical and molecular subtype of ALL (Ferrando et al., 2002).

1.1.3 Chronic Myeloid Leukemia (CML)

Chronic myeloid leukemia (CML) is a disease of the hematopoietic stem cell characterized by the overproduction of granulocytes. The annual incidence of CML is $1/10^6$ in childhood and $30/10^6$ after the age of 60 years *[http://atlasgeneticsoncology.org/Anomalies/CML.html]*. In contrast to the situation in AML, in CML the maturation proceeds in an orderly manner in the different lineages without any differentiation arrest or block. After the initial, relatively benign “chronic phase”, which on an average lasts for about 3 years, the disease typically enters a more malignant “accelerated phase” and eventually the terminal “blast crisis”. Almost all chronic phase CML patients have a t(9;22)(q34;q21) as their sole cytogenetic abnormality, which results in the formation of the BCR/ABL fusion gene. However, when the disease progresses to the accelerated phase and blast crisis, 75-80% of the cases acquire additional chromosomal abnormalities, which may serve as valuable prognostic indicators (Sverre Heim, 1995c). The morphologic characterisics of the leukemic cells in the terminal CML stages vary; myeloblastic or lymphoblastic features may predominate.

1.1.4 Chronic Lymphocytic Leukemia (CLL)

Chronic Lymphocytic Leukemia is characterized by the malignant proliferation and accumulation of small B-cells in the bone marrow and the peripheral blood. CLL of T-cell origin is exceptionally rare. The annual incidence of CLL is $30/10^6$ at a median age of 60-80 years *[http://atlasgeneticsoncology.org/Anomalies/CLL.html].* The disease represents 70% of all lymphoid leukemias and one-fourth of all leukemias. The clinical course of the disease is very

benign, with some forms of CLL having a survival, which is similar to age matched controls even without therapy.

Trisomy 12, deletion 13q14.3, deletion of 11q22-q23 and loss of p53 gene are the commonly observed cytogenetic abnormalities in CLL. Deletion 11q22-q23, involving ATM gene and deletion 17p13, involving p53 are the two most important cytogenetic prognostic markers (Hamblin, 2007).

1.2 GENETIC ALTERATIONS IN CANCER

As mentioned above, we are more and more moving into an era, in which cancer diagnostics and cancer therapies are based on the specific genetic aberrations found in the cancer to be treated. Some of the genetic aberrations serve as efficient prognostic markers in a particular disease, while others are even used as targets for therapeutic agents. Looking at the history of genetic aberrations and cancer, the idea that chromosomal alterations might be the cause of cancer was already put forward by Theodor Boveri in 1914 (Boveri, 1914).

This prescient hypothesis could only be verified after half a century of technical improvements, which led to the reliable visualization of human chromosomes (Tijo and Levan, 1956). The hypothesis was clearly supported by the consistent observation of a very small chromosome, named „Philadelphia chromosome“, in the cells of Chronic Myeloid Leukemia (CML) patients by Nowell and Hungeford (Nowell and Hungerford, 1960). A decade later, after the introduction of chromosome banding techniques (Caspersson et al., 1970), Janet Rowley discovered that the Philadelphia chromosome found in CML was the result of a balanced t(9;22) translocation (Rowley, 1973). In the early 70ies many specific and recurring chromosomal translocation were described in various tumor types especially in leukemias.

The last two decades have seen the molecular characterization of a large number of balanced chromosomal translocation with the discovery of fusion gene and aberrant gene regulation. These efforts have provided us with the conclusive verification of Boveri’s hypothesis. Now it is well accepted that cancer is a genetic disease with two major types of initiating genetic events: (1) The inactivation of tumor suppressor genes by deletions, point mutations

or maybe even by epigenetic mechanisms and (2) the activation or deregulation of oncogenes by point mutations, amplifications or balanced cytogenetic abnormalities like inversion, insertion or translocation.

In addition to onocogene activation and tumor suppressor gene inactivation, a few other general priniciples underlying malignant transformation have been postulated such as a block in differentiation, increased proliferation, resistance towards apoptosis resulting in prolonged cell survival, and enhanced self-renewal capacity of tumor propagating cells (or tumor stem cells) (Weissman, 2000; Warner et al., 2004). It is also important to note that malignant transformation in general and also in leukemia is a multistep process for which several somatic mutations are required rather than only a single event. It has been postulated that for leukemic transformation at least two genetic events are required with one of this genetic lesions driving increased cellular proliferation (class I mutation) and the second mutation leading to a differentiation block (class II mutation) (Kelly and Gilliland, 2002).

To better understand the concept of class I and class II mutations, we have to consider that the hematopoietic organ, like any organ with a high cellular turn-over, has a very defined and orderly differentiation and proliferation hierarchy. The production of new blood cells (erythrocytes, granulocytes and lymphocytes) starts from the hematopoietic stem cells, which divide asymmetrically. One daughter cell retains the stem cell phenotype and continues to reside in the stem cell niche. While the other daughter cell starts a hierarchical differentiation program turning into a progenitor cell for one of the many blood lineages. Lineage specific transcription factors play an important role in these differentiation processes from primitive progenitors to mature blood cells. Interestingly, mutations of these key transcription factors have been identified in several leukemias (e.g. mutations in the granulocyte lineage specific transcription factor (TF) *CEBPA*). Several leukemia-associated fusion genes either directly affect lineage specific TFs or disrupt the function of lineage specific TFs thus leading to a block of differentiation. These are called class II mutations. However, several studies using mouse models could clearly demonstrated that such a "class II" fusion protein (eg. the fusion proteins PML/RARA or RUNX1/RUNX1T1, wich are class II

mutations) is in most cases not sufficient to cause a full blown leukemia (Grisolano et al., 1997; Pollock et al., 1999; de Guzman et al., 2002). However, the coexpression of a protein with a class I mutation (eg. an activated tyrosine kinase like the FLT3-ITD), which drives cellular proliferation, will lead to leukemic transformation. It was shown that mutations in the receptor tyrosine kinase FLT3 collaborate with PML/RARα (Kelly et al., 2002) and with *AML1/ETO* (Schessl et al., 2005) to cause leukemia. Similarly, many activated tyrosine kinases are sufficient to induce a myeloproliferative disease on their own in murine models (Pear et al., 1998; Zhang and Ren, 1998; Li et al., 1999; Million et al., 2002), but for the development of a leukemia they require the help from other oncogenes which cause a differentiation block (Schessl et al., 2005).

As with all theories and concepts it should be kept in mind that these are just concepts which are more beneficial for the human mind and which can only be an approximation of the true nature of things. Thus, it is quite often not possible to clearly group a mutation or fusion gene found in a patient sample clearly into one of the two classes proposed by Gilliland and colleagues. Even if the grouping of a mutation seems obvious, it might not behave as predicted. There are bona fide class I mutations (eg. activated tyrosine kinases like the BCR/ABL protein) which are capable of inducing an aggressive leukemia in certain mouse models without a long latency period. Similarly, there are also bona fide class II mutation like the CALM/AF10 fusion or the MLL/AF9 fusion which lead to the rapid onset of leukemia in bone marrow transplantation models without the apparent help from a class I mutation (Deshpande et al., 2006; Somervaille and Cleary, 2006).

To fully understand the complicated process of malignant transformation in cancer and in leukemia in particular it is therefore necessary to discover and study in detail as many primary genetic lesions in these diseases as possible. One of the most fruitful methods to discover new genetic lesions in leukemia has been the study of chromosomal translocations.

1.2.1 Chromosomal Translocations In Leukemia

In human leukemia, the study of balanced chromosomal translocations has contributed greatly to our understanding of the pathogenesis of this disease in particular and, in addition, provided us with many paradigms for carcinogenesis in general. Balanced chromosomal translocations have pinpointed those genes, which are the important players in leukemia.

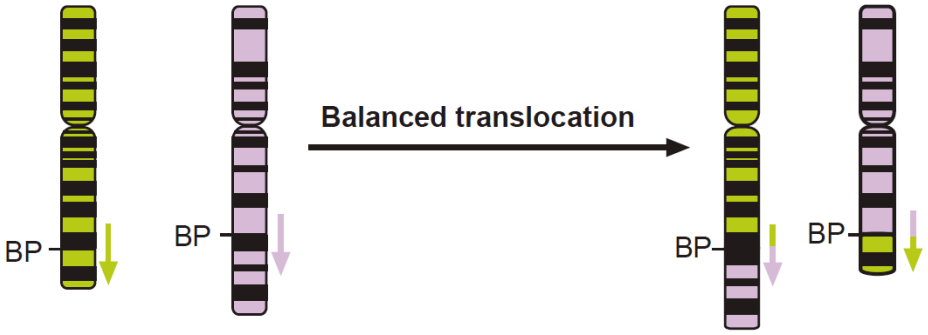


Figure 1.1: Balanced chromosomal translocation: The two non-homologous chromosomes are indicated by different colour patterns. The "BP" indicates the breakpoint region on the two non-homologous chromosomes.

Balanced chromosomal translocations lead to the exchange of genetic material between two non-homologous chromosomes *(Figure: 1.1)*, resulting in the alteration of the function of the genes in the vicinity of the translocation breakpoints. Basically, translocations alter gene function by two distinct mechanisms: (1) Overexpression of a gene at the translocation breakpoint by the juxtapposition of the promoter of another gene *(Figure 1.2 A)* and (2) Creation of a fusion gene, which encodes for a fusion protein with new, oncogenic properties *(Figure 1.2 B)*.

The classic example of the first type of molecular mechanism is the t(8;14)(q24;q32), where the MYC gene from chromososme 8 is juxtapposed to the IGH enhancer on chromososme 14 and becomes constitutively expressed owing to the influence of regulatory elements of the IGH promoter (Taub et al., 1982; ar-Rushdi et al., 1983). This mechanism is more commonly found in gene fusions in lymphoid malignancies but has recently also been discovered in the majority (80%) of prostate cancer (Tomlins et al., 2005).

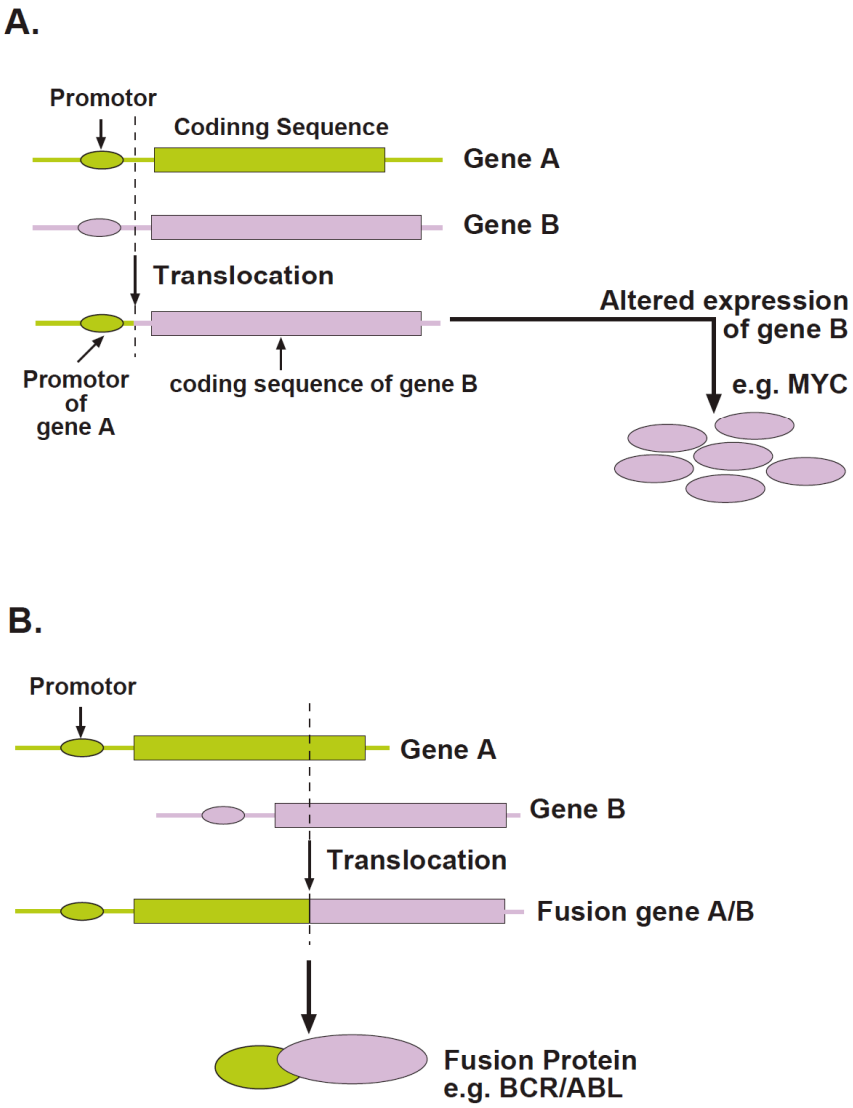
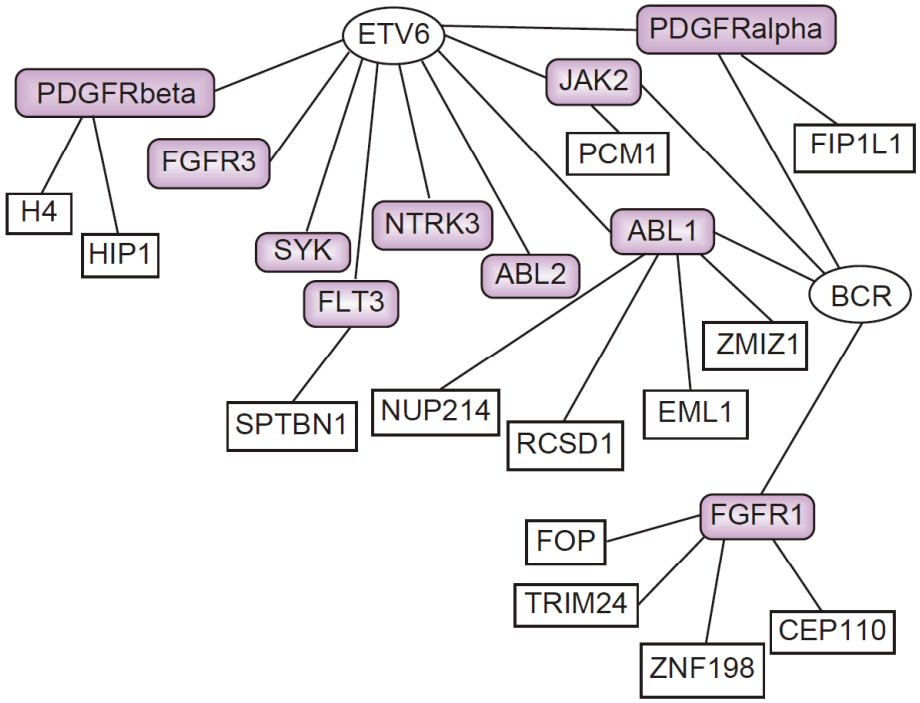


Figure 1.2: Consequences of balanced chromosomal translocation: **(A)** Overexpression of gene B by the juxtaposing the promoter of gene A to the whole coding sequence of gene B **(B)** Expression of a fusion protein by a chimeric gene formed by the in frame fusion of the parts of coding sequence of gene A and gene B.

The second type of molecular mechanism is exemplified by the t(9;22)(q34;q11) observed in CML, AML and ALL, which brings together the 5' part of BCR (Breakpoint Cluster Region) gene on chromosome 22 and the 3' part of ABL1 (Abelson) tyrosine kinase gene from chromosome 9, leading to the formation of the BCR/ABL1 fusion gene (Shtivelman et al., 1985) and subsequently to the production of a chimeric BCR/ABL1 protein with constitutive tyrosine kinase activity (Witte et al., 1980; Konopka et al., 1984).

During the last two decades an increasing number of chromososmal translocations have been characterized at the molecular level, leading to the identification of many genes involved in the induction of malignant transformation. Up to now a total of 264 gene fusions, involving 238 different genes, have been described in haematologic disorders including malignant lymphomas (Mitelman et al., 2007). As a consequence, a network of fusion genes has emerged in which several genes are involved in more than one translocation forming fusion genes with several partner genes (Bohlander, 2000; Mitelman et al., 2007). One of the subsets of this fusion gene network are the tyrosine kinase fusion genes (Cross and Reiter, 2002; Bohlander, 2005) *(Figure 1.3)*.



Adapted from Bohlander, et.al 2005 and Cross, et al 2002

Figure 1.3: Network of tyrosine kinase fusion genes associated with hematologic malignancies. The colored boxes indicate tyrosine kinases.

1.3 TYROSINE KINASES

Tyrosine kinases (TK) are enzymes that are essential for many cellular signaling pathways and regulate key cellular functions like growth, proliferation, differentiation, cell shape, cell adhesion and programmed cell

death. TKs phosphorylate other proteins by transferring a phosphate group from ATP to a tyrosine residue in the substrate protein. This phosphorylation changes the function of the protein usually resulting in the activation of the downstream signaling pathways. TKs can be divided into two major classes: Receptor Tyrosine Kinases (RTK) and cellular or non-receptor tyrosine kinases (NRTK). An RTK contains an extracellular ligand binding domain, a trans-membrane domain, which anchors the receptor in the plasma membrane, and an intracellular domain, which includes the tyrosine kinase domain and multiple regulatory domains, which include tyrosines for auto phosphorylation.

1.3.1 Normal activation of tyrosine kinases

In its inactive state the RTK is present as a monomer, but when a ligand (growth factors or hormones) binds to the extracellular domain of two RTKs, the RTKs are dimerized. The dimerized RTKs then phosphorylate each other's tyrosine residues by cross-phosphorylation. This cross-phosphorylation results in stably active RTKs providing phosphotyrosine-docking sites for other proteins which transduce downstream signals into the cell. In contrast to the RTKs, non-receptor tyrosine kinases are located in the cytoplasm, nucleus, or are anchored to the inner surface of the plasma membrane. They do not have a ligand binding domain or a transmembrane domain, but they use similar mechanism of activation like the RTKs; i.e. cross-phosphorylation via dimerization or oligomerization, but they are also regulated by phosphorylation through other (upstream) TKs.

1.3.2 Constitutive activation of tyrosine kinases in oncogenic tyrosine kinase fusion proteins

ABL, JAK2, FGFR3, PDGFR-alpha, PDGFR-beta, and NTRK are some of the tyrosine kinases involved in the formation of tyrosine kinase fusion proteins in leukemia and solid tumors. In these fusion proteins, the partner protein is joined to the entire catalytic domain of the tyrosine kinase. All these partner proteins contribute one important protein domain to the fusion protein: a dimerization or oligomerization domain. This structural feature of the fusion

partner leads to the dimerization or oligomerization of the fusion protein, which mimics the process of ligand-mediated dimerization and results in the constitutive activation of the tyrosine kinase portion of the fusion protein.

1.3.3 Tyrosine kinase inhibitors as cancer drug

The first TK fusion protein discovered and studied extensively was the BCR/ABL fusion resulting from the reciprocal translocation t(9;22)(q34;q11). The abnormal TK activity of the BCR/ABL fusion protein was studied in great detail. One of the aims of these studies was the development of specific competitive tyrosine kinase inhibitors. Among the various tyrosine kinase inhibitors, Imatinib (formerly known as CGP 57148B and STI571) was selected as being the best suited drug for the treatment of BCR/ABL positive CML. Imatinib, which was developed by Brian Druker and colleagues (Buchdunger et al., 1996; Druker et al., 1996) was the first targeted cancer therapy. Imatinib is a competitive inhibitor of ABL, c-Kit and the PDGFR-beta tyrosine kinase. Imatinib, which has a 2-phenylaminopyrimidine structure, occupies the ATP-binding pocket in the TK domains of ABL1, c-Kit and PDGFR-beta inhibiting the kinase function of these proteins. Imatinib is used for the treatment of aberrantly activated ABL1, c-Kit and PDGFR-beta. This targeted therapy has proved to be extremely beneficial for patients with CML, improving greatly the quality of life for these patients (Druker et al., 2001a; Druker et al., 2001b; Sawyers et al., 2002). Although long-term remissions in CML patients can be achieved with Imatinib, it does not appear to cure CML. When the treatment with Imatinib is discontinued the patients relapse, and even while Imatinib is given resistance mutations can arise in the BCR/ABL fusion gene.

Other compounds, which specifically inhibit other tyrosine kinases such as FGFR1 (eg. SU5402 and PD173074) but are inactive against BCR/ABL have recently been developed (Mohammadi et al., 1997; Demiroglu et al., 2001).

1.4 ABL1 FUSION PROTEINS

Up to now 6 different fusion partners of the *ABL1* tyrosine kinase gene have been reported in hematological malignancies *(Figure 1.3)*. Other than

BCR/ABL1, the ETV6/ABL1 fusion (Papadopoulos et al., 1995) is also well characterized and responds well to Imatinib treatment. In addition, three other *ABL1* fusion partners: the nuclear pore complex protein 214 kDa protein NUP214 (9q34) (Graux et al., 2004), *EML1* (14q32) (De Keersmaecker et al., 2005), *RCSD1* (1q24) (De Braekeleer et al., 2007) and ZMIZ1 (Soler et al., 2008) have been reported in different subtypes of leukemia.

Fusion	Translocation	Disease	di or oligomerization domain contributed by the ABL1 partner
BCR/ABL1	t(9;22)(q34;q11)	CML, ALL, AML	Coiled-coil
ETV6/ABL1	t(9;12)(q34;p12)	ALL, AML, CML	Helix-loop-helix
NUP214/ABL1	t(9;9)(q34;q34) Amplified episomes	T-ALL	Coiled- coil
EML1/ABL1	t(9;14)(q34;q32)	T-ALL	Coiled-coil
RCSD1/ABL1	t(1;9)(q24;q34)	B-ALL	Unknown at present
ZMIZ1/ABL1	t(9;10)(q34;q22.3)	B-ALL	Unknown at present

Table 1.4: List of known ABL1 fusions with the di- or oligomerization domains within the fusion partner. The chromosomal rearrangements generating the respective ABL1 fusion are indicated. Relative disease is also indicated.

All these *ABL1* fusion partners activate the ABL1 tyrosine kinase by providing a di- or oligomerization domain *(Table 1.4)*. For example, in the BCR/ABL fusion protein the coiled-coil domain of BCR serves as a dimerization/oligomerization domain facilitating the constitutive activation of the ABL tyrosine kinase.

In addition to the five known fusion partners of ABL1, we have discovered a novel fusion partner of *ABL1*, the *SHIP1* gene. The *SHIP1* gene (SH2 containing Inositol Phosphatase 1), named officially inositol polyphosphate-5-phosphatase (*INPP5D*) is located on the long arm of chromosome 2 (2q37).

This novel *SHIP1/ABL1* fusion gene was discovered in the leukemic cells of an 18-year old female patient with B-ALL.

It is the aim of this doctoral thesis to:

(1) Characterize this novel fusion at the genomic and transcript level.

(2) Study the transformation potential of the SHIP1-ABL1 fusion protein.

(3) Test whether SHIP1/ABL1 is responsive to Imatinib.

(4) Analyze the protein domains of SHIP1, which are responsible for the TK activation SHIP1/ABL1 and its transforming potential.

2 Materials and Methods

2.1 MATERIALS

2.1.1 Reagents

Reagent	Company
1,4- Diaminobenzol	Sigma-Aldrich, USA
1-kb-DNA Ladder	Invitrogen, Karlsruhe, Germany
3-amino-1,2,4-triazole (3-AT)	Sigma, Taufkirchen, Germany
Acetic acid	Merck, Darmstad, Germany
Acrylamid Rotiphorese® Gel 30 (37, 5:1)	Carl Roth, Karlsruhe, Germany
Agar	Carl Roth, Karlsruhe, Germany
Agarose	ICN Biomedicals Inc.
Amino acids	Sigma, Taufkirchen, Germany
Ammonium acetate (NH_4Ac)	Sigma, Taufkirchen, Germany
Ammonium chloride (NH_4Cl) solution	CellSystems, Vancouver, Canada
Ammonium persulfate (APS)	Sigma, Taufkirchen, Germany
Ampicillin Na-Salt	Pan Biotech, Aidenbach, Germany
Antibiotic-Antimycotic	Gibco, Invitrogen corp.
Aqua ad iniectabilia	Braun B Melsungen AG, Germany
ß-Mercaptoethanol	Sigma, Taufkirchen, Germany
Biocoll separating solution	Biochrom AG, Berlin, Germany
Big dye terminator™ Mix (V 1.1)	PE Applied biosystems, Foster city, CA
Bovine serum albumine (BSA)	Sigma, Taufkirchen, Germany
Bradford Reagent (BioRad Protein Assay reagent)	BioRad Laboratory GmbH, Munich, Germany
Bromophenol blue	Carl Roth, Karlsruhe, Germany
Calcium cloride ($CaCl_2$)	Sigma, Taufkirchen, Germany
Chloramphenicol	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Cot-1 DNA	Vysis Inc., USA
D(+)-glucose-monohydrate	Merck, Darmstadt, Germany
d_2H_2O	Millipore, Eschborn, Germany
DAPI	Sigma, Taufkirchen, Germany

Reagent	Company
Deoxyribonuclease I, Amplification Grade	Invitrogen, Karlsruhe, Germany
Dextran sulfate	Carl Roth, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Carl Roth, Karlsruhe, Germany
DNA molecular weight marker II	Boehringer Manheim GmbH, Germany
DNA molecular weight marker III	Boehringer Manheim GmbH, Germany
DNA molecular weight marker VI	Boehringer Manheim GmbH, Germany
dNTP Set, PCR Grade	Invitrogen, Karlsruhe, Germany
DPBS	Pan Biotech, Aidenbach, Germany
ECL™ Plus Western Blotting Detection Reagent	Amersham, Freiburg, Germany
EDTA (ethylenediaminetetraacetic acid)	Carl Roth, Karlsruhe, Germany
EGTA (Ethylen Glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid)	Sigma, Taufkirchen, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Carl Roth, Karlsruhe, Germany
Fetal bovine serum (FBS)	Gibco Invitrogen Cell Culture, Karlsruhe, Germany
FISH probes; BCR-ABL DCDF	Abbott-Vysis, Germany
Fluorescein-12-dATP	Perkin Elmer Life Sc. Inc., USA
Fluorescein-12-dCTP	Perkin Elmer Life Sc. Inc., USA
Formaldehyde 37%	Carl Roth, Karlsruhe, Germany
Formamide	Carl Roth, Karlsruhe, Germany
Gelatin	Merck, Darmstadt, Germany
Glass beads	Sigma, USA
Glycerol	Carl Roth, Karlsruhe, Germany
Glycine	Merck, Darmstadt, Germany
Heparin	Sigma, Taufkirchen, Germany
HEPES (N-(2-Hydroxyethyl) piperazine-N´- (2-ethanesulfonic acid))	Sigma, Taufkirchen
Hydrochloridric acid 37%	Merck, Darmstadt, Germany
Hydrochloridric acid (0.1N)	Merck, Darmstadt, Germany
Hydrogen peroxide (H_2O_2) solution 35 %	Merck, Darmstadt, Germany

Reagent	Company
IPTG	Roche, Mannheim, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Kaisers Glyceringelatine	Merck, Darmstadt, Germany
Kanamycin	Pan Biotech, Aidenbach, Germany
Klenow Fragment	MBI Fermentas, St. Leon-Rot, Germany
L-Glutamine	Pan Biotech, Aidenbach, Germany
Lithium acetate	Sigma, Taufkirchen, Germany
LSI/WCP hybridization buffer	Abbott Molecular Inc., USA
Magnessium Chloride (MgCl2)	Sigma, Taufkirchen, Germany
Maleic acid	Sigma, Taufkirchen, Germany
Methanol	Merck, Darmstadt, Germany
Milk powder	Carl Roth, Karlsruhe, Germany
NP40 (nonyl phenoxylpolyethoxylethanol)	Roche, Mannheim, Germany
Penicillin/streptomycin	Pan Biotech, Aidenbach, Germany
Pepsin	Sigma-Aldrich, USA
Phenol/chloroform/isoamyl (25:24:1)	Invitrogen, Karlsruhe, Germany
Phenylmethylsulfonylfluorid (PMSF)	Sigma, Taufkirchen, Germany
Platinum® *Pfx* DNA Polymerase	Invitrogen, Karlsruhe, Germany
Platinum® Taq DNA Polymerase	Invitrogen, Karlsruhe, Germany
Polyethylenglycol (PEG) 4000	Merck, Darmstadt, Germany
Polyethylineimine (PEI)	Sigma, Taufkirchen, Germany
Potassium chloride (KCl)	Calbiochem, San Diego, USA
Propidium iodide	Sigma, Taufkirchen, Germany
Protamine sulphate	Sigma-Aldrich, St. Louis, MO
Protein A-Agarose	Roche, Mannheim, Germany
Protein G-Agarose	Roche, Mannheim, Germany
Proteinase inhibitor cocktail	Sigma, Taufkirchen, Germany
Random examer primers $p(dN)_6$	Roche Diagnostics, Mannheim, Germany
Recombinant murine IL3 (rm IL-3)	Immunotools GmbH, Germany
Restriction enzymes	MBI Fermentas, Germany
Restriction enzymes	NewEngland Biolabs, Schwalbach, Germany

Reagent	Company
Rnasin Plus Rnase inhibitor	Promega, WI, USA
SDS (sodium dodecyl sulfate)	Carl Roth, Karlsruhe, Germany
SeeBlue® Plus2 pre-stained standard	Invitrogen, Karlsruhe, Germany
Sequenase version 2.0 DNA polymerase	USB corporations, USA
S.O.C. medium	Invitrogen, Carlsbad, USA
Sodium acetate, Anhydrous (NaAC)	Calbiochem, San Diego, USA
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Sodium citrate ($Na_3C_6H_5O_7$)	Carl Roth, Karlsruhe-, Germany
Sodium deoxycholate	Sigma, Taufkirchen, Germany
Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
Sodium orthovanadat (Na_3VO_4)	Sigma, Taufkirchen, Germany
Sodium phosphate dibasic anhydrous (Na_2HPO_4)	Sigma, Taufkirchen, Germany
Sodium phosphate monobasic monohydrate (NaH_2PO_4)	Calbiochem, San Diego, USA
Spectrum green- dUTPs	Vysis Inc., USA
Spectrum orange- dUTPs	Vysis Inc., USA
STI571 (Imatinib)	Novartis, Basel, Switzerland
Sucrose	Sigma, Taufkirchen, Germany
T4 DNA Ligase	NewEngland Biolabs, Schwalbach, Germany
Tetramethylethylendiamin (TEMED)	Serva, Heidelberg
Tetrazole salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromid (MTT),	Sigma, Taufkirchen, Germany
Texas-Red-5-dATP	Perkin Elmer Life Sc. Inc., USA
Texas-Red-5-dCTP	Perkin Elmer Life Sc. Inc., USA
Trichloroacetic acid (TCA)	Sigma, Taufkirchen, Germany
Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol)	Carl Roth, Karlsruhe, Germany
Triton X-100	Carl Roth, Karlsruhe, Germany
Trypan blue	Invitrogen, Karlsruhe, Germany
Trypsin-EDTA	Gibco™, Germany
Tween® 20	Sigma, Taufkirchen, Germany
X-alpha-gal (5-Bromo-4-Chloro-3-indolyl a-D-galactopyranoside)	Clontech, Heidelberg, Germany

Reagent	Company
Yeast extract	Sigma, Taufkirchen, Germany
Yeast nitrogen base without aa	Difco, Detroit MI, USA

2.1.2 Materials and Kits

Name	Company
Blotting paper GB003 and GB002	Schleicher & Schuell, Dassel, Germany
Cell Strainer 40 µm Nylon Falcon®	BD Biosciences, Palo Alto, CA
CENTRI Sep8 well strips	Princeton Separations, USA
Coverslips	Menzel-Gläser®, Braunschweig, Germany
Cryotube™ vials	Nalge Nunc Internacional, Denmark
Electroporation cuvettes 2 mm	EquiBio, Kent, UK
Endofree® Plasmid Maxi kit (10)	Qiagen, Hilden, Germany
Eppendorf tubes	Eppendorf, Hamburg, Germany
FACS Polystrene round-bottom tubes	Becton Dickinson, Meylan, France
Ficoll (type 400)	Sigma, Taufkirchen, Germany
Filter unit 0.22 µm	Millipore, Belford, USA
Filter unit 0.45 µm	Millipore, Belford, USA
Freezing container (Cry0 1° C)	Nalgene ™, USA
Glass beads	Sigma, Taufkirchen, Germany
Glassware	Schott, Jena, Germany
Herring Testes Carrier DNA denatured DB	Biosciences Clontech, Heidelberg, Germany
Hybond-P PVDF membrane	Amersham, Freiburg, Germany
Hypercassete™ 18x24 cm	Amersham, Freiburg, Germany
Hyperfilm ECL high performance	Amersham, Freiburg, Germany
MagNaPure LC mRNA Isolation Kit	Roche Diagnostics Mannheim Germany
Marabu Fixogum Rubber Cement	Marabuwerk GmbH, Tamm, Germany
Microcentrifuge tube (1.5 ml)	Eppendorf, Hamburg, Germany
Microscope slides	Menzel-Gläser®, Braunschweig, Germany
pGEM-Teasy vector system	Promega, WI, USA
Pipette tips	Carl Roth, Karlsruhe, Germany

Name	Company
Plasmid Midi kit (25)	Qiagen, Hilden, Germany
Plastic cuvettes for biophotometer	Carl Roth, Karlsruhe, Germany
Plastic ware for cell culture	Greiner Labortechnik, Frickenhausen, Germany
Plastic ware for cell culture	Sarstedt, Nümbrecht, Germany
Plastic ware for cell culture	Corning, USA
Polypropylene conical tubes	Becton Dickinson, Meylan, France
QIAshredder™ columns	Qiagen, Hilden, Germany
QIAquick gel extraction kit	Qiagen, Hilden, Germany
S.O.C. medium	Invitrogen, Carlsbad, USA
SuperScript™ II RNase H⁻ Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
Surgical blades	Feather Safety Razor Co. Med. Div., Japan
Syringes	Braun, Melsungen, Germany

2.1.3 Buffers and solutions

2.1.3.1 Buffers and solutions used in microbiology

Name	Components	
10x TE buffer	0.1 M	Tris-HCl
	10 mM	EDTA
		pH 7.5, Autoclave.
		Storage: RT
10x LiAc solution	1 M	Lithium acetate
		Adjust to pH 7.5 with acetic acid and autoclave
		Storage: RT
1x TE/LiAc solution	1 vol.	10x TE buffer
Prepared fresh	1 vol.	10x LiAc solution
	8 vol.	ddH_2O

Name	Components	
PEG/LiAc solution	8 vol.	50 % PEG 4000
Prepared fresh	1 vol.	10x TE buffer
	1 vol.	10x LiAc solution
Resuspension buffer for plasmid preparations	50 mM	Tris·Cl, pH 8.0
(Buffer P1, Qiagen)	10 mM	EDTA
	100 µg/ml	RNase A
		Storage: 2-8° C
Alkaline lysis buffer for plasmid preparations	200 mM	NaOH
(Buffer P2, Qiagen)	1% (w/v)	SDS
		Storage: 15-25° C
Neutralization buffer for plasmid preparations	3.0 M	Potassium acetate
(Buffer P3, Qiagen)		pH 5.5
		Storage: 15-25° C

2.1.3.2 Buffers and solutions used in molecular biology

Name	Components	
1X TBE (Tris-Borate-EDTA) buffer	0.1 M	Tris
	90 mM	Boric acid
	1mM	EDTA
	10X TBE buffer was purchased from Invitrogen Life technology and diluted to 1X.	
		Storage: RT
3x loading dye	15 g	Ficoll 400
(For agarose gel electrophoresis)	0.1 mg	Bromophenol blue
	ad 100 ml	ddH_2O
		Storage: 4° C

Name	Components	
10x T4 DNA ligation buffer (Supplied with the T4 DNA ligase)	400 mM	Tris-HCl (pH 7.8 at 25°C) for pH maintenance
	100 mM	$MgCl_2$ for Mg^{++}
	100 mM	DTT, reducing agent
	5 mM	ATP as co-factor
		Storage: -20° C

Restriction enzyme buffers:

The restriction enzyme buffer contains salt ($MgCl_2$, KCl or NaCl) for ionic stregth and proper enzyme function, Tris for the maintainance of the required pH for the reaction and bovine serum albumine for the stability of the restriction enzyme. Different enzmes have different preferences for ionic strength. We used the recommended buffers for the optimum activity of each restriction enzyme, supplied by the manufacturers. 1x Tango buffer is one of the universal buffers which can be used for a large number of restriction enzymes and for double digests.

Name	Components	
1x Tango buffer; Fermentas (Restriction enzyme buffer)	33 mM	Tris-acetate pH 7.9 at 37° C
	10 mM	Mg-acetate
	66 mM	K-acetate
	0.1 mg/ml	BSA
		Storage: -20° C
10x PCR amplification buffer without $MgCl_2$ (Invitrogen)	200 mM	Tris-HCl (pH 8.4)
	500 mM	KCl
		Storage: -20° C
Resuspension buffer for plasmid preparations (Buffer P1, Qiagen)	50 mM	Tris·Cl, pH 8.0
	10 mM	EDTA
	100 µg/ml	RNase A
		Storage: 2-8° C
Alkaline lysis buffer for plasmid preparations (Buffer P2, Qiagen)	200 mM	NaOH
	1% (w/v)	SDS
		Storage: 15-25° C

Name	Components	
Neutralization buffer for plasmid preparations (Buffer P3, Qiagen)	3.0 M	Potassium acetate pH 5.5 Storage: 15-25° C

2.1.3.3 Buffers and solutions used in cell culture

Name	Components	
Sodium phosphate buffer (required solutions for 1 l)	684 ml 316 ml	1 M Na_2HPO_4 1 M NaH_2PO_4 pH 7.2 Storage: RT
2x HBS	50 mM 12 mM 12 mM 280 mM 1.5 mM	HEPES KCl Dextrose NaCl Na_2HPO_4 pH 7.0-7.1 Storage: -20° C
FACS buffer	1x 2% 1 mg/l	PBS FBS propidium iodide Storage: 4° C
Freezing medium	10% (v/v)	DMSO in FBS Storage: 4° C

2.1.3.4 Buffers and solutions used in protein biochemistry

Name	Components	
Tris-HCl	1.5 M, pH 8.8 1 M, pH 6.8 1 M, pH 8.0 1 M, pH 7.5	Tris powder in d_2H_2O, adjusted pH with HCL Tris powder in d_2H_2O, adjusted pH with HCL Tris powder in d_2H_2O, adjusted pH with HCL Tris powder in d_2H_2O, adjusted pH with HCL Storage: RT

Name	Components	
EDTA	0.5 M, pH 8.0	EDTA in d_2H_2O, adjusted pH Storage: RT
10 % SDS	10 % (w/v)	Sodium dodecylsulfate in d_2H_2O Storage: RT
10% APS	10 % (w/v)	Amonium Persulphate in d_2H_2O aliquoted and stored at -20° C
NTE (Na-Tris-EDTA) buffer	150 mM 10 mM 1% 5 mM	NaCl Tris, pH 7.5 NP40 EDTA Storage: 4° C
Lysis buffer No. 1	50 mM 150 mM 1 mM 10% 1% 100 mM 10 mM	HEPES pH 7.5 NaCl EGTA Glycerol Triton-X 100 NaF $Na_4P_2O_7$-$10H_2O$ Storage: 4° C
2.5x Laemmli stacking buffer	0.3 M 0.25 %	Tris/HCl pH 6.8 SDS Storage: up to 1 month at 4°C
2x Laemmli loading buffer	10% 6% 20% 0.2 mg/ml 0.025x	β-mercaptoethanol SDS Glycerol Bromophenol blue Laemmli stacking buffer (optional) Storage: RT, up to 2 months

Name	Components	
10x Tris-Glycine electrophoresis buffer	250 mM 1.92 M 1% Storage: RT	Tris Glycine SDS
1x Gel Transfer buffer (For western blotting)	25 mM 192 mM 20% Storage: 4° C	Tris Glycine Methanol
1x TBS	10 mM 150 mM Storage: RT	Tris/HCl pH 8.0 NaCl
1x TBST washing buffer	1x 0.1 % Storage: RT	TBS Tween® 20
Stripping solution for WB	62.5 mM 0.1 M 2% Storage: RT	Tris/HCl pH 6.8 β-mercaptoethanol SDS
20% TCA (for yeast lysis)	20% w/v Storage: 4° C	TCA in d_2H_2O
TCA Buffer Use deionized H_2O	20 mM 50 mM 2 mM 60 µl/ ml PIC was added immediately prior to use	Tris-HCL (pH 8) Ammonium acetate EDTA PIC (Protease inhibitor cocktail, Sigma)

2.1.3.5 Buffers and solutions used for cytogenetics and FISH

Name	Components	
Saline sodium citrate buffer (SSC) 20x	3 M 0.3 M	NaCl Sodium citrate pH 7.0 Autoclave, Storage: RT
10x MAP	400 mM 100 mM 500 mM 50 mM	Tris HCl pH 7.5 $MgCl_2$ NaCl DTT Storage: -20° C
10x BP buffer	66 mM 550 mM 0,11 % (w/v)	Tris HCl pH 9.0 KCl_2 Gelatin Storage: -20° C
10x DNase adjusting buffer	0.5 M 50 mM	Tris HCl pH 7.5 $MgCl_2$ Storage: 4° C
DNase stock solution	50% 20 mM 1 mM	Glycerol Tris HCl pH7.5 $MgCl_2$ Storage: -20° C
DNase buffer solution	2.0 ml 0.1 ml 97.9 ml	1M Tris HCl pH7.5 1M $MgCl_2$ d_2H_2O Storage: RT
Pepsin solution (stock)	0.2 gm 10 ml	Pepsin (Sigma, #P7012) d_2H_2O 500 µl aliquots, stored at -20° C
Pepsin solution (working) Prepared fresh	100 ml 500 µl	0.01 N HCl, preheated at 37° C Pepsin solution (stock)

Name	Components	
Post fixation solution	1x	PBS
	1% (v/v)	Formaldehyde
	50 mM	$MgCl_2$
		Storage: Up to 3-5 days at 4° C
FISH hybridization buffer	11 ml	Formamide
	2 gm	Dextransulphate
	1 ml	20x SSC
		dissolved at 70°C O/N, adjusted pH7.0, Adjust volume to 14 ml with d_2H_2O; Storage: -20° C
FISH Wash solution I	0.4x	SSC
	0.3% (v/v)	Triton-X 100
		pH 7.0-7.5, Storage: RT
FISH Wash solution II	2x	SSC
	0.1% (v/v)	Triton-X 100
		pH 7.0-7.5, Storage: RT
FISH Wash solution III	4x	SSC
	0.05% (v/v)	Triton-X 100
		Storage: RT
Blocking solution (FISH) Prepared fresh	3% (w/v)	BSA in FISH wash solution III
Antifade-solution A	1x	PBS
	1% (w/v)	1,4 diaminobenzol
		Used immediately for antifade (Working solution)
Antifade (Working solution)	5 ml	Antifade-solution A
	45 ml	Glycerol
		Mixed well, 1ml aliquots
		Storage: -20° C
DAPI counter stain	125 ng/ml	DAPI in the Antifade (Working solution)
		Storage: -20° C

2.1.4 Equipment

Name	Company
AxioCam HR digital camera	Carl Zeiss, Jena, Germany
Axioplan 2 Imaging microscope	Carl Zeiss, Jena, Germany
Axiovert 200M microscope	Carl Zeiss, Jena, Germany
BioPhotometer 6131	Eppendorf, Hamburg, Germany
Centrifuge 5417 C	Eppendorf, Hamburg, Germany
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Centrifuge RC5B Plus	SORVALL®, Langenselbold, Germany
Centrifuge Rotanta 460R	Hettich zentrifugen, Germany
Centrifuge Super T21	SORVALL®, Langenselbold, Germany
Electroporator (Easyject Prima)	EquiBio Peqlab, Erlangen, germany
Sunrise™ ELISA reader	TECAN group Ltd., Switzerland
FACSVantage SE System	BD Biosciences, Palo Alto, CA
Film developing machine M35X-OMAT Processor	Kodak AG, Stuttgart, Germany
Fluorscence microscope	Carl Zeiss, Jena, Germany
Fridge (4°C, -20°C)	Siemens AG
Fridge (-80°C) UF80-450S	Colora Messtechnik GmBH, Lorch
Gel electrophoresis systems	Bio-rad, Munich
Genetic analyzer automated DNA sequencer ABI PRISM 3100	PE Applied Biosystems, Foster City, CA
GS Gene Linker™ UV Chamber	Bio-Rad Laboratories, Hercules, CA
Hybridization incubator GFL 7601	GFL incubators, Czech Republic
HYBriteTM	Vysis GmbH, Germany
Incubator (For mammalian cell culture: CO_2 control)	Heraeus Instruments, Langenselbold, Germany
Incubator (with shaker): Certomat-R, Certomat-H	Sartorius Stedim biotech GmbH, Göttingen, Germany
Incubator	BE 200- Memmert, Schwabach, Germany

Name	Company
Innova™ 4400 Incubator Shaker	New Brunswick Scientific, Nürtingen, Germany
Liquid nitrogen tank	Cryoson, Schöllkrippen, Germany
MagNaPure LC	Roche Diagnostics Mannheim Germany
Mini Trans-Blot Electrophoretic Transfer Cell	Bio-Rad Laboratories, Hercules, CA
NanoDrop ND 1000 spectrophotometer	Thermo scientific, Delaware, USA
Orbital shaker (KS 250.3 basic)	IKA-labortechnik, Germany
pH-meter 766	VWR International, Ismaning, Germany
Thermocycler: Cyclone 25	PeQlab, Erlangen, Germany
Thermocycler: PTC-200 DNA engine cycler	MJ Research Inc., Nevada, USA
Thermomixer 5436	Eppendorf, Hamburg, Germany
Trans-Blot® SD Semi -Dry Transfer Cell	Bio-Rad Laboratories, Hercules, CA
Vortexer	Cenco, Breda, The Netherlands
Water bath	HAAKE, Karlsruhe
XCell *SureLock*™ Mini-Cell	Invitrogen Ltd., UK
X-Ray Automatic Processor Curix 60	Agfa, Köln Germany
Printer P91D	MITSUBISHI, Japan
Gel Jet imager	INTAS

2.1.5 Software

Name	Company	Usage
ISIS FISH imaging system	Metasystems, Germany	For capturing and processing FISH images
MS-Office	Microsoft Corporation, USA	For text editing, data analysis and graphs generation
Adobe Photoshop® 7.0	Adobe Systems, Unterschleißheim	Image processing
EndNote 8.0	Thompson ISI, Carlsbad, CA, USA	Management of bibliography

Name	Company	Usage
Mac Vector™ 9.0	Oxford Molecular Group	Used for primer design, restriction enzyme mapping, construct design and sequence annotation an display
Sequencher 4.7	Oxford Molecular Group	Used for generating sequence contigs
Macromedia Freehand 11.2	Freehand Systems, USA	High end illustrations
CellQuest Version 3.1(f)	BD Biosciences, Palo Alto, CA	Analysis of flow cytometry data

2.1.6 Culture medium

2.1.6.1 Tissue culture media

The following cell culture media were used and handled under sterile conditions and stored at 4°C.

Type of cell culture Medium	Company
Dulbecco´s Modified Eagle medium (DMEM)	Gibco® Invitrogen cell culture
Roswell Park Memorial Institute culture medium (RPMI 1640)	Gibco® Invitrogen cell culture

2.1.6.2 Bacterial culture medium

LB medium contents:

1 %	Tryptone
0.5 %	yeast extract
1 %	NaCl
1.5 %	agar (for plates only)
	pH 7.0

A ready to use mixture of the above components for LB-broth or LB-agar was used from Carl Roth GmbH, Germany. An appropriate amount of the powder was desolved in d_2H_2O and autoclaved.

In order to select transformants, ampicillin, kanamycin or chloramphenicol was added to the medium at final concentration of 50 µg/ml, 25 µg/ml or 12.5 µg/ml, respectively. The LB-agar medium with antibiotic was poured into Petri dishes and stored at 4° C.

2.1.6.3 Yeast culture medium

All yeast media and solutions were prepared and handled under sterile conditions.

YPD medium

20 g/l	peptone
10 g/l	yeast extract
20 g/l	agar (for YPD agar plates only)
950 ml	d_2H_2O

The pH was adjusted to 6.5 (with NaOH), if required followed by autoclaving. The medium was allowed to reach approx. 55°C, followed by the addition of 50 ml of 40 % glucose stock solution (2% final concentration). At the same temperature YPD-agar medium was poured into Petri dishes and stored at 4°C.

A combination of a minimal SD base and a dropout (DO) solution was used to produce a defined minimal yeast medium lacking one or more specific nutrients. The nutrients were added separately for each selective SD/DO medium according to the desired selection. A 10x stock dropout solution and SD medium lacking amino acids adenine, histidine, leucine, and tryptophan were prepared as follows.

10x DO, -Trp, -Leu, -His, -Ade	
200 mg	Arginine
300 mg	Isoleucine
300 mg	Lysine
200 mg	Methionine
500 mg	Phenylalanine
2000 mg	Threonine
300 mg	Tyrosine
200 mg	Uracil
1500 mg	Valine
1000 ml	ddH_2O, autoclave

SD -Trp, -Leu, -His, -Ade (per liter)	
6.7 g	yeast nitrogen base without aa
20 g	agar (for SD plates only)
850 ml	d_2H_2O
100 ml	10X dropout solution (-Trp, -Leu, -His, -Ade)

For SD -Trp, -Leu, -His, 200 mg Adenine was added to 1 l of the SD -Trp, -Leu, -His, -Ade.

For SD -Trp, -Leu, 200 mg Adenine and 200 mg Histidine were added to 1 L of the SD -Trp, -Leu, -His, -Ade.

Adjusted the pH to 5.8 when required and autoclaved. Allowed the medium to reach approx. 55°C and added 50 ml of 40 % glucose stock solution. For SD -Trp, -Leu, -His, -Ade plates supplemented with 3-AT appropriate amount of 1 M 3-AT stock solution was added to the SD-agar medium, mixed well and poured into Petri dishes. The petridishes with SD-agar medium were subsequently stored at 4°C.

2.1.7 Bacterial strain (*Escherichia coli*)

Bacterial strain	Usage	Genotype	Source
XL1 Blue	Host for plasmid amplification	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^{r})]	Stratagene, USA
JM109	Host for plasmid amplification	*endA1, recA1, gyrA96, thi, hsdR17* (r_K–, m_K+), *rel*A1, *sup*E44, Δ(*lac-proAB*), [F´, *tra*D36, *pro*AB *lac*I^{q}*Z*Δ*M15*]	Promega GmbH, Germany
SCS110	Host for plasmid amplification	*rpsL (Str^{r}) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ(lac-proAB) [F´ traD36 proAB lacI^{q}Z ΔM15]*	Stratagene, USA

2.1.8 Yeast strain

Strain	Reporters	Transformation markers	Source
AH 109	HIS3, ADE2, lacZ, MEL1	trp1, leu2	BD Biosciences Clontech, Heidelberg, Germany

2.1.9 Mammalian cell lines

All cell lines were originally obtained from the Cell Culture Collection from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), unless otherwise noted.

Name	Cell type/ Origin
NIH3T3	Swiss mouse embryo fibroblast
Ba/F3	mouse pro B-cells, IL-3 dependent line established from peripheral blood
Phoenix Ecotropic	Packaging cell line producing gag-pol and envelop proteins for ecotropic viruses; derivative of 293T cell line
293T	Human embryonic kidney cell line

2.1.10 Human sample

The peripheral blood and bonemarrow samples were obtained from an 18-year-old female patient diagnosed with acute leukemia in the Laboratory for leukemia diagnostics, Klinikum Grosshadern.

2.1.11 Plasmids

pBluescript II KS (+/-)	Cloning vector, Stratagene, La Jolla, USA; used as transformation control.
pGEM®-T Easy	Cloning vector with 3'-T overhangs at the cloning site for direct cloning of PCR products, Promega, Madison, USA.
pEYFP-C1	Expressing yellow (YFP) fluorescent proteins, BD Biosciences Clontech, Heidelberg, Germany; used as transfection control.
pMSCV-IRES-GFP	A modified MSCV vector containing a bicistronic expression cassette with an internal ribosomal entry site followed by GFP.
pGBKT7	Shuttle vector, expressing GAL4-DBD (amino acids 1-147) fusion proteins in the yeast system, DB Biosciences Clontech, Heidelberg, Germany.
pGADT7	Shuttle vector, expressing GAL4-AD (amino acids 768-881) fusion proteins in the yeast system. DB Biosciences Clontech, Heidelberg, Germany.

2.1.12 Constructs

Construct	Detail	Source
pcDNA3-BCR-ABL	Expression construct for BCR-ABL	Ying Chen
pACT2-IKAROS	Expressing a GAL4-AD-IKAROS fusion protein	Belay Tizazu
pGBKT7-AF10 (658-1027)	Expressing GAL4-DBD-AF10 (658-1027) fusion protein	Belay Tizazu
pMIY-BCR/ABL1	Retroviral expression construct for BCR-ABL	Dr. Karsten Spiekermann

2.1.13 Oligonucleotides

BCR-ABL1 primers used during the diagnostic screening

1. diagnostic p190 F 70 5'-ACCATCGTGGGCGTCCGCAAGA-3'
2. diagnostic p190 R 20 5'-TGATTATAGCCTAAGACCCGGA-3'
3. diagnostic p210 F 60 5'-GAGCGTGCAGAGTGGAGGGAGAACATCCGG-3'
4. diagnostic p210 F 40 5'-CCATTTTTGGTTTGGGCTTCACACCATTCC-3'

BCR-ABL1 primers used for the sequencing of the aberrant transcript

5. p190 nested 7i 5'- AGATCTGGCCCAACGATGGCGAGGGC -3'
6. p191 nested 2i 5'- ATCTCCACTGGCCACAAAATCATACA -3'
7. p210 nested 6i 5'- GAAGAAGTGTTTCAGAAGCTTCTCC -3'
8. p210 nested 4i 5'- TGTGATTATAGCCTAAGACCCGGAGCTTTTC -3'

Primers for the confirmation of presence of 5'SHIP1/3'ABL1 transcript

9. INPP5D-ABL1F997-1015 5'- TTGCTGCACGAGGGTCCTG -3'
10. INPP5D-ABL1B1474-1454 5'- TCTCCAGACTGTTGACTGGCG -3'

Oligonucleotides for the detection of 5'ABL1/3'SHIP1 transcript

11. ABL1-INPP5D402-421F 5'- CCAAGAAGGGGCTGTCCTCG -3'
12. ABL1-INPP5D741-721B 5'- GGGATGTAGTCCGCAGAGTCG -3'

Oligonucleotides for the cloning of the full length coding sequence of SHIP1/ABL1

13. SHIP-ABL-F20-38 5'- TAAGCTGGTGGCAGCAGCC -3'
14. SHIP-ABL-B1520-1502 5'- TACTCAGCGGCATTGCGGG -3'
15. SHIP-ABL1-F38-57 5'- CGAGGCCACCAAGAGGCAAC -3'

16. SHIP-ABL1-B1508-1489 5'- TTGCGGGACACAGGCCCATG -3'
17. SHIP-ABL1-HindIII-MunI-F39-59 5'-GCACAAGCTTCAATTGAGGCCAC -CAAGAGGCAACGG-3'

Oligonucleotides for the cloning of full length SHIP1/ABL1 with 5' epitope tags

18. Hind3-Mun1-Flag-SHIP1No3 5'-GCACAAGCTTCAATTGCCACCATGGACTAC-3'
19. Hind3-Mun1-Flag-SHIP1No2 5'-TGCCACCATGGACTACAAGGACGACGATGACA-3'
20 Hind3-Mun1-Flag-SHIP1No1 5'-AGGACGACGATGACAAGATGGTCCCCTGCTGG-3'
21. Hind3-Mun1-HA-SHIP1No3 5'-GACAAGCTTCAATTGCCACCATGGTCTACCCA-3'
22. Hind3-Mun1-HA-SHIP1No2 5'-CCATGGTCTACCCATATGACGTCCCAGACTAC-3'
23. Hind3-Mun1-HA-SHIP1No1 5'-ACGTCCCAGACTACGCTATGGTCCCCTGCTGG-3'

Sequencing primers for pGEM-T-easy inserts

24. pGEM-T-easy SP6 5'-ATTTAGGTGACACTATAGAATAC-3'
25. pGEM-T-easy T7 5'-TAATACGACTCACTATAGGGCGA-3'

Oligonucleotides used for the sequencing of full length SHIP1/ABL1 fusion cloned in to the pMSCV-IRES-GFP back bone

26. pMIG-F-1368-1389 5'-TCCCTTTATCCAGCCCTCACTC-3'
27. pMIG-B-1572-1548 5'-GCTTCCTTCACGACATTCAACAGAC-3'
28. [2] pMIG-SHIP-ABL(2)1859-1880 5'-CTGAGGAGGACACAGTAGAAAG-3'
29. [33] pMIG-SHIP-ABL(33)3175-3194 5'-AAGTGGGAGATGGAACGCAC-3'
30. [34] pMIG-SHIP-ABL(34)3626-3644 5'-TGAGCAGGTTGATGACAGG-3'
31. [43] pMIG-SHIP-ABL(43)3980-3999 5'-TCTCAGACGAAGTGGAAAAG-3'
32. [45] pMIG-SHIP-ABL(45)4287-4308 5'-CAAGAAGAAGAAGAAGACAGCC-3'
33. [46] pMIG-SHIP-ABL(46)4713-4732 5'-GTTTGACTCGTCCACATTTG-3'
34. [54] pMIG-SHIP-ABL(54)5148-5165 5'-ATTGTCCAGGCTCAAACC-3'
36. [60] pMIG-SHIP-ABL(60)5689-5708 5'-GATTCCATCCAGCAAATGAG-3'
37. [69] pMIG-SHIP-ABL(69)1924-1905 5'-TCAGCGGGATGTTTCTTGGG-3'
38. [101] pMIG-SHIP-ABL(101)3268-3250 5'-TGTATTTCTTCCACACGCC-3'
39. [126] pMIG-SHIP-ABL(126)5844-5826 5'-TTCCTTCACCGAACTGAGG-3'

Oligonucleotides used for the preparation of bacterial-Yeast shuttle vector constructs for Yeast-two-hybrid experiments

40. SHIP-SH2-BamH1F(161-176) 5'- CTAGGATCCCCTGCTGGAACCATGG -3'
41. SHIP-SH2-Pst1R(480-465) 5'- GTACTGCAGCTAGCCTGTGTCCTCTTCC-3'
42. SHIP-D1-EcoR1F(442-457) 5'- GTAGAATTCCTGCAATACCCTGTGC-3'
43. SHIP-D1-BamH1R(900-886) 5'- GTAGGATCCTAACGTGGACGGAGGCC-3'

44. SHIP-D2-EcoR1F(829-844) 5'- GTAGAATTCCGGACCCTCCCATCCC-3'
45. SHIP-D2-BamH1R(1188-1170) 5'- CGAGGATCCTACAGGATTTTCTTGTGGCTG-3'
46. SHIP-D2-Not1R(1182-1164) 5'- CGAGCGGCCGCTATTTCTTGTGGCTGTAGAAC-3'
47. SHIP-SH2-Xho1R(480-465) 5'- GTACTCGAGCTAGCCTGTGTCCTCTTCC-3'
48. SHIP-D2-Xho1R(1182-1164) 5'- CGACTCGAGCTATTTCTTGTGGCTGTAGAAC-3'

Primers for the cloning of the SHIP1/ABL1 deletion constructs

49. Hind3-Mun1-Flag-SHIPD1No1(460-474) 5'-AGGACGACGATGACAAGCTGGAGGAAGAGGAC -3'
50. Bcl1-SHIPD1 (898-884)B* 5'- GTACTGATCAGTGGACGGAGGCCCG-3'
51. Bcl1-ABL1BP(1184-1197)F 5'- CGACTGATCAAAGCCCTTCAGCGGCCA-3'
52. SHIP-ABL-Apa1-Spe1-R1508-1494 5'- ACAGTGGGCCCTTGCGGGACACAGGC-3'
53. HpaI-SHIP-D2 F903-919 5'- GCACGTTAACTCAGGTTCCTGGTGAGG-3'
54. Bpu10I-SHIP-D2 F904-921 5'- GCACCCTGAGCAGGTTCCTGGTGAGGCC-3'
55. Hind3-Mun1-HA-SHIPD1No1(460-474) 5'- ACGTCCCAGACTACGCTCTGGAGGAAGAGGAC-3'
56. Hind3-Mun1-SHIPD1(463-478)F 5'-CAAGCTTCAATTGCCACCATGGAGGAAGAG -GACACAG-3'
57. HindIII-Mun1-D2-F(829-844) 5'-CAAGCTTCAATTGCCACCATGCGGACCCTC -CCATCCC -3'
58. Hind3-Mun1-Flag-SHIPD2No1 5'- AGGACGACGATGACAAGCGGACCCTCCCATCCC -3'
59. Hind3-Mun1-HA-SHIPD2No1 5'- ACGTCCCAGACTACGCTCGGACCCTCCCATCCC -3'

Primers for sequence independent amplification (SIA)

(Bohlander et al., 1992)

60. Primer A 5'-TGGTAGCTCTTGATCANNNNN-3'
61. Primer B original SKB 5'- AGAGTTGGTAGCTCTTGATC-3'

2.1.14 Antibodies

Antibody	Company	Working dilution
Alexa Fluor 488, donkey-anti-goat IgG (H+L)	Molecular probes, Invitrogen	1:100
Alexa Fluor 488, goat-anti-rabbit IgG (H+L)	Molecular probes, Invitrogen	1:100
Anti-c-Abl (Ab-3) Mouse mAb (24-21)	Calbiochem, Merck KGaA, Germany	1:3000

Antibody	Company	Working dilution
anti-FITC/OG, Rabbit IgG, Alexa fluor 488 conjugate	Molecular probes, Invitrogen	1:50
Anti-Flag M2 Monoclonal (mouse)	Sigma-Aldrich, USA	1:3000-5000
Anti-GAL4 (DBD) (RK5C1), Mouse monoclonal IgG2a	Santa Cruz biotech, USA	1:3000
Anti-GAL4-TA (C-10), Mouse monoclonal IgG2b	Santa Cruz biotech, USA	1:3000
Anti-HA-high affinity (Rat monoclonal antibody, clone 3F10)	Roche applied science, Germany	1:3000
Goat-anti-mouse IgG-HRP conjugated	Santa Cruz biotech, USA	1:3000
Goat-anti-rat IgG-HRP conjugated	Santa Cruz biotech, USA	1:3000
Normal mouse IgG	Santa Cruz biotech, USA	Used for IP, 2µg for 400 µg total cell lysate
Normal rat IgG	Santa Cruz biotech, USA	

2.1.15 Bacterial artificial chromosomes

Clone name	Location	Source
RP11-400N9	3' to SHIP1	BACPAC resources center, CHORI, Oakland
RP11-512N9	5' to SHIP1	"
RP13-497I2	5' end of SHIP1	"
RP13-916J2	3' end of SHIP1	"
RP11-17L7	5' to ABL1, spanning the FUBP3 gene	Prof. Mariano Rocchi, Bari, Italy
RP11-57C19	5' end of ABL1	BACPAC resources center, CHORI, Oakland
RP5-1132H12	5' part of ABL1	Prof. Mariano Rocchi, Bari, Italy
RP5-835J22	3' par of ABL1	"
RP11-544A12	3' to ABL, corresponds to the NUP214 gene	BACPAC resources center, CHORI, Oakland

2.2 METHODS

2.2.1 Microbiology Techniques

2.2.1.1 Bacterial cultures

For routine bacterial cultures the frozen cells from the glycerol stocks were streaked out on to LB-agar plates containing appropriate selective antibiotics and incubated O/N (over-night) at 37°C to obtain single colonies. A single colony was then inoculated in the desired volume of LB medium containing appropriate antibiotic and incubated O/N at 37°C in a shaking incubator at 190 rpm. To prepare competent bacterial cells, LB agar and medium without antibiotics were used.

2.2.1.2 Preparation of bacterial stocks

850 µl of an overnight bacterial culture was mixed with 150 µl of glycerol and stored at -20°C or at -80°C for long-term storage.

2.2.1.3 Preparation of electro competent bacteria

A single bacterial colony was inoculated in 10 ml of LB medium and incubated O/N with vigorous shaking at 37°C. The O/N culture was diluted into 400 ml of fresh LB medium and incubated at 37°C for approximately 2-3 hours until the cell density reached an OD_{600} of 0.5-0.7. In order to stop the bacterial growth, the cultures were put on ice immediately. The cells were harvested by centrifugation at 4°C. The cells were washed twice with 40 ml ice cold, double distilled water (ddH_2O), twice with 20 ml ice-cold 10 % glycerol in ddH_2O and finally resuspended in 800 µl of ice-cold 10 % glycerol in ddH_2O. The competent cells were aliquoted (40 µl vol.) and stored at -80°C. All centrifugation steps were carried out at 4500 rpm (6000 x g) for 5 min at 4°C.

2.2.1.4 Delivery of plasmid DNA into bacterial cells

2.2.1.4.1 Electroporation

Electrocompetent cells (XL1 blue, 40 µl aliquots) *(2.2.1.3)* were thawed on ice for about 5 minutes. 1 µl plasmid DNA (0.1-2.0 ng) or 2-5 µl ligation reaction

(2.2.2.4.6) was mixed with the competent cells by gentle pipetting, incubated on ice for 1 min and transferred to a pre-chilled electroporation cuvette (2 mm electrode gap). The cuvette was placed in an electroporator (Easyject Prima, Equibio) and the cells were electroporated at 2.5 kV (12.5 kV/cm, 15 µF, 335 Ω, 5 ms pulse duration). After electroporation, 1 ml pre warmed LB medium (37°C) was immediately added to the cells, the suspension was transferred to a fresh 1.5 ml tube and incubated for 1 hr with vigorous shaking (190-210 rpm) at 37°C. A 100 µl aliquot from this 1 ml culture was plated onto LB agar containing appropriate selective antibiotic (10% plate) and the remaining 900 µl were briefly centrifuged (3000 g, 2 minutes) to pellet down the cells, 800 µl of the supernatant was removed, the cell pellet was resuspended in the remaining 100 µl medium and spread onto a second LB agar plate (90% plate). The plates were incubated O/N at 37°C. Thus each transformation reaction was plated on two plates: one with 10% and the other with 90% volume of the reaction. This allowed us to pick single transformant colonies even in cases of very efficient transformation experiments with a large number of transformants. The transformants were further analyzed either by the so called Colony PCR using the DNA obtained after direct thermal denaturation of the bacterial cells *(2.2.2.4.1)* or by restriction enzyme digestion after plasmid minipreparations *(2.2.2.4.5)*.

In case of the pGEM-Teasy constructs, the transformants were initially identified by the blue-white selection provided by the pGEM-Teasy vector system. The high copy-number pGEM-T easy vectors contains T7 and SP6 RNA polymerase promotors flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of α-peptide allows recombinant clones to be directly identified by a blue/white selection on indicator plates containing IPTG, X-Gal and ampicillin. Recombinant clones result in white colonies, because of the insertional inactivation of the α-peptide. White colonies were further analyzed either by the colony PCR or by plasmid minipreparation and restriction enzyme digestion.

All the PCR fragments generated for construct cloning were first cloned into pGEM-Teasy vectors in order to avoid repetitive amplifications in case of

experimental failures and to verify the sequences before proceeding with the subsequent cloning steps.

2.2.1.4.2 Transformation of chemically competent bacteria

For some cloning experiments, highly efficient chemically competent bacterial cells were purchased. The transformation was performed largely according to the manufacturer's suggestions. Briefly, the competent cells (strain JM109) were thawed on ice for 5-10 minutes. 1 µl plasmid DNA (0.1-2.0 ng) or 2-5 µl ligation reaction *(2.2.2.4.6)* was pipetted in a prechilled 1.5 ml Eppendorf tube and 50 µl of the competent cells were added to the tube, mixed by gentle flicking, incubated on ice for 20 minutes, heat-shocked at 42°C for 45 seconds, put on ice for two minutes, and resuspended in S.O.C. medium to a volume of 1 ml. The cultures were incubated at 37°C, 190 rpm for 60-90 minutes. Then 10% and 90% volume of the transformation reactions was plated on LB agar containing appropriate antibiotics as described in the previous section *(2.2.1.4.1)*.

2.2.1.5 Delivery of plasmid DNA into yeast cells

2.2.1.5.1 Preparation of competent yeast cells

Yeast cells were transformed using the Lithium acetate method. One or more yeast colonies (≤ 4 weeks old and more than 2 mm in diameter) were resuspended in 1 ml YPD medium, mixed by vortexing and transferred to 20 ml YPD medium *(2.1.6.3)*, incubated O/N (16-18 hrs) at 30°C and 250 rpm (stationary phase OD_{600} >1.5). The culture was then transferred to 300 ml YPD and incubated for approx. 3 hrs until an OD_{600} of 0.4-0.6 was reached. The cells were harvested by centrifugation at 1000 x g for 5 min at RT and resuspended in 30 ml of ddH_2O. After a second centrifugation step, the supernatant was discarded, and the cells were resuspended in 1.5 ml freshly prepared 1x TE/LiAc *(2.1.3.1)*. These freshly prepared competent cells were used directly for the transformation *(2.2.1.5.2)*.

2.2.1.5.2 Transformation of yeast cells

100 µl of the freshly prepared yeast competent cells *(2.2.1.5.1)* were mixed

with 10 µl carrier DNA (10 mg/ml of herring sperm DNA), 100 ng of the plasmid to be introduced and 600 µl PEG/LiAc. For the yeast two hybrid interaction assay both the bait and the prey plasmids were added together in the reaction at a molar ratio of 2:1 (200 ng prey plasmid encoding the GAL4-DNA-Binding Domain (GAL4DBD) fusion, and 100 ng bait plasmid encoding the GAL4-Activation Domain (GAL4AD) fusion). The reaction was incubated for 30 min shaking at 30°C (200 rpm). 70 µl DMSO was added and gently mixed by inversion. The cells were heat shocked for 15 min in a 42°C water bath, chilled on ice for 2 min, pelleted by centrifugation at 14000 rpm (>20000 g) at RT for 5 sec and resuspended in 1 ml YPD. In order to allow cells to recover from the stress, they were incubated for 1 hr at 30°C, shaking at 230 rpm. After that, cells were washed twice in 1 ml TE buffer (>21000 x g, 5 sec at RT) and finally plated on SD plates lacking tryptophan and/or leucine (selection for the presence of plasmid expressing the GAL4DBD and GAL4AD fusions, respectively) and incubated at 30°C for 2 to 4 days. Colonies positive for both bait and prey plasmids, which were able to grow on the double selection plates, were then assayed for protein interaction by re-plating on appropriate SD drop out plates. Plates were incubated at 30°C and colony growth was monitored for several days.

2.2.1.6 Preparation of yeast stock

Glycerin stocks were prepared for long-term storage of the yeast clones. A single colony was inoculated into 3 ml YPD medium *(2.1.6.3)* and incubated O/N shaking at 30°C. 1 ml of the O/N yeast-culture plus 300 µl glycerol were pipetted into a fresh 1.5 ml cryo tube, mixed by vortexing and stored at -80°C.

2.2.2 Molecular biology techniques

2.2.2.1 Plasmid DNA isolation

Plasmid and BAC DNA was isolated from bacteria using the alkaline lysis method (Sambrook, 1989). To obtain plasmid DNA for transfection into mammalian cells, the DNA was purified by an affinity column purification step using Qiagen kits *(2.1.2)*.

2.2.2.1.1 Small scale preparation of plasmid or BAC DNA from E. coli

This method was used to isolate small amounts of DNA (mini prep) from a large number of clones simultaneously, i.e., during cloning. First, a single *E. coli* colony was inoculated into 3 ml of LB medium containing selective antibiotics and incubated O/N with vigorous shaking at 37°C. From the O/N culture, 1.5 ml was centrifuged for 2 min at 8000 rpm (6000 x g) and the supernatant was discarded. The pellet was resuspended in 150 µl of P1 buffer *(2.1.3.2)* and the cells were lysed by adding 150 µl P2 lysis buffer *(2.1.3.2)* followed by gentle mixing and 5 min incubation at RT (room temperature). The lysate was then neutralized by adding 150 µl of buffer P3 *(2.1.3.2)*, gently mixed and incubated on ice for 10 minutes. The reaction was centrifuged for 10 min at 21000 x g, and the supernatant containing the plasmid DNA was transferred to a fresh 1.5 ml tube. The DNA was precipitated by adding 300 µl (0.7 vol of the collected supernatant) of RT isopropanol, mixing and incubating on ice for 20-30 minutes followed by centrifugation at 4°C, 19000 g for 20 minutes. The DNA pellet was washed with 90 µl 70 % ethanol (10 min; 19000 x g, 4°C), air dried and dissolved in 50 µl TE. The DNA was stored at -20°C.

For the BAC (Bacterial artificial chromosomes) DNA mini preps, the DNA was isolated from 4 ml of O/N bacterial cultures following the above mentioned protocol using 200 µl of the buffer P1, P2 and P3 and the volume of the other reagents was scaled up accordingly. The BAC DNA minipreps were used for the preparation of FISH probes by SIA (Sequence independent amplification) and PCR labeling *(2.2.5.2.1)*.

2.2.2.1.2 Large scale preparation of plasmid from E. coli.

Endotoxin free plasmid DNA (maxi preparations) were prepared for cell culture applications, i.e., delivery of plasmid DNA into mammalian cells *(2.2.3.3, 2.2.3.4)*. This was done using the endotoxins free plasmid DNA isolation kit from Qiagen according to the manufacturer's instructions.

2.2.2.2 RNA extraction

Poly-A mRNA was isolated from human blood or bone marrow mononuclear cells using the MagNA Pure LC mRNA isolation kit and the MagNa Pure instrument from Roche Applied science.

2.2.2.2.1 Isolation and lysis of mononuclear cells

Mononuclear cells were isolated by gradient density centrifugation, using the Biocoll separating solution (Biochrom AG, Berlin, Germany.) The Biocoll separating solution contains Ficoll 400® (Polysucrose 400), a polymer with a molecular weight of approx. 400 kD. Ficoll has a higher density than lymphocytes or monocytes and a lower density than erythrocytes and granulocytes. By centrifugation with Ficoll, the mononuclear cells, which include monocytes, lymphocytes and natural killer cells, are enriched in the interphase layer between the whole blood/ bone-marrow and the Ficoll solution and can be recovered by pipetting.

To obtain the mononuclear cells, 15 ml of Biocoll separating solution (density = 1.077 gm/ml at +20°C) was pipetted into a 50 ml centrifugal tube. In parallel, equal amounts of heparinized whole blood or bone-marrow and phosphate buffer saline (PBS) were mixed in another 50 ml centrifugal tube and slowly added to the tube containing the Biocoll separating solution with a sterile 10 ml pipette, taking care not to disturb the Ficoll layer. The tube was centrifuged for 20 minutes at 1200 x g at RT to allow the formation of density gradients. Using a 10 ml pipette the layer of enriched (70 - 100%) mononuclear cells (between plasma and Biocoll) was aspirated and carefully transferred to a new 50 ml centrifuge tube. The cells were washed with 1x PBS (10 min at 300 x g) and a cell count was obtained by a Microcell counter (Sysmex, Germany). Aliquots of $5x10^6$ cells were prepared and the cells were immediately lysed in 300 µl of RLT buffer. The RLT lysates were stored in 1.5 ml centrifuge tubes at -80°C until mRNA isolation.

2.2.2.2.2 Isolation of mRNA

For mRNA isolation, the RLT lysates were thawed on ice and homogenized by passing them through a QIAshredder spin column (Qiagen, Germany) at

21000 x g for 2 min. The flow through was used to extract the mRNA by the MagNA Pure LC mRNA isolation kit in a MagNa Pure machine (Roche Applied science) according to the manufacturer's instructions.

The following steps are performed automatically by the MagNa Pure machine, the homogenized RLT lysates are mixed with Biotin-labeled oligo(dT) in hybridization buffer, to allow the hybridization of the oligo(dT) to the 3' poly(A+) tails of the mRNA. The oligo(dT)-mRNA complexes are removed from the solution by Streptavidin-coated-magnetic particles (SMPs), which bind to the complex through the Biotin-labeled oligo(dT). Residual genomic DNA is removed from the mixture by a DNase I treatment and the contaminants are removed by repeated washing steps. The purified mRNA is eluted from the mRNA-Oligo(dT)-SMP complexes in elution buffer at 70ºC and the Biotin-labeled Oligo(dT)-SMP complexes are removed by a magnet.

We eluted the mRNA in 30 µl of elution buffer and the yield was determined by measuring the RNA concentration with a spectrophotometer for small volumes (Nanodrop, Thermo Scientific, Schwerte Germany). The isolated mRNA was stored at -80°C until cDNA synthesis.

2.2.2.3 DNA Analysis and purification

2.2.2.3.1 Agarose gel electrophoresis

Non-denaturing gel electrophoresis was employed for DNA separation, which was used for both analysis and isolation of the DNA fragments. The agarose concentration of the gels was determined according to the desired range of separation of linear DNA according to the table shown below:

Agarose (%)	Range of separation of linear DNA	Used for
0.8	500 bp to 15 kb	>3.5 kb
1.0	250 bp to 12 kb	
1.2	150 bp to 6 kb	1.5 kb to 3.5 kb
1.5	80 bp to 4 kb	
1.8	50 bp to 3 kb	50 bp to 1.5 Kb

The gel was prepared by boiling the desired amount of agarose in 1x TBE buffer *(2.1.3.2)*. After the gel had cooled to approx. 50°C, 3.5 µl Ethidium

bromide (Ethidium bromide stock: 10 mg/ml) *(2.1.1)* was added per 100 ml of gel volume. The gel was poured onto a horizontal gel bed. After the gels had completely cooled they were transferred into appropriate gel boxes. The DNA samples were loaded in the gel slots with loading dye added to 1x final concentration *(2.1.3.2)*. Then the DNA fragments were separated by applying an electrical field of approximately 700 V/m. Under these conditions the negatively charged DNA molecules would migrate to the anode at speeds inversely proportional to the length of the individual DNA fragments. The gels would typically be run for 1 to 2 hours.

2.2.2.3.2 Isolation of DNA fragments from agarose gels

DNA fragments that would be used in subsequent cloning experiments were cut out from the gels without exposing them to the UV light of the transilluminator. For this, a fraction (indicator sample) of the same DNA sample was run in adjacent wells on both sides of the main DNA sample in parallel on a gel without Ethidium bromide. After the run, the gel was cut apart in such a way that the main sample lane was separated from the flanking lanes containing the indicator sample. The gel pieces with the indicator samples were stained with Ethidium bromide for approx. 20 minutes. Then the desired DNA band was excised from the indicator lanes on the UV transilluminator. After this, the gel was reassembled and the cuts in the indicator lanes on either side of the gel fragment containing the main sample were used to guide the excision of the desired band in the main sample under normal light.

Cutting the DNA fragments (vectors and inserts) in the absence of UV increased the cloning efficiency in the subsequent experiments by a factor of approximately 1000. The excised gel slice was then transferred to a 1.5 ml Eppendorf tube and the DNA fragments were extracted from the gel using the Gel Extraction kit from Qiagen *(2.1.2)* according to the instruction manual. The DNA fragments were eluted in 30 µl Elution Buffer provided with the kit.

2.2.2.3.3 Determination of DNA and RNA concentration

DNA concentration was measured with a Nanodrop spectrophotometer by using 1.7 µl of the undiluted DNA sample. The purity of the sample was determined by the OD_{260}/OD_{280} ratio. For pure DNA sample this ratio should be between 1.8 to 2.0.

Alternatively, the an Ethidium bromide stained agarose gel was used to estimate the DNA concentration. For this, the intensity of the sample DNA band was compared to the bands of a DNA maker of known concentration loaded on the same gel. The concentration of the band of the marker with the same intensity was estimated to be the concentration of the sample DNA.

2.2.2.4 Enzymatic manipulation of DNA and RNA

2.2.2.4.1 Polymerase chain reaction (PCR)

The PCR technique is employed to amplify DNA fragments of interest in an exponential fashion. The amplification is achieved using synthetic oligonucleotides, so called primers, flanking the sequence to be amplified. These primers, under appropriate conditions, anneal to the denatured target sequence and, in the presence of a heat stable DNA polymerase enzyme and dNTPs, direct the synthesis of a complementary strand of the target sequence. As a template 1 µl cDNA (about 10 to 200 ng), 1 µl genomic DNA (0.1 to 1 µg) or 0.5 ng plasmid DNA was used. The PCR reactions were prepared as follows:

PCR reagents	**Amount in µl (Per 25 µl reaction)**	**Final concentration**
10X PCR Buffer	2.0	**1 x**
50 mM $MgCl_2$	0.6	**1.5 mM**
10 mM dNTP mix	0.4	**0.2 mM**
10 µM primers F	1.0	**0.5 µM**
10 µM primers R	1.0	**0.5 µM**
DNA (0.5 ng/µl to 1 µg/µl)	1-5	**N. A.**
DNA Polymerase (5 U/µl)	0.1	**1U**
ddH_2O	**up to 20.0**	

Alternatively, reactions were scaled up to 50 µl vol.

The reaction was cycled through a temperature profile in a thermocycler with the following basic program:

Steps	***Temperature (°C)***	***Time (min:sec)***	***No. of cycles***
Initial denaturation	94	4:00	1
Subsequent denaturations	94	0:45	20 to 35
Annealing	60	0:45	
Elongation	72	3:00	
Final elongation	72	7:00	1

The annealing temperatures varied between 42º and 65°C depending on the primer pair used and the extension times varied according to the expected size of the PCR product allowing about 1 minute extension time per 1000 bp of product size.

For the so called Colony PCR, which was used to identify bacterial clones carrying the DNA sequence of interest during the cloning procedure, DNA templates were obtained by boiling the bacterial cells at denaturing temperature. In detail, a single bacterial colony from an LB-agar plate *(2.1.6.2)* was picked with a tooth-pick, put into 40 µl of TE in an Eppendorf tube. The genomic DNA and plasmid DNA was then liberated by boiling at 95°C for 10 minutes. The cell debris were pelleted by centrifuging at 16000 g for 1 minute and 5 µl from the supernatant was used as a template in the colony PCR reaction (typically 20 µl total PCR volume).

2.2.2.4.2 Tailing of the PCR products amplified by proof reading polymerases

The pGEM-Teasy vector has single 3'-T overhangs at the cloning site, greatly increasing the efficiency of ligating PCR products with 3'-A overhangs with the plasmid. Thermostable DNA polymerases, such as *Taq*, *Tfl* and *Tth*, generate 'A' overhangs at the 3' ends of the amplified fragments, providing compatible ends for the pGEM-Teasy vectors. In contrast, thermostable polymerases

with proof reading activity (*Pfx* and *Pfu* DNA polymerase) generate blunt ended fragments during PCR amplification. For efficient ligation of the PCR fragments amplified by these proof reading enzymes, 3'-A overhangs have to be added to the blunt ended PCR products. This process is called tailing.

Briefly, for the tailing of the blunt ended PCR products, the products were first gel purified and eluted in 30 µl of elution buffer (EB) *(2.2.2.3.2)*. A 40 µl reaction containing, 30 µl of the eluted PCR product, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 unit of *Taq* DNA polymerase was set up. The reaction was incubated at 72° C for 15 minutes, chilled on ice and an aliquot was used for the ligation reactions.

2.2.2.4.3 Reverse transcriptase reaction (cDNA synthesis)

Reverse transcriptase reaction followed by PCR (RT-PCR) is a sensitive technique used to determine the presence or absence of specific RNA transcript or to quantify the level of gene expression. It is also a useful tool for the amplification of genes of interest. In the reverse transcriptase PCR (RT-PCR), a cDNA strand, complementary to the RNA template is synthesized first using random hexamer primers by using the reverse transcriptase. The cDNA is then used as template in subsequent PCR reactions.

Reagents	**Amt (µl) per 50 µl reaction**	**Final concentration**
100 mM dNTP Mix	4.4	8.8 mM
5x First-Strand Buffer (Supplied with the Superscript™ II RNase H⁻ Reverse Transcriptase, Invitrogen)	10.0	1x
Random Primer p(dN)6 (1µM) (Roche diagnostics, Germany)	2.5	50 nM
RNasin® RNase inhibitor (40 U/µl) (Promega, USA)	1.25	1 U/µl
SuperScript II RT (200 U/µl) (Invitrogen, Germany)	1.9	7.6 U/µl
Template RNA	1-2 µg	-
RNase-free water	up to 50 µl	-

For cDNA synthesis, 1-2 µg of the eluted mRNA *(2.2.2.2.2.)* was denatured at

70° C for 7-8 min. The denatured RNA was then added to the reverse transcriptase reaction as given in the above table. The reaction mix was incubated at 37° C for 60 min to allow for reverse transcription and cDNA synthesis. The reaction was stopped by thermal inactivation of the reverse transcriptase enzyme at 95° C for 5 min. The cDNA was stored at -20° C.

2.2.2.4.4 Sequencing

DNA Nucleotide sequencing was employed to confirm the identity of a clone and to check the fidelity of PCR products or of ligation junctions. For all sequencing the Sanger chain termination method was used. For most of the sequencing, we used a commercial service (“sequiserve", Ebersberg). Some of the sequencing was also performed by our molecular biology diagnostic group in the Leukemia Diagnostic Laboratory, Med III, Klinikum Großhadern, München. The commercial and the in-house sequencing was performed with a cycle-sequencing dye-dideoxy chain termination protocol.

The cycle sequencing reaction consists of successive rounds of denaturation, annealing and extension in the presence of polymerase, dNTPs and dye-labeled terminator dideoxy nucleotides. For the sequencing reaction 4 µl BigDye terminator was added to the DNA to be sequenced together with 1 µl primer (10 µM) in a 10 µl reaction. 10-100 ng PCR products (0.2-1.5 kb) or 1 µg plasmid DNA was used as template in each sequencing reaction. The reaction was performed in a thermocycler with the following program:

Steps	Temperature (°C)	Time (min)	No. Of cycles
Initial denaturation	96	2.0	1
Subsequent denaturations	96	0.30	25
Annealing	50	0.15	
Elongation	60	4.00	

After cycle sequencing had been completed, 10 µl ddH_2O was added to reach a 20 µl volume. This mixture was passed through a CENTRI Sep column (CENTRI Sep 8 well strip, Princeton, USA) at 750 x g for 2 minutes according to the manufacturer’s instructions. This step efficiently removes the excess dye terminators from the completed DNA sequencing reactions. 10 µl of the

purified sequencing reaction was then loaded in a single well of a 96-well sample plate of a Genetic Analyzer automated sequencer (ABI PRISM 3100, PE Applied Biosystems) for sequence analysis.

2.2.2.4.5 Digestion of DNA with restriction enzymes

Restriction endonucleases cut double stranded DNA at a short specific nucleotide recognition sequence. These endonucleases are widely used for the modification and manipulation of DNA. For site-specific digestion (cutting) of DNA, type II restriction endonucleases (enzymes) are required. These enzymes are capable of recognizing, binding and cleaving the phosphodiester bond at short specific nucleotide sequences within the target DNA. The restriction digestion of DNA was carried out with specific buffers that had different compositions of salts and buffering agents depending on the restriction enzyme used. When multiple enzymes were used, the Five Buffer Plus System such as Y+/Tango™ (MBI Fermentas) was used according to the manufacturer's recommendation. For single digestion the following components were mixed and the digestion was allowed for 2 hrs to O/N at an optimal temperature as recommended for each enzyme.

Digestion reaction:

Components	**Amount**
DNA	0.5-1.0 µg
Appropriate restriction enzyme buffer	1x final concentration
Restriction enzyme	1-4 units (depending upon the enzyme activity)
ddH_2O	To make up the total vol of 10 µl

Alternatively, the reactions were scaled up to 50 µl vol.

2.2.2.4.6 Ligation

To join DNA fragments after restriction digests in a covalent fashion, ligation reactions were used. For the ligations, both vector (V) and inserts (I) having compatible ends were prepared by restriction digestion *(2.2.2.4.5)* and gel purification *(2.2.2.3.2)*. The vector and the insert were used at a molar ratio of 1:3 (V:I) in the ligation reaction as described in the following table. The

reaction was incubated for 1-2 hrs at RT or overnight at 4°C and then stored at -20°C. Alternatively, the ligation reactions were incubated in an open ice water-filled styrofoam container on the lab bench overnight. The ice would slowly melt, and the temperature of the ligation reaction would reach room temperature over a course of 12 to 16 hours.

Ligation Reaction

Components	**Amount**
Vector DNA (eg 3 kb in size)	50 ng
Insert DNA (eg 1.5 kb in size)	75 ng
T4 DNA ligase buffer (with ATP)	To 1x final concentration
T4 DNA ligase (5 U/µl)	1 µl
d_2H_2O	Adjust to 10 µl reaction volume

The ligations of PCR products with the pGEM-T-easy vector *(2.1.2)* were performed according to the manufacturer's instructions.

2.2.2.5 Yeast-two-hybrid system

2.2.2.5.1 Principle

The yeast-two-hybrid (Y2H) assay is an in vivo technique for the detection of protein-protein interactions. This technique is based on a transcriptional assay for the detection of protein-protein interactions in yeast. In yeast, GAL80 and GAL4 are the two regulatory proteins in galactose metabolism. When galactose is present, the GAL4 protein binds to GAL4 responsive elements (called Upstream Activating Sequences or UAS) upstream of several genes involved in galactose metabolism and activates transcription. When galactose is absent, the GAL80 protein binds to GAL4 and blocks GAL4-mediated transcriptional activation. The tight regulation of the GAL UASs by GAL4 makes it an important tool for controlling the expression of reporter genes in the Y2H system. This technique can be used to confirm expected interactions between two known proteins or to discover an unknown interacting partner (prey) of a known protein (bait). If the two proteins interact, the transcription of a reporter gene is activated *(Figure 2.1)*.

The Y2H system is based on the fact that transcription factors bind to specific DNA sequences in the promoter region of genes and then activate the transcription of these genes. For example, GAL4 regulates galactose metabolism by binding to the UAS in the promoter regions of genes involved in galactose metabolism. When the GAL4 protein binds to GAL4-UASs, the downstream genes are activated and transcribed. The GAL4 protein consists of two important domains: a DNA binding domain (GAL4-DBD) by which it binds to the UAS and a transcription activation domain (GAL4-AD), which activates the transcription of downstream genes.

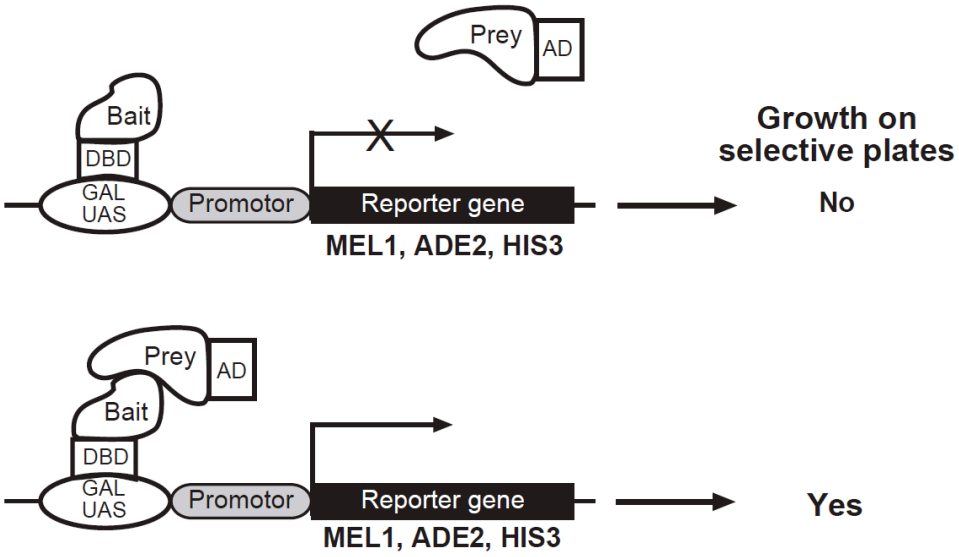


Figure 2.1: The Yeast-two-hybrid system. The DBD is amino acids 1-147 of the yeast GAL4 protein, which binds to the GAL4 UAS upstream of the reporter genes. The AD is amino acids 768-881 of the GAL4 protein and functions as a transcriptional activator. If the proteins fused to the DBD and the AD portion interact the transcription of the reporter gene is turned on.

2.2.2.5.2 Adjusting for background HIS3 reporter gene expression

In the yeast strain AH109, which was used for the Y2H assays, the complete HIS3 promoter (including both TATA boxes) is replaced by the entire GAL1 promoter, leading to tight regulation of the HIS3 reporter. However, the expression of some GAL4-DBD-bait proteins produces background growth on SD plates lacking tryptophan and histidine (SD/-Trp/-His) plates because of a low amount of HIS3 expression. The effect of this low level HIS3 expression on the SD/-Trp/-His plates can be suppressed with 3-aminotriazol (3-AT), a

competitive inhibitor of the enzymatic activity of the HIS3 protein. The concentration of 3-AT in the SD medium, required to suppress the effect of the low level HIS3 expression, was titrated by plating the transformed yeast cells on SD/-Trp/-His plates containing 0, 3, 5, 10, 15, and 20 mM 3-AT. The lowest concentration of 3-AT, which after one week, allowed small colonies to grow was then used in the Y2H assays.

2.2.2.5.3 Yeast two hybrid assay

For Y2H assays, the coding sequence for the bait and the prey proteins were cloned into pGBKT7 and pGADT7 vectors separately to generate GAL4DBD and GAL4AD fusion genes, respectively. The two constructs were then used to co-transform the yeast strain AH109 *(2.2.1.5.2)* and the co-transformants were identified by plating the transformations onto SD plates lacking tryptophan and Leucine (SD/-Trp/-Leu), which are the selection markers on the pGBKT7 and pGADT7 plasmids, respectively. The double transformants, were replated on SD/-Trp/-Leu/-His plates *(2.1.6.3)* containing the appropriate concentration of 3-AT *(2.2.2.5.2)* to assess the potential interaction between the bait and the prey proteins by monitoring colony growth. Alternatively, SD/-Trp/-Leu/-His/-Ade plates can also be used for more stringent experimental conditions, because the additional activation of the reporter gene ADE2 is required for colony growth. Those bait prey combinations that showed interaction under the most stringent experimental conditions, were later verified in co-immunoprecipitation experiments.

2.2.3 Cell culture techniques

2.2.3.1 Cultivation of mammalian cells

All the mammalian cell lines used in the study were cultivated in CO_2 incubators at 37°C in presence of 95% relative air humidity and 5% CO_2. All the cell culture media used in the study *(2.1.6.1)* were supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (final concentration: penicillin: 100 U/ml and streptomycin: 100 µg/ml).

2.2.3.1.1 Cultivation of adherent cells

All the adherent cell lines were grown in complete Dulbecco's Minimum Essential medium (DMEM) under abovementioned conditions. For harvesting the adherent cells, the confluent cultures were washed once with pre-warmed (37°C) phosphate buffered saline (PBS) and treated with trypsin-EDTA *(2.1.1)* at 37°C for 5 minutes to achieve detachment of the cells from the surface of the culture flask and separation of the cells from each other. Adding complete DMEM stopped the activity of the trypsin and the cell suspension was either used for subculturing or for the preparation of frozen stocks. The cell lines were propagated and maintained by dividing the confluent cultures at a ratio of 1:3, 1:5 or 1:10 every 3-5 days depending on the growth characteristics of the cell lines.

2.2.3.1.2 Cultivation of suspension cells

The suspension cells used in the study (WEHI and Ba/F3) were cultivated in complete RPMI-1640 medium in the culture conditions mentioned above. For the IL-3 dependent murine pro-B cell line Ba/F3, the complete RPMI-1640 was supplemented with 10% IL3 (WEHI) conditioned medium *(2.2.3.1.3)* as a source of murine IL3. Alternatively, recombinant murine IL3 (rmIL3) was used at a final concentration of 10 ng/ml. The Ba/F3 cell cultures were divided at a ratio of 1:5 or 1:10 every 2-4 days.

2.2.3.1.3 Preparation of IL3 conditioned medium

The murine myelomonocytic leukemic cell line WEHI-3B produces IL-3, which was used to supplement the growth medium for the Ba/F3 cells. The WEHI-3B cells grow adherently and partially in suspension and are very sensitive to trypsin. To obtain the IL3 conditioned medium, WEHI-3B cells were grown in complete RPMI-1640 medium *(2.1.6.1)* and as they are trypsin sensitive, while sub-culturing and freezing, the cells were incubated with DPBS (without Ca^{2+} or Mg^{2+}) for 10 minutes at 37°C and detached from the surface of the culture flask with the help of scrapers. The cells were then sub-cultured at a 1:5 ratio every 3-5 days. The supernatant from the confluent cultures was filtered through a 0.22 µm pore size filter (Millipore, USA) and stored at -20°C

until used to supplement the culture medium for the IL-3 dependent Ba/F3 cells.

2.2.3.2 Freezing and thawing of cells

Mammalian cells can be maintained for years by storing them in liquid nitrogen. They can be revived by proper thawing. For freezing, approximately $1x10^7$ cells were pelleted, resuspended in 1 ml of fetal calf serum containing 10% vol DMSO (dimethyl sulphoxide) and transferred in a 1.5 ml cryotube (Nunc, Roslilde, Denmark). DMSO serves as a cryoprotective agent and is used to minimize the cellular injury induced by the freezing and thawing procedures. The cryotubes were then put in a cryo freezing container (NALGEN™, USA) in a -80° C freezer to gently freeze cells at a cooling rate of about 1° C/min. While short-term preservation of cell lines at -80°C is possible, storage in liquid nitrogen (-196°C) or its vapor (-120°C) is much preferred.

For optimal recovery and viability of the cells, rapid thawing is essential. Therefore, the cells were thawed quickly in a 37°C water bath and, as DMSO in the freezing medium are toxic to the cells, the cells were subsequently washed by diluting them with 10-20 ml of DPBS or culture medium and centrifugation at 1000 rpm (~300 x g) for 5 min. The supernatant was discarded and the pellet resuspended in fresh culture medium. All the thawed cell lines were cultured for 3 days (split once) before being used for experiments.

2.2.3.3 Transient transfection of adherent cells

Different mammalian cell lines exhibit greatly varying transfection efficiency. The HEK293 cells (derived from human embryonal kidneys) can be transfected with high efficiency. Hence, these cells were transiently transfected for the overexpression of proteins. Transient transfection of adherent cells was performed by either using the cationic polymer, polyethylenimine (PEI), as a transfection reagent or using the $CaPO_4$ precipitation method. The day before the transfection, 1.5-2 x 10^6 cells were seeded in a 10 cm plate with 10 ml of appropriate complete growth medium.

The cells were then incubated for 16-24 hrs at 37°C in a CO_2 incubator until they were about 70 % confluent. For the transfection using the cationic polymer, 10 µg of plasmid DNA *(2.2.2.1.2)* + 400 µl plain medium (DMEM without any supplements) and 15 µg PEI *(2.1.1)* were mixed thoroughly and incubated at RT for 10-60 min to allow DNA-cationic polymer complex formation. Meanwhile cells were washed once with DPBS and 3 ml antibiotic-free medium with serum was added to the cells. The DNA-polymer complexes were added to the cells and the DNA was allowed to enter the cells by endocytosis at 37°C, 5% CO_2 for about 16 hours (usually overnight). Then 7 ml of complete growth medium was added to the cells, which were incubated for another 20-48 hrs. After that, cells were harvested for protein extraction *(2.2.4.1)*. The $CaPO_4$ transfection method was mainly used to obtain virally conditioned medium *(2.2.3.4.1)*.

2.2.3.4 Preparation of stably transduced Ba/F3 cells

To establish stably transduced Ba/F3 cell-lines, the gene of interest is cloned into a retroviral expression vector and expressed in fibroblast cell line in presence of retroviral packaging proteins (packaging cell line), to obtain a high titer viral supernatant which is then used to transduce the Ba/F3 cells.

2.2.3.4.1 Production of high titer virally-conditioned medium

For the production of high titer retroviral supernatant, the ecotropic retroviral packaging cell line Phoenix Eco *(2.1.9)* was transiently transfected with the DNA of interest using the calcium phosphate precipitation method. 12-15 x 10^6 Phoenix Eco cells were plated in a 15 cm dish, one day prior to the transfection and the medium was replaced with fresh medium 4 hours prior to the transfection. Per plate 30 µg of plasmid DNA of interest and water were mixed to make a total volume of 900 µl in a tube followed by drop wise addition of 100 µl of 2 M $CaCl_2$ to the DNA-water mixture. This mixture was then added drop wise to 1 ml of 2x HBS, pH 7.0 and mixed gently followed by incubating at room temperature for 3-4 minutes. After incubation, this mixture was added drop wise to the cultures covering the whole plate and distributed evenly by gently swirling the plate, taking care not to disturb the monolayer. After 14-16 hours, the medium was changed. After an additional 24 hours the

first supernatant containing retroviral particles carrying the gene of interest (virally conditioned medium, VCM) was collected and fresh medium was added to the cells. This procedure of VCM collection and medium addition was repeated two more times at intervals of 10-12 hours. The VCMs were stored at -80° C until used for viral transduction of the Ba/F3 cells *(2.2.3.4.2)*.

2.2.3.4.2 Stable transduction of Ba/F3 cells

For the preparation of stably transduced Ba/F3 cells, the appropriate VCM aliquot *(2.2.3.4.1)* was thawed and filtered with 0.45 µm pore size Millipore filter. 1.5 ml of the filtered VCM was mixed with 1.5 ml of Ba/F3 cell suspension containing 7.5 x 10^5 cells and 30 µg (final concentration 10 µg/ml) Protamine sulphate. This mixture (3 ml in total) was put in one well of a 6-well plate. The pipetting scheme was scaled up accordingly, when more than one well was used. The cells were allowed to express green or yellow fluorescence protein from the transduced retroviruses and expanded to establish a stable cell line with repeated rounds of fluorescence activated cell sorting (FACS).

2.2.3.4.3 Establishment of the stable Ba/F3 cell line by FACS sorting

4-6 days after the viral transduction of Ba/F3 cells, the cells expressing YFP/GFP were sorted using the Vantage SE Flowcytometer equipped with a turbo-sort DEVICE (capable of sorting 20,000 cells/s), and the Cellquest 3.3 software. For cell sorting, cells were pelleted at 1000 rpm (~300 x g) for 10 minutes and resuspended in 1 ml of FACS buffer (2% FBS in PBS + Propidium iodide at a final concentration of 1 µg/ml). The sorted YFP/GFP positive cells were expanded and resorted if required to attain > 95% purity. The established stably transduced Ba/F3 cells carrying the gene of interest were stored frozen at -80°C or in liquid nitrogen for long-term storage *(2.2.3.2)*, until used for further experiments.

2.2.3.5 Determination of cell viability

Determination of viable cell number is an important aspect of cell culture especially for seeding the cells and for proliferation experiments. We mainly

used the following two methods for the determination of the number of viable cells.

2.2.3.5.1 Trypan blue exclusion assay

Trypan blue stain distinguishes viable cells from non-viable cells. Viable cells actively exclude the stain, whereas the non-viable cells absorb the stain. For the determination of the number of viable cells, the cell suspension was diluted with trypan blue at a ratio of 1:1 to 1:9 (cell suspension:trypan blue) and the numbers of viable (unstained) cells in a 4 x 1 mm^2 area (4 x 100 nl of the diluted cell suspension) were counted with the help of a Neubauer haemocytometer under a phase contrast microscope. The total number of viable cells per ml of the cell suspension (non-diluted) was calculated using the following formula.

$$\text{Total number of viable cells /ml} = \left(\frac{\text{number of viable cells in 4x 1mm}^2\text{ area}}{4}\right) \text{x dilution factor x } 10^4$$

2.2.3.5.2 MTT assay

As an alternative to the trypan blue exclusion assay, the MTT was also used to determine cell viability and cell number. The MTT viability test is a colorimetric assay based on the metabolism of a yellow Tetrazole salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromid (MTT), to an insoluble purple formazan salt by an enzyme produced in the mitochondria of living cells. The purple insoluble formazan crystals are disolved in a solubelization solution (0.04 M HCl in isopropanol or 10% acidified SDS). The cell viability is then quantified by the absorption of the colored cell suspension measured with the help of ELISA reader at 570 nm with reference to the absorption at 650 nm.

We used the MTT assays for studying the sensitivity of the SHIP/1ABL1 fusion protein to the ABL1 tyrosine kinase inhibitor, Imatinib. For these assays, the cells were seeded at a density of $4x10^4$/ml without IL3 and with or without Imatinib at different concentrations in quadruplicates in a micro-titer 96-well plate (100 µl/well). After 48 or 72 hours 10 µL of MTT (stock: 5 mg/ml) was added per well and the cells were incubated for 4 hours. The reaction

was stopped by adding 200 µL of 0.04M HCl in isopropanol to each well and the purple crystals were dissolved by vigorous pipetting. The viability was measured by determining the OD with the help of a Sunrise^{TM} ELISA plate reader (TECAN, Switzerland).

2.2.3.6 Cell proliferation assay

Proliferation assays allow the determination of the number of proliferating cells in the presence or absence of certain factors affecting the proliferation, i.e., cytokines or inhibitors. We performed proliferation assays with Ba/F3 cell lines, to study the proliferative potential of the genes being studied and to demonstrate their sensitivity to the ABL1 tyrosine kinase inhibitor, STI571 (Imatinib, Novartis). For these assays, Ba/F3 cells were seeded at a density of 4 x 10^4 cells/ml in the presence or absence of IL-3, or inhibitors as required. The number of viable cells was determined either by the Trypan blue exclusion method or using the MTT assay at 24, 48 and 72 hours. To study the sensitivity of the genes of interest to Imatinib, the cell viability was assayed after 72 hours by MTT method. The concentration of STI517 at which 50% of cells were viable compared to cells grown in the absence of inhibitor, defined as $IC^{50Imatinib}$ was also determined after 72 hours. All proliferation assays were performed in triplicates or in quadruplicates and repeated three times to confirm the reproducibility.

2.2.4 Protein Biochemistry

2.2.4.1 Protein extraction from mammalian cells

Protein extraction from transiently transfected and non-transfected native HEK293T cells and the stably transduced and non-transduced Ba/F3 cells was performed as follows. The cells were harvested and washed twice in ice-cold PBS (600 g for 5 min). The cell pellets were resuspended in an appropriate volume of lysis buffer No. I *(2.1.3.4)*. A protease inhibitor cocktail (Sigma) and 0.1 M orthovanadate were added fresh to the lysis buffer at 1:100 ratio (for a final concentration of 1 mM orthovanadate). To allow optimum lysis, the lysis reactions were incubated at 4° C for 30 min with gentle agitation (10 rpm). After the incubation, the whole cell extracts were

cleared by centrifugation at 16 000 g for 10 min at 4º C. The supernatant containing most of the soluble cellular proteins was transferred into a new tube and stored at -80° C until further studies.

2.2.4.2 Protein extraction from yeast cells

Yeast cells growing in log phase, at a density corresponding to an OD_{600} of 0.4-0.5 were used for protein extraction. After attaining the desired cell density, 10 ml of the culture was immediately put in a prechilled tube containing approximately the same amount of ice. The tubes were then centrifuged at 1000 g and 4° C for 5 minutes followed by resuspension of the pellet in 10 ml of ddH_2O and the centrifugation step was repeated one more time. The yeast cell pellet was resuspended in 100 µl of TCA buffer *(2.1.3.4)* per OD_{600} units of cells and was transferred into a 1.5 ml Eppendorf tube containing a mix of 100 µl glass beads *(2.1.1)* and 100 µl 20% TCA *(2.1.3.4)* for each 7.5 OD_{600} units of cells. To disrupt the cells, the tubes were vortexed for 1 minute 4 times at RT. To keep the temperature low the tubes were put on ice for 30 sec between each vortexing. Then the tubes were put on ice for 5 minutes to let the glass beads settle down, and the supernatant was collected in a prechilled fresh Eppendorf tube. The glass beads were then washed by adding 500 µl of 1:1 mixture of 20% TCA and TCA buffer and vortexing twice for 1 min at RT and putting them on ice for 30 sec in between each vortexing. The glass beads were allowed to settle down. The supernatant was then pooled with the previously collected supernatants. The proteins in the collected supernatant pools were pelleted down at 16000 g at 4° C for 10 min. The protein pellet was resuspended in 10 µl of Laemmli loading buffer per 1 OD_{600} unit of cells and denatured at 100° C for 10 min. The samples were centrifuged at 16000 g for 10 min at RT and the supernatant containing the proteins were collected in a new tube and stored at -80° C until further use.

2.2.4.3 Protein quantification

The protein concentration in cell lysates was determined by the Bradford method (Bradford, 1976), which is a colorimetric protein assay. The principle of this assay is based on an absorbance shift in the Coomassie dye, when the

red form of the dye changes and stabilizes into a blue form, Coomassie blue, by binding to protein. Thus the concentration of the total protein in the lysate can be measured by reading the absorbance at 595 nm with a spectro-photometer.

For the measurement of the protein amount, a standard regression curve was established first by preparing a series of protein dilution using BSA (stock: 1 µg/µl in ddH_2O) at a known concentration as follows:

BSA (µl)	0	1	5	10	15	20	25
d_2H_2O (µl)	800	799	795	790	785	780	775
Bradford reagent (µl)	200	200	200	200	200	200	200

In parallel, for each protein lysate, 1 µl of the lysate was diluted in 799 µl of ddH_2O followed by the addition of 200 µl of Bradford reagent and mixing. The measurements were performed with an Eppendorf BioPhotometer 6131.

2.2.4.4 Immunoprecipitation

Immunoprecipitation is a technique in which a known protein is precipitated from a solution containing many different proteins (typically a cellular lysate) using an antibody that is specific for that particular protein. We used this technique to study the homotypic di- or oligomerization of our fusion protein.

For immunoprecipitation, 400 µl of total cell lysate were incubated with approximately 5 µg of the specific anti-body at 4° C for 3 hours with continuous gentle agitation at 15 rpm. This was done in order to form specific protein-anti-body complexes. In parallel, a control reaction was set up in which, 400 µl of the total cell lysate was mixed with 2 µg of non-specific anti-IgG antibody and allowed to form protein-antibody complexes. Mean while 40 µl of Protein A- or G-Agarose beads were prepared (washed). For the washing of the beads, 40 µl of chilled PBS was added to the 40 µl of bead slurry and centrifuged at 1600 rpm (300 g) and 4° C for 5 min. The supernatant was carefully removed and this procedure was repeated once more followed by an additional wash with the lysis buffer mix used for cell lysis. The washed beads were then added to the protein-antibody complexes

(obtained after 3 hours of incubation) and the reactions were incubated for 1.5 hours at 4° C with gentle agitation at 11 rpm. This allows protein-antibody-agarose beads complexes to form. After the incubation these complexes were washed three times with 1 ml of the lysis buffer No. 1 to remove unbound or weakly bound proteins at 1600 g, 4° C for 5 min. After the last wash, the complexes were resuspended in 20 µl of 2x Laemmli loading dye and incubated at 95° C for 5 min. This releases the proteins from the anti-body-agarose beads complexes. After denaturation at 95°C, the tubes were briefly centrifuged to settle down the beads and the supernatant containing the precipitated proteins was loaded on an SDS-PAGE gel.

2.2.4.5 Polyacrylamide gel electrophoresis (PAGE)

An SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their molecular weight under denaturing conditions. SDS-PA gels of appropriate concentration of polyacrylamide were prepared depending on the size of the proteins to be separated. We mainly used 8%, 10% and 12% gels. The SDS-PA gel is prepared in two layers of gel of two different concentrations. These layers are known as separating gel and stacking gel. As its name says, the separating gel separates the proteins and the stacking gel concentrates the proteins as a thin band before they enter the separating gel. This results in sharper protein bands in the separating gel. The separating gel is poured first and then the stacking gel is poured on top of the separating gel. The polyacrylamide concentration of the stacking gel is always the same (5%). For the preparation of both gels the following reagents were mixed as follows:

Separating Gel

For 1 Gel (5 ml)	**8 % Gel**	**10 % Gel**	**12 % Gel**
ddH_2O	2.3 ml	1.9 ml	1.6 ml
30 % Acrylamide mix	1.3 ml	1.7 ml	2.0 ml
1.5 M Tris/HCl (pH 8.8)	1.3 ml	1.3 ml	1.3 ml
10 % SDS	50 µl	50 µl	50 µl
10 % APS	50 µl	50 µl	50 µl
TEMED	3 µl	2 µl	2 µl

Stacking Gel

For 1 Gel (2 ml)	5 % Gel
ddH$_2$O	1.4 ml
30 % Acrylamide mix	330 µl
1 M Tris/HCl (pH 6.8)	250 µl
10 % SDS	20 µl
10 % APS	20 µl
TEMED	2 µl

TEMED and APS were always added last to the gel mixtures and just before the gel was poured. The separating gel mix was poured first into the gel cassette *(2.1.2)* and allowed to polymerize at RT. After the polymerization of the separating gel, the stacking gel mix was prepared and poured on top of the polymerized separating gel. Then the comb was positioned in the upper portion of the stacking gel to form the loading wells. The total cell lysates or immunocomplexes were denatured by boiling at 95°C for 5 minutes in SDS-Laemmli buffer. The SDS-Laemmli buffer confers a negative charge to each protein in proportion to its mass, resulting in the movement of the protein towards the anode (+) at a speed that is roughly proportional to the molecular weight of the protein in a specific weight range. The proteins were resolved by applying 60-120 V (1 kV/m) for 4-8 hours at 4°C in 1x Tris-Glycine electrophoresis buffer *(2.1.3.4)*. 10-50 µg of each protein sample was loaded in each well. A commercial protein size marker (See blue plus 2, Invitrogen) was also loaded so that the molecular weight of the proteins could be estimated.

2.2.4.6 Western blot analysis

After the separation of the proteins by SDS-PAGE, the proteins in the gel were transferred to an activated Polyvinylidene Fluoride (PVDF) membrane using a wet transfer system (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad). The transfer assembly was prepared according to the manufacturer's instruction. Briefly, a sandwich of the provided fiber pad-filter paper-the membrane- the gel-filter paper-fiber pad (all soaked in transfer buffer) was placed in the transfer cell filled with chilled transfer buffer. A

cooling element (called "ice bucket") was also placed in the transfer cell. The transfer of the proteins from the gel to the membrane occurred under 350 mA constant electric current from the negative to the positive pole at for 1.5 hours.

After the transfer, the membranes were incubated in blocking solution (5 % nonfat dried milk in TBST) for 1 hr at RT (alternatively, O/N at 4°C) to block nonspecific binding sites. Immunoblotting was carried out with specific antibodies diluted in 5 % nonfat dried milk in TBST. The dilution of the primary antibody varied depending on the antibodies used *(2.1.14)*. The incubation with the primary antibody was carried out for 1 hr at RT (alternatively, O/N at 4°C) in most cases and was followed by extensive washing with TBST washing buffer *(2.1.3.4)* (rinsed twice, wash 3 x 5 min at RT). Secondary antibodies conjugated to horseradish peroxidase (HPR) were used for the detection of the primary antibody. The membranes were incubated for 1 hour at RT followed by extensive washing with TBST buffer as described above. Finally, the secondary antibodies were detected by incubating the membrane with enhanced chemiluminescence reagent for 3- 5 min at RT (ECL, Amersham Pharmacia biotech). The membranes were then placed in an X-ray film cassette and autoradiography films were exposed for 5 sec to 10 min. In order to probe the same membrane with a different antibody, the immunoblots were stripped. Immunoblots were stripped by incubating in stripping solution at 56° C for 30 min, followed by two washes in TBST buffer for 10 min at RT. The membranes were then blocked again and probed with a different primary antibody.

2.2.5 Cytogenetic Techniques

2.2.5.1 Conventional Cytogenetics

Conventional cytogenetic techniques are mainly used for the preparation of chromosomes from different tissues and the study of genetic aberrations at the chromosome level.

2.2.5.1.1 Short-term culture of human bone marrow and blood samples

An appropriate amount of blood or bone marrow sample containing 0.4 -1.0 x 10^7 lymphocytes was added to 5 ml of RPMI-1640 medium with 20% FBS and 1% antibiotic-antimycotic solution (Gibco, Invitrogen: 10,000 U/ml Penicillin G sodium, 10,000 µg/ml Streptomycin sulphate, 25 µg/ ml Amphotericin B in 0.85% saline). The cells were allowed to grow for 24 hours in a closed culture system at 37° C. After 24 hrs of incubation, colcemid (stock: 10 µg/ml) was added to the cultures to a final concentration of 300 ng/ml and the cells were harvested after 2 more hours of incubation. To obtain a high mitotic index, the cells could also be harvested 24 hrs after the addition of colcemid.

2.2.5.1.2 Harvesting and fixation

After the incubation with colcemid, the cells were pelleted down at 700 x g for 10 min at RT. The cell pellet was gently resuspended with 6 ml of pre-warmed hypotonic solution (0.075 M KCl) and incubated at 37° C for 15-20 min. The cells were centrifuged at 600 x g for 10 min at RT and resuspended very gently by drop wise addition of Carnoy's fixative (1:3; acetic acid:methanol). The fixed cells were incubated at 4° C or -20° C for 30 minutes followed by several washing steps with the fixative to remove cell debris. The centrifugation for the washing was done at 700 x g for 7 min at 4° C.

2.2.5.1.3 Preparation of metaphase spreads

After the last wash, the cell pellet was resuspended in about 1 ml of fixative and mixed gently. The fixed cell suspension was then dropped (4-6 drops) onto a clean-chilled- wet slide held at an angle of about 45°. The cells were allowed to roll over the surface of the slide. The cell suspension was then allowed to dry evenly by keeping the slide horizontal, thus the cells burst and the metaphase spreads were obtained by surface tension. Optimum spreading of the chromosomes can be obtained by controlling the speed of the drying by using a hot plate or a humidified chamber. For fluorescence *in*

situ hybridization (FISH), the slides were aged at least for 1 day and then stored at -20° C if not used immediately.

Cytogenetic preparations were obtained from cultured cell lines in a similar way with slight modifications in the protocol.

2.2.5.2 Fluorescence *in-situ* hybridization

Fluorescence In-Situ hybridization (FISH) allows the localization of specific fluorescently labeled DNA sequence in interphase nuclei or on metaphase chromosomes. Among other things, this technique is used to characterize and analyze chromosomal aberrations and fine map translocation breakpoints.

2.2.5.2.1 Probe preparation

We prepared “dual color dual fusion” (DCDF) FISH probes *(Figure 2.27 A, B)* for the detection of the SHIP1/ABL1 rearrangement. The FISH probes were prepared from selected Bacterial Artificial Chromosome (BACs) from the SHIP1 and ABL1 locus *(2.1.15)*by “Sequence independent amplification” (SIA).

2.2.5.2.1.1 Sequence independent amplification (SIA) of BACs-DNA.

To generate sufficient quantities of BAC DNA for the preparation of FISH probes, we used the technique of Sequence Independent Amplification (SIA) (Bohlander et al., 1992). By SIA, we converted the small amounts of BAC DNA that were obtained from mini preparations (3 to 6 ml culture volume of the low copy BACs) into PCR amplifiable DNA fragments that could be labeled conveniently.

The SIA technique was originally developed to amplify extremely small amounts of DNA from micro dissected chromosome fragments to generate chromosomal band specific DNA libraries or chromosome band specific FISH painting probes (Bohlander et al., 1992).

In this method, short random DNA fragments (200 to 1500 bp in length) from the input DNA are flanked by a known primer (Primer A) sequence *(2.1.13)*. This is called “preamplification”. The random DNA fragments flanked by the known 5’ sequence introduced by primer A are then amplified with a normal

PCR using Primer B *(2.1.13)*. Primer B has the defined sequence of Primer A at its 3' end and additionally 5 nucleotides at the 5' end (Figure 2.2).

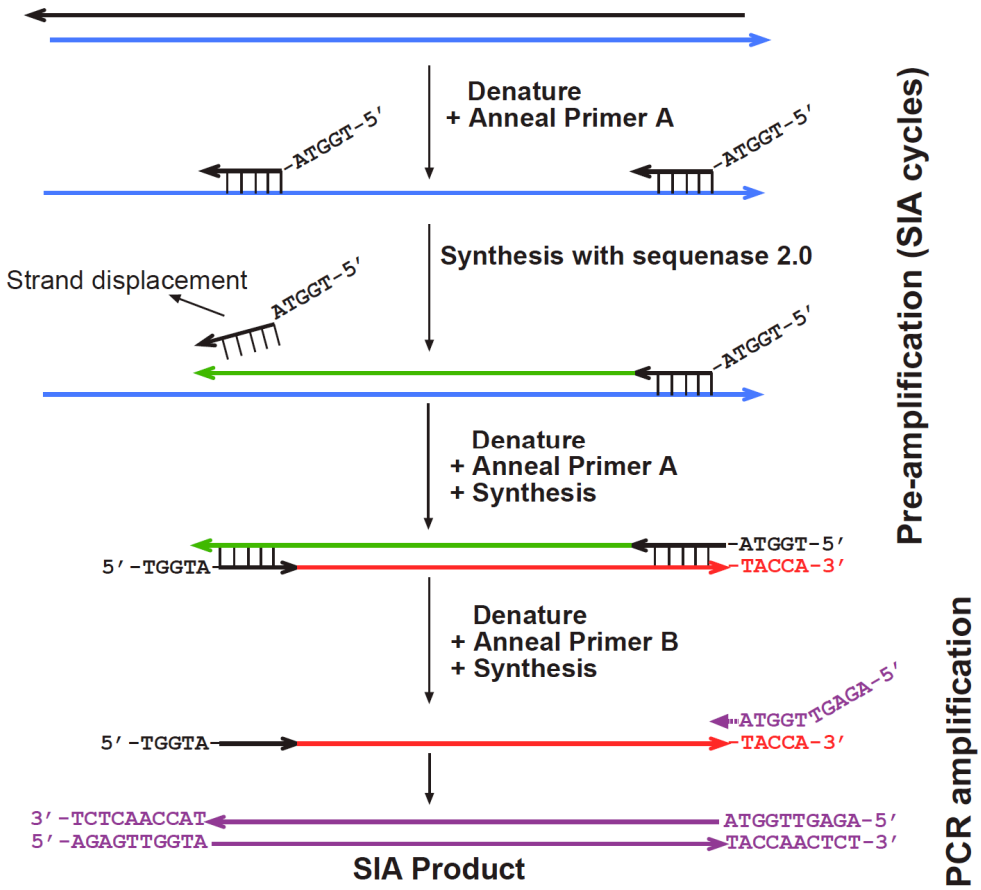


Figure 2.2: Diagram of SIA principle

The flanking of the DNA fragments with the primer sequences and the subsequent PCR amplification are performed in a single Eppendorf tube. The procedure is described in detail in the following paragraphs.

After an initial denaturation, a special primer (primer A) (2.1.13) is annealed to the single stranded template DNA. Primer A has a random pentanucleotide sequence at its 3' end and is capable to anneal in a sequence independent fashion to single stranded DNA with this 3' end. The 5' end of Primer A is a defined 15 bp long sequence. In the initial denaturation step, 0.5 µl of the BAC DNA template *(2.2.2.1.1)* is added to the a 4µl of reaction buffer A (50 mM NaCl, 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 ng/µl BSA, 300 µM of each dNTP, 5 mM DTT, and 0.625 µM of Primer A) and denatured at 94°C for 4 min.

After the denaturation, 0.5 µl of reaction buffer A containing 0.5 U of the T7 DNA polymerase Sequenase 2.0 (United States Biochemical, USB) are added to the tube (denatured template) and the polymerase is allowed to work by slow increase in the temperature from 13°C to 37°C over a period of 3 minutes, followed by an incubation at 37°C for an additional 2 minutes. In this so-called preamplification cycle, DNA fragments containing the defined sequence introduced by Primer A at their 5' ends are generated. This preamplification cycle is stopped by another denaturation step at 94°C for 1 minute. Then a fresh aliquot of buffer A (0.5µl) containing 0.5 U of Sequenase 2.0 is added for another preamplification cycle with the same temperature profile as the first preamplification cycle. After this second preamplification step and the denaturation, single-stranded DNA fragments are produced that have the known sequence of Primer A at their 5' end and the reverse complement of this sequence at their 3' end. These DNA fragments could now be amplified in PCR reaction with a primer (Primer B) *(2.1.13)* that has the known sequence of Primer A and additional 5 nucleotides at the 5' end. However, to increase the yield of the sequence-independent amplification, the preamplification cycle is repeated two more times for a total of four preamplification rounds, each time adding fresh 0.5 µl aliquot of buffer A containing Sequenase 2.0 enzyme. Thus, the final reaction volume after 4 preamplification cycles would be 6 µl.

After the 4^{th} preamplification round, the 6 µl preamplification reaction is added to 54 µl of buffer B (55 mM KCl, 6.6 mM Tris-HCl pH 9.0, 11 ng/µl gelatin, 133 µM of each dNTP, 1.25 µM of Primer B, and 0.03 U/µl *Taq* Polymerase) to attain the total reaction volume of 60 µl. The addition of buffer B adjusts the reaction conditions to PCR conditions suitable for *Taq* polymerase. Primer B has the defined sequence of Primer A at its 3' end and additional 5 nucleotides at the 5' end. Then a normal PCR reaction is performed with an initial 5 cycles of low stringency annealing (from 44°C to 70°C; ramp 0.2°C/sec) followed by 22 cycles of high stringency annealing (56°C)

The detailed protocol for the sequence independent amplification is given below.

1. Initial denaturation of the template DNA:

    - The reaction buffer A was pipetted as follows and 4 µl of the mix was mixed with 0.5 µl BAC-DNA template and incubated at 94° C for 90 seconds.

    Buffer A

Reagents	**Amount (µl)**	**Final concentration**
10x MAP	0.600	1x
1 mg/ml BSA	0.300	50 µg/ml
3 mM each dNTP	0.600	300 µM
10 µM Primer A	0.375	0.625 mM
ddH_2O	3.985	up to 5.860 µl
Sequenase 2.0 (13 U/µl)	*0.140*	*0.3 U/µl*
Added later (Step 2)		
Total	**6.000**	

2. Pre-amplification:

    - To the rest of the buffer mix A (1.860 µl), 0.140 µl Sequenase was added and
    - From the above mix 0.5 µl was added to the reaction tube (step 1) after denaturation and a sequenase cycle was run as given in the following table.
    - The sequenase cycle was repeated three more times preceded by the addition of 0.5 µl of the Buffer A with Sequenase. The reaction volume was 6 µl after the 4^{th} preamplification cycle.

Preamplification Cycle (Sequenase Cycle)

Steps	*Temperature (°C)*	*Time (min)*
1	13°C	1
2	13-37°C	2 min, ramp with 0.2° C/ sec
3	37°C	2
4	94°C	1
5	4°C	soak

3. PCR amplification using specific primers

   - After the preamplification, 54 µl of buffer B was added to each tube to bring the volume to 60 µl and the reactions were cycled through the temperature profile as shown below (SIA PCR cycles).

   - **Buffer B**

Reagents	**Amount (µl)**	**Final concentration**
10x BP buffer	5.400	1x
3 mM each dNTP	2.400	133 µM
20 µM Primer B	3.375	1.25 mM
ddH_2O	42.525	to 54 µl total
Taq Pol (5 U/µl)	0.300	
Total	54.000	

The products of the SIA procedure are examined on a 1.5% agarose gel. The procedure yields a smear of fragments ranging from 200 bp to 1500 bp from 1 ng of BAC DNA as template. The BAC clones contained inserts ranging in size from 100 kbp to 200 kbp.

SIA PCR cycles

Steps	***Temperature (°C)***	***Time (min:sec)***	***No. of cycles***
Initial denaturation	94	3:00	1
Denaturation	94	0:45	5 cycles, Ramp 0.2° C/s
Initial annealing	44	1:00	
Annealing	44-72	2:00	
Elongation	72	2:00	
Denaturation	94	0:45	22 cycles
Annealing	56	0:45	
Elongation	72	2:00	
Final elongation	72	7:00	
Soak	4		1

2.2.5.2.1.2PCR labeling of SIA products

After the initial SIA amplification from a BAC, the DNA fragments were fluorescently labeled in a second PCR by the incorporation of nucleotides that were chemically modified with fluorochromes. We used dATP and dCTP labeled with FITC or Texas Red and Spectrum Orange or FITC conjugated dUTPs.

In this secondary labeling PCR (usually 50 µl in volume), primer B was used at 1 µM concentration. Depending on which fluorescently-labeled nucleotide was used the concentration of the same non-labeled nucleotide was reduced accordingly. Typically we used the labeled nucleotide at a final concentration of 40 µM, eg. 40 µM of Spectrum Orange dUTP. Then the concentration of dTTP was reduced to 110 µM so that the total concentration of dUTP and dTTP would be 150 µM. The individual concentration of the other three nucleotides would also be 150 µM. 1 µl from the final SIA product *(2.2.5.2.1.1)* containing the fragments from a specific BAC was used as template.

The following cycle profile was used to label SIA products:

Steps	*Temperature (°C)*	*Time (min:sec)*	*No. of cycles*
Denaturation	94	1:00	20 cycles
Annealing	56	1:00	
Elongation	72	3:00	

2.2.5.2.1.3 DNase treatment of labeled SIA products

In order to achieve the right size range (300-500 bp) of DNA fragments for optimum hybridization kinetics and results, the labeled SIA products were mildly treated with DNase I.

The DNase I stock solution was diluted in DNase dilution buffer at an empirically determined ratio (usually 1:1000). Then 1/10 volume of the labeled PCR reaction *(2.2.5.2.1.2)* was removed for later analysis and 1/10 volume of diluted DNase I solution was added. The reaction was incubated at RT for 10 minutes and then the DNase was inactivated at 65°C for 10 minutes. To monitor the efficiency of the DNase digestion, the aliquot (1/10 of the labeled PCR reaction) removed before the digestion and an aliquot of the sample after the digestion were run side by side on a 1.8% agarose gel. Typically a distinct reduction in the average size of the DNA fragments should be visible. However, since DNase I introduces single stranded nicks into the DNA, even a slight reduction in the average molecular weight of the smear indicated substantial digestion of the DNA.

2.2.5.2.1.4 Precipitation of labeled probes

After labeling and DNase treatment (in the case of the SIA products), the FISH probes were precipitated in order to remove un-incorporated labeled nucleotides, to concentrate the probe and to add non-labeled highly repetitive human DNA (Cot-1-DNA). Cot-1-DNA, which would hybridizes to the labeled repeat DNA, was used to reduce non-specific background hybridization signals. For precipitation, 3 µg Cot-1 DNA (1 µg/l), 0.1 v/v 3 M sodium acetate and 2.5 v/v of 100% ethanol were added to the labeled probes in an Eppendorf tube. After vortexing, the tube was put at -80°C for 20 min to allow

the DNA to precipitate. The precipitated DNA was pelleted at 16000 x g, 4°C for 20 min. After air drying the pellet was resuspended in 7 µl hybridization buffer (Abbott, Germany) and 3 µl d_2H_2O. This mix was used for the hybridization.

2.2.5.2.2 Protein digesting pre-treatment and hybridization

The cytoplasmic proteins of the cells may obstruct the denaturation of the chromosomal DNA and interfere with hybridization. This may result in a high background fluorescence and poor quality fluorescence signals. To improve hybridization results, we pretreated the chromosomal preparations *(2.2.5.1.3)* with pepsin to digest the cytoplasmic proteins, when required.

For pepsin treatment, the slides were immersed in working pepsin solution *(2.1.3.5)* at 37° C for 3-5 min and washed with 1x PBS for 3 minutes at RT. After the wash, 100 µl of the post fixation *(2.1.3.5)* solution was applied onto the slide, overlaid with a coverslip and incubated for 10 min at RT. The slides were washed by 1x PBS for 3 min, RT and subjected to dehydration in 70%, 90% and 100% ethanol baths for 2 min each. When the protein digestion was not required, the slides were directly dehydrated through the ethanol series before hybridization.

The slides were air dried and 8-10 µl of the probe mix was applied for a 24x24 mm^2 area, covered with a coverslip and sealed with rubber cement. Alternatively for a 9x9 mm^2 area 1-1.5 µl of probe mix was applied. The slides were subjected to denaturation at 76°C for 3 min followed by hybridization at 37° C for 12-16 hrs using an automated FISH hybridization chamber (Hybrite^{TM}, Vysis, Germany)

2.2.5.2.3 Post-hybridization washes, staining and microscopy

After hybridization, the slides were washed in Wash solution I *(2.1.3.5)* at 75°C for 2 min (high stringency wash), followed by an additional wash in Wash solution II *(2.1.3.5)* for 1 min at RT (low stringency wash). The slides were mounted with DAPI (1000 ng/ml in antifade solution) for counter staining and the fluorescent signals were visualized with an epi-fluorescence microscope (Axioplan2 imaging system; Carl Zeiss, Jena, Germany),

equipped with a CCD camera. The images were captured and processed using the Isis software (Metasystems, Altlussheim, Germany).

2.2.5.2.4 Fluorescent signal amplification

If the fluorescent signals were weak, the signals were amplified with a sandwich technique employing fluorescently labeled antibodies. For the amplification, the slide was washed in wash solution II for 1 min at RT to remove the DAPI counter stain, followed by an additional wash in Wash solution III *(2.1.3.5)* for 3 min at RT. The slide was then incubated in freshly prepared blocking solution *(2.1.3.5)* for 10-20 min at RT and washed with Wash solution III for 3 min at RT. The slides were then incubated with 100 µl of appropriate antibody (e.g., anti-FITC, Rabbit IgG, Alexa Fluor 488 conjugated antibody) diluted in blocking solution, at 37° C in a moist chamber for 30 min. The slides were washed in Wash solution III for 3 min, 4 times at RT. When stronger signals amplification was required, an additional round of amplification was performed using appropriate secondary and tertiary antibodies, followed by washing with wash solution III as described earlier. The slides were then washed twice with PBS for 5 min at RT, dehydrated through a 70-80-90-100% ethanol series, air-dried and mounted with the DAPI counter stain *(2.1.3.5)*.

2.2.6 Statistical methods

Standard statistical parameters (eg. mean, median and standard deviation) were calculated using the built in statistics functions of spreadsheet programs like Excel (Microsoft). The IC50 (inhibition cell growth by 50%) concentration, the concentration of a drug at which 50% of the cells die, was derived manually by performing a standard Probit analysis using Finney's table (Finney 1952) (Finney, 1952).

3 Results

3.1 DISCOVERY OF A NOVEL FUSION PARTNER OF ABL1

3.1.1 Case History

An 18-year-old female patient was admitted to the University of Munich hospital because of anemia, thrombocytopenia and general weakness and was diagnosed with Acute Lymphoblastic Leukemia (ALL). The peripheral blood showed the presence of 23% of small lymphoid blasts with narrow cytoplasm (white blood cell count: 7000/µl). In the bone marrow (BM) about 70% of the cells were blasts. The flow cytometry analysis of the BM sample revealed an immature lymphoid blast population, which was positive for CD19, CD10, HLA-DR, CD34 as well as the cytoplasmic markers TdT and CD22. There was no expression of IgM and no co-expression of myeloid markers (CD13, CD33, CD65, CD15, CD14, CD64, CD61 or myeloperoxidase), which was compatible with the diagnosis of common Acute Lymphoblastic Leukemia (cALL).

As a part of the routine diagnostic procedure for ALL patients, the samples were screened for the presence of the BCR-ABL1 fusion gene. An RT-PCR was performed to detect the BCR/ABL1 fusion transcript, and fluorescence in situ hybridization (FISH) was performed for the detection of the BCR/ABL1 fusion gene at the genomic level. In addition, routine cytogenetic techniques revealed a normal female karyotype of 46,XX at the 250 band resolution level in standard GAG-banding.

The patient was treated according to the GMALL 07/2003 protocol in the very high risk arm. She was given Imatinib, since our FISH results suggested an *ABL1* rearrangement (3.1.6). The treatment was uneventful, and she achieved a complete remission within 11 days of the start of therapy. She was transplanted with the bone marrow from an unrelated male donor 5 months after the initial diagnosis and continues to be in complete remission (follow-up 15 months).

3.1.2 Detection of unexpected products after RT-PCR to detect BCR/ABL1

For the detection of the BCR/ABL1 fusion transcript, RT-PCR *(2.2.2.4.3)* with the mRNA derived from the patient's BM and peripheral blood samples was performed using primer pairs *(2.1.13)* specific for the p190 and p210 BCR-ABL1 fusion transcripts. These PCRs did not result in the amplification of DNA fragments of the expected sizes corresponding to the p190 or p210 fusion transcripts. However, in both the RT-PCR reactions unexpectedly weak bands of higher molecular weight were seen after agarose gel electrophoresis *(Figure 3.1)*. To characterize these bands further, we excised them from the agarose gels and sequenced them with the primers used for amplification.

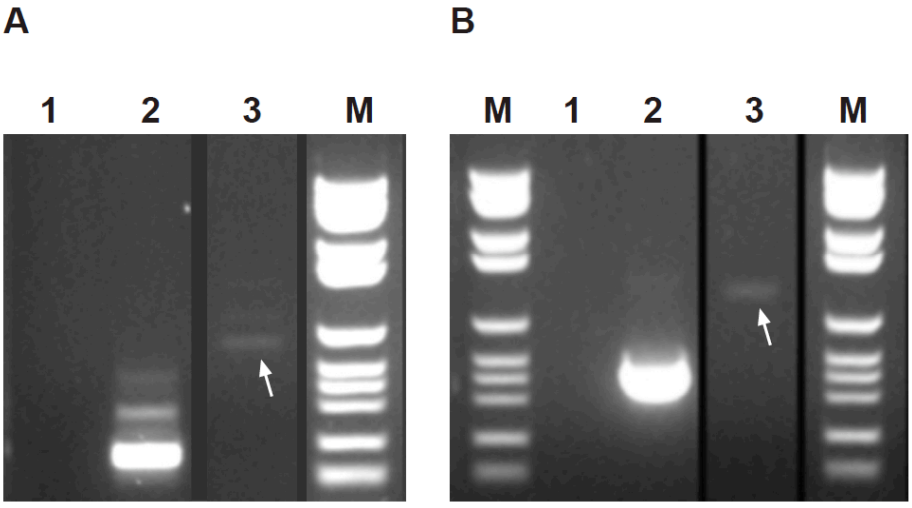


Figure 3.1: Detection of higher molecular weight PCR bands after RT-PCR for the BCR-ABL1 fusion transcripts: (A) PCR with primers specific for the p190 BCR/ABL fusion: Lane 1: Negative control (no template); lane 2: positive control (template: cDNA of the patient with the p190 fusion transcript); lane 3: patient sample. **(B)** PCR with primers specific for the p210 BCR/ABL fusion: Lane 1: Negative control; lane 2: positive control; lane 3: patient sample.

3.1.3 Sequence analysis of the unexpected PCR fragment

To sequence the higher molecular weight bands, the following primers were used: for the p190 product the P190 F70 and p190 R20 primers and for the p210 product the P210 F60 and the p210 R40 primers *(2.1.13)*. A BLAST

(Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov) analysis of the sequences showed homologies to *ABL1* and, unexpectedly, to the *INPP5D* gene (inositol polyphosphate-5-phosphatase) from chromosome 2 band q37. A detailed analysis revealed a fusion of the *INPP5D* (NM_001017915.1) gene at nucleotide 1183 to the *ABL1* gene (NM_007313.2) at position 576. The fusion conserved the open reading frame of both genes, with an in-frame fusion between the 9th exon of the *INPP5D* gene and the 2nd exon of the *ABL1* gene *(Figure 3.2)*. This initial sequence covered *INPP5D* from nt 1035 to 1183 and *ABL1* from nt 576 to 701. Since

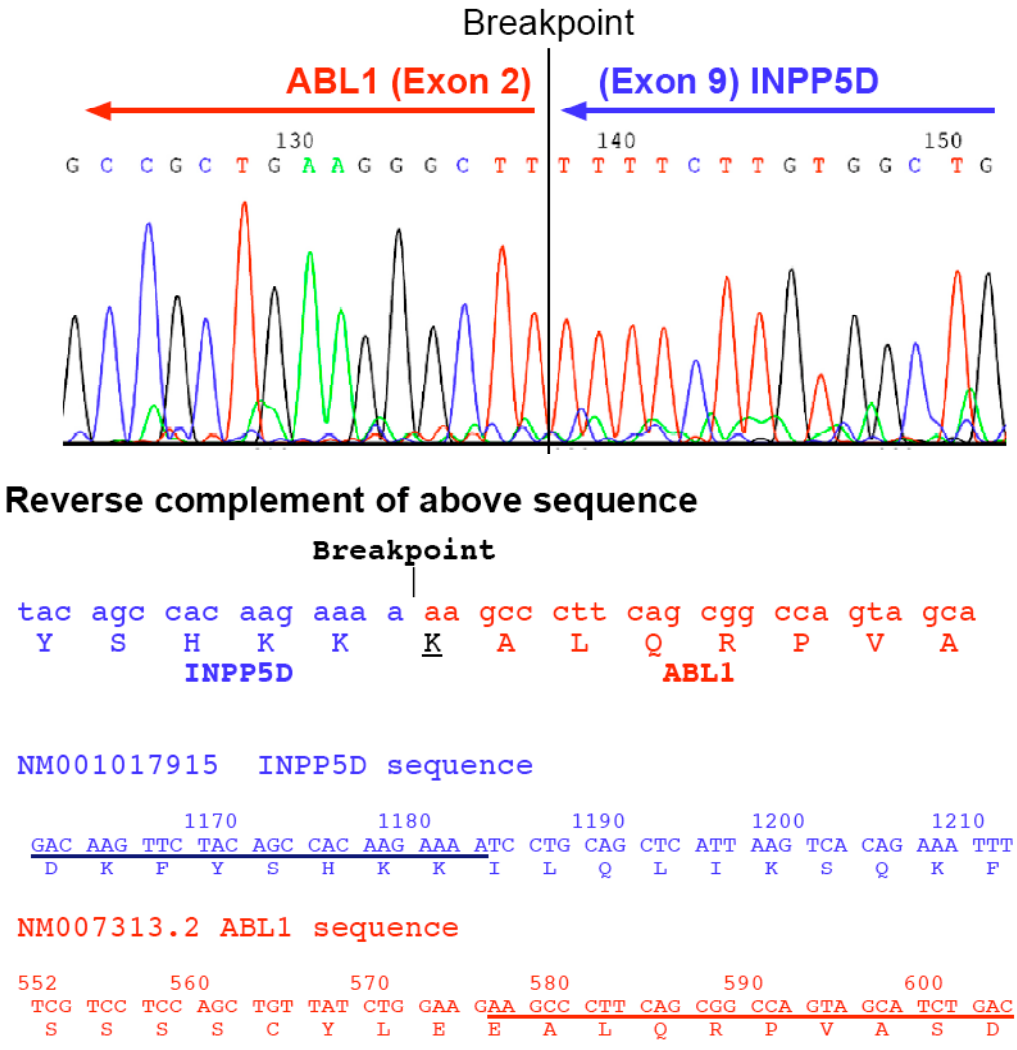


Figure 3.2: Sequence of the in-frame fusion between the 5'-*INPP5D* and 3'-*ABL1* genes: The sequence was obtained by sequencing the unexpected higher molecular weight RT-PCR product amplified by the BCR-ABL fusion transcript specific primers. The underlined sequences in the individual sequences of INPP5D and ABL1 fuse to form an INPP5D/ABL1 fusion gene.

there was no proper intron-exon annotation of the INPP5D at the time of our analysis due to a gap in the NCBI reference genome assembly in the middle of the *INPP5D* gene, we determined the exon-intron boundaries of the *INPP5D* gene ourselves using the NCBI-BLAST tool and the Celera genome assembly.

3.1.4 Confirmation of the presence of the *INPP5D/ABL1* fusion transcript in the patient sample.

To confirm the presence of an *INPP5D/ABL1* fusion transcript in the patient sample, a putative *INPP5D/ABL1* fusion sequence was constructed using *INPP5D* (Genbank accession no: NM_001017915) and *ABL1* (NM_007313.2) sequences, based on the breakpoint identified from the PCR product *(Figure 3.2, Appendix I)*. Corresponding to this putative *INPP5D/ABL1* sequence, a specific primer pair (*INPP5D-ABL1F997-1015 and INPP5D-ABL1B1474-1454*) *(2.1.13)* flanking a 477 bp region spanning the breakpoint (nt 1183 of NM_001017915) was designed *(Figure 3.3A)*. Using this primer pair, we were able to amplify a specific 477 bps fragment from the patient's cDNA sample, thus confirming the presence of the *INPP5D/ABL1* fusion transcript in the patient sample *(Figure 3.3B)*. The identity of this *INPP5D/ABL1* fusion transcript was verified by sequence analysis.

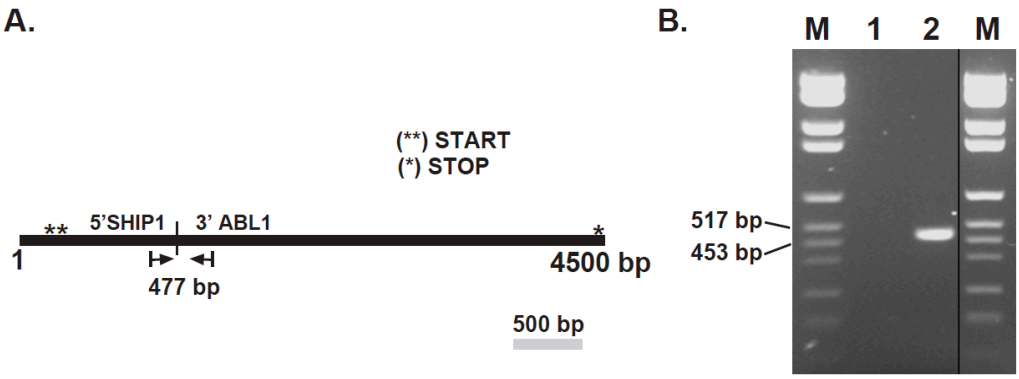


Figure 3.3: Confirmation of the presence of the *INPP5D/ABL1* fusion transcript in the patient sample: (A) Diagrammatic representation of the position of the breakpoint spanning primer pair in the putative INPP5D/ABL1 fusion transcript. The primer pair covers a breakpoint spanning region of 477 nucleotides. **(B)** Detection of the specific *INPP5D/ABL1* fusion transcript (477 bp fragment) in the patient sample: M: size marker; lane 1: negative control; lane 2: patient sample.

3.1.5 Absence of the reciprocal *ABL1/INPP5D* fusion transcript in the patient sample.

To detect the presence of the reciprocal ABL1/INPP5D fusion, a hypothetical *ABL1/INPP5D* sequence was generated. A primer pair (*ABL1-INPP5D402-421F and ABL1-INPP5D741-721B*), flanking a 339 bps region of the hypothetical ABL1/INPP5D sequence spanning the breakpoint of the reciprocal fusion transcript was designed *(2.1.13)*. Using this primer pair we were unable to amplify an *ABL1/INPP5D* fusion transcript from the patient sample. Taken together with our data from the FISH experiments (see below: *3.1.6*), this negative result strongly suggests that there is no reciprocal *ABL1/INPP5D* transcript in the patient sample

3.1.6 Analysis of the genomic rearrangement using BCR and ABL Fluorescence In-situ Hybridization (FISH) probes

Considering the presence of the 5'INPP5D/3'ABL1 in the patient's sample and that the transcriptional direction of both the ABL1 and SHIP1 genes is centromere to telomere *(3.2)*, we expected that a simple balanced translocation had occurred between chromosome 9 (with the breakpoint in ABL1 at 9q34) and chromosome 2 (with the breakpoint in INPP5D at 2q37) *(Figure 3.4)*. The fact that we were unable to amplify the reciprocal fusion transcript might have been due to alternative splicing or the presence of a small deletion at the breakpoint. To confirm the presence of this translocation we performed a fluorescence in-situ hybridization (FISH) experiment on the patient's interphase and metaphase cells using a commercially available BCR/ABL-dual-color-dual-fusion (DCDF) probe *(2.2.5)*. To better understand the FISH results, the BCR/ABL-DCDF probe is described in *Figure 3.5 A* and the results of the hybridization for interphase and metaphases in the case of the classical t(9;22)(q34;q11) translocation, which generates the BCR/ABL1 fusion, is described in *Figure 3.5 B.* The expected hybridization results for this probe for both metaphase and interphase cells in the case of a balanced t(2;9)(q37;q34) is shown in *Figure 3.5 C*. We would expect three orange signals for the ABL1 probes: one signal for the intact ABL1 locus, and one signal each for the 5' and 3' portion of the rearranged ABL1 allele.

Unexpectedly, the patient's metaphase cells showed a normal signal pattern for the BCR/ABL-DCDF probe with 2 green signals for the *BCR* gene, one on each chromosome 22, and 2 orange signals for *ABL1* gene, one on each chromosome 9, without any indication of any rearrangements involving *BCR* or *ABL1 (Figure 3.6 A)*. However, when the same probe was hybridized on the interphase cells from the patient, the orange signals for both the *ABL1* loci were split *(Figure 3.6 B) resulting in two pairs of closely spaced orange signals*.

Based on the RT-PCR and FISH results, we speculate that the *INPP5D/ABL1* fusion was generated by an insertion of the 5' part of *INPP5D* into the first intron of the *ABL1* gene *(Figure 3.6 C)*. In addition, to explain the fact that both ABL1 signals appear split in the interphase FISH analysis, we have to assume that the normal chromosome 9 was lost and that the chromosome 9 with the insertion of the *INPP5D* sequences in the *ABL1* locus was duplicated.

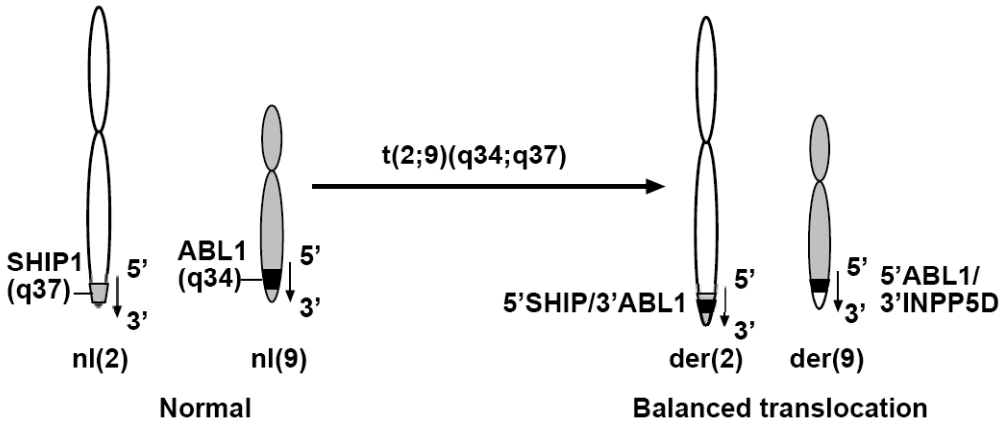


Figure 3.4: Putative genomic rearrangement leading to the SHIP1/ABL1 fusion: Diagram of the chromosomal location of SHIP1 and ABL1 and the balanced translocation resulting in the formation of the 5'SHIP1/3'ABL1 fusion on the derivative chromosome 2 and the reciprocal fusion 5'ABL1/3'SHIP1 on the derivative chromosome 9.

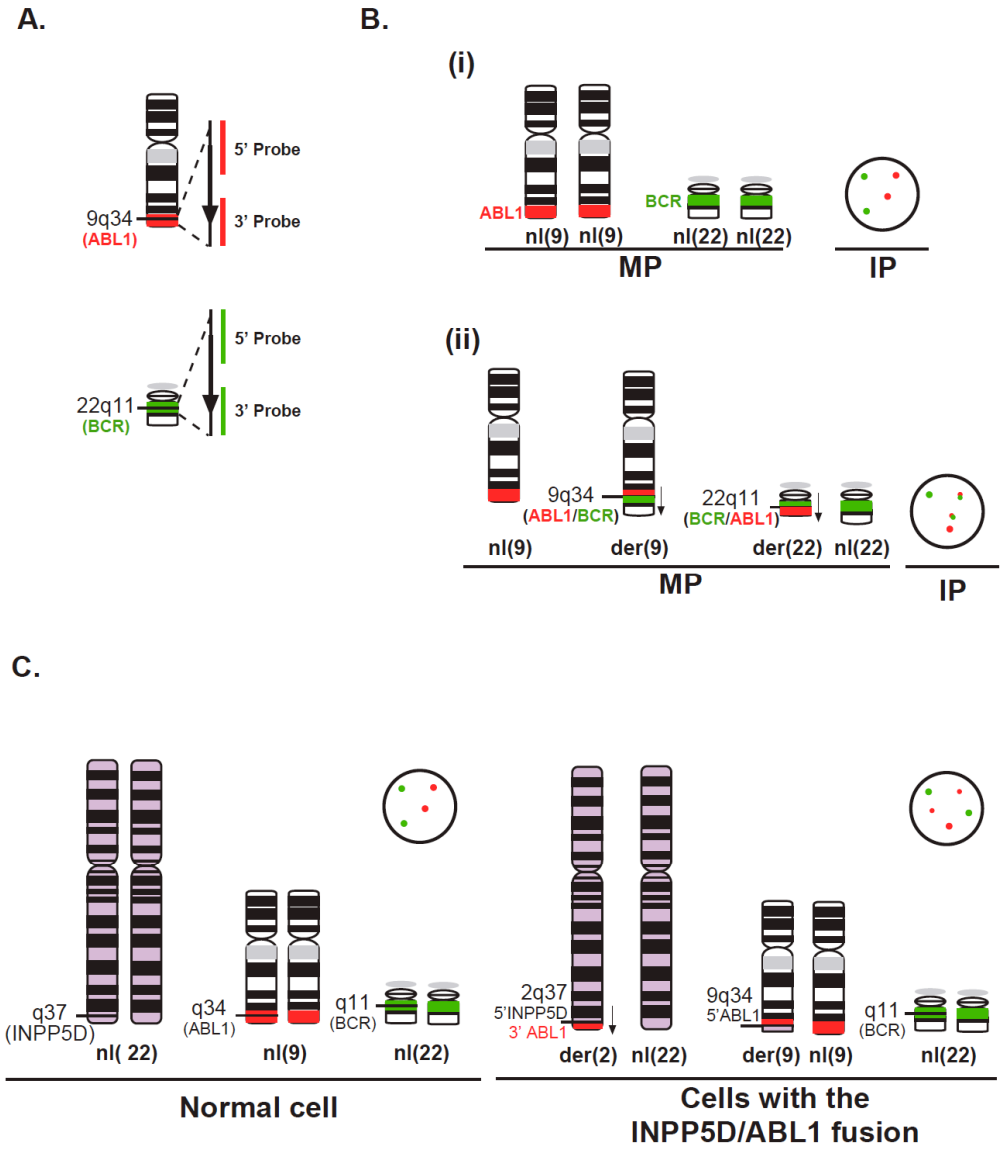


Figure 3.5: The BCR/ABL1-DCDF probe: (A) Design of the BCR/ABL1 DCDF FISH probe: *BCR* is labeled with FITC (green signals) and *ABL1* is labeled with Spectrum Orange (orange signals) **(B)** Possible BCR/ABL DCDF FISH signal patterns: (i) Signal pattern indicating absence of the BCR/ABL1 fusion: 2 orange and 2 green signals corresponding to the normal ABL1 and BCR loci both in interphase (IP) cell and in metaphase (MP) cells (ii) Signal pattern indicating a classical translocation t(9;22)(q34;q11), on metaphase (MP) chromosomes and in interphase (IP) cells: The resulting two reciprocal fusions, BCR/ABL1 and ABL1/BCR, give rise to two yellow signals in addition to 1 orange and 1 green signal for the normal ABL1 and BCR loci, respectively**. (C)** Expected signal pattern with the BCR/ABL1 DCDF FISH probes in cells with the t(2;9)(q37;q34). In metaphase cells: 2 green signals on the two chromosomes 22 for the normal BCR loci, 1 orange signal on the normal ABL1

loci, 1 orange signal for the 3'ABL1 portion on the long arm of chromosome 2 and one orange signal on the other chromosome 9 for the 5'ABL1 portion; In interphase nuclei: two green signals and 3 orange signals would be expected.

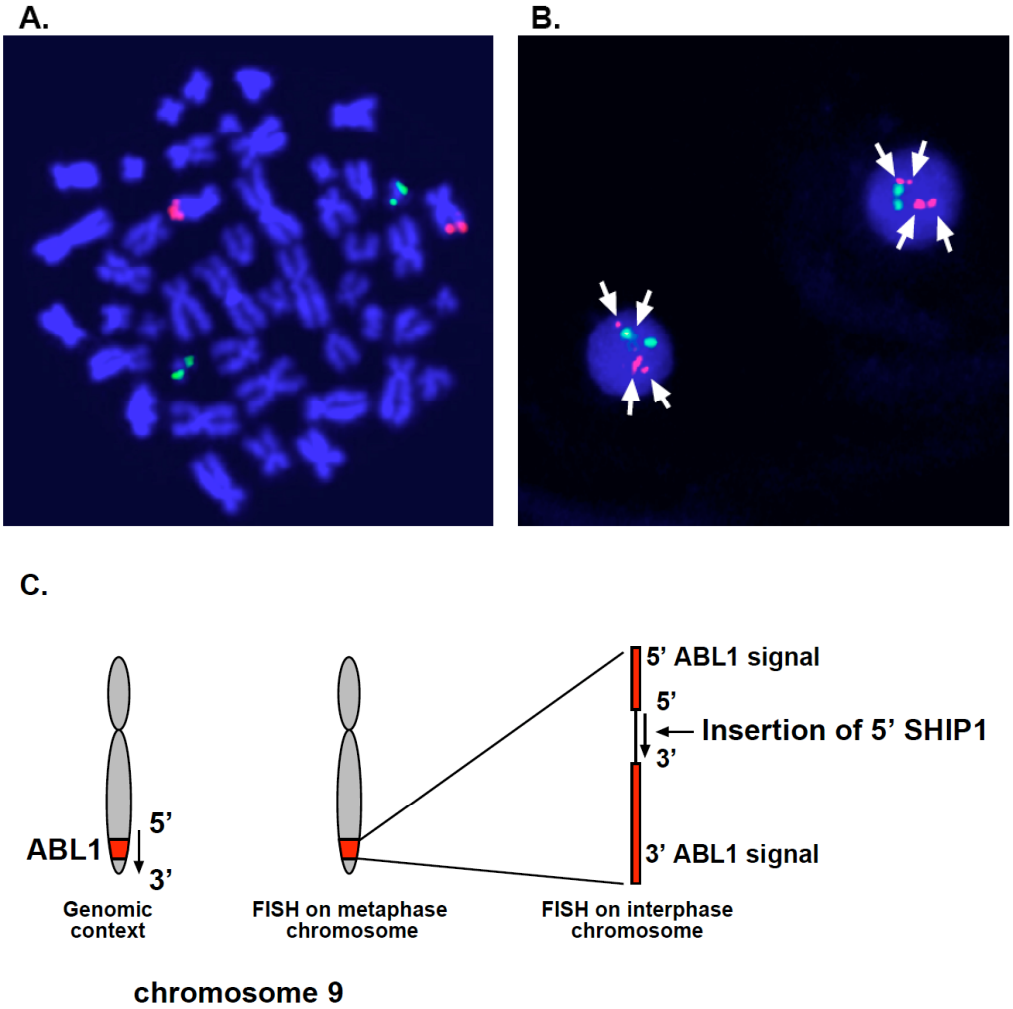


Figure 3.6: FISH assays performed on the patient's sample: (A) Hybridization of the BCR/ABL1 DCDF FISH probes. (i) Representative metaphase cell showing the absence of the BCR/ABL1 fusion: 2 orange signals corresponding to the two ABL1 loci, 2 green signals corresponding to the two BCR loci and no yellow signal; (ii) Representative interphase cells showing the absence of BCR/ABL1 fusion (no yellow signal), two normal BCR loci (2 green signals) and split ABL1 at both the ABL1 loci (two split orange signals). **(B)** Diagrammatic explanation of the ABL1 split signal pattern in the FISH assays: An insertion of the 5' portion of INPP5D within the ABL1 genomic locus could generate an INPP5D/ABL1 fusion and the observed signal pattern.

3.2 INPP5D *(SHIP1): THE NEW FUSION PARTNER OF* ABL1

The inositol polyphosphate-5 phosphatase (*INPP5D*) gene, the novel fusion partner of *ABL1*, is also known as SH2 containing inositol phosphatase 1 (*SHIP1*). *INPP5D (SHIP1)* is located in the telomeric region of the long arm of chromosome 2 (2q37) and is transcribed 5' to 3' in a centromere to telomere direction. The *SHIP1* gene encodes a protein of 1188 amino acids (aa) (145 kDa molecular weight) with an N-terminal SH2 (scr-homology) domain, an inositol phosphatase domain, and a C-terminal proline rich region containing two protein interaction motifs. These motifs, INPNY and ENPLY, are target sequences for the phosphotyrosine binding domains of several other proteins *(Figure 3.7)*. The expression of the SHIP1 protein is restricted to haematopoietic cells where its movement from the cytosol to the plasma membrane is controlled by tyrosine phosphorylation (Damen et al., 1996; Ware et al., 1996). *SHIP1* functions as a negative regulator of myeloid cell proliferation and survival (Helgason et al., 1998). Mice with a targeted disruption of the *SHIP1* locus develop a myeloproliferative syndrome and show an increased number of NK cells and increased rate of mast cell degranulation (Helgason et al., 1998). The hematopoietic specific expression of SHIP1 and its negative regulatory role in important signaling pathways made the *SHIP1/ABL1* fusion a very interesting subject for further characterization.

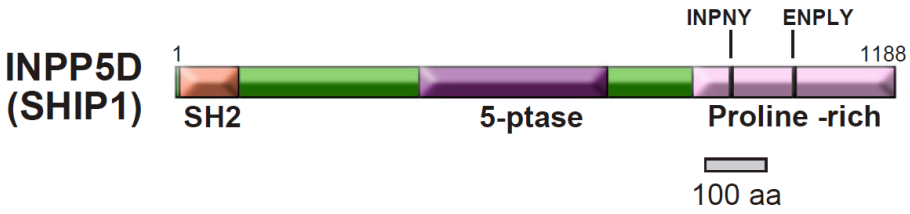


Figure 3.7: Defined protein domains within the SHIP1 protein. SH2: Src homology-2 domain; 5-ptase: Inositol 5-phosphatase domain; INPNY and ENPLY: Target sequences for the phospho tyrosine binding domains of other proteins. (Derived from Damen et al., 1996)

3.3 THE PUTATIVE SHIP1/ABL1 FUSION PROTEIN

As mentioned earlier *(3.1.3)*, in the SHIP1/ABL1 fusion, the breakpoint in ABL1 is in the 1st intron, in the same location where it is observed in all the other known ABL1 fusions. The resulting SHIP1/ABL1 fusion protein comprises the first 343 amino acids of the INPP5D protein and the whole ABL1 protein except for the first 27 amino acids (in the case of ABL1a: NP_005148.2) or the first 46 aa (in the case of ABL1b: NP_009297.2) *(Figure 3.8)*. Thus, the putative INPP5D/ABL1 fusion protein is predicted to contain the SH2 domain SHIP1 (amino acids 2 to 102) and the SH3, SH2, tyrosine kinase and the actin-binding domain of the ABL1 protein.

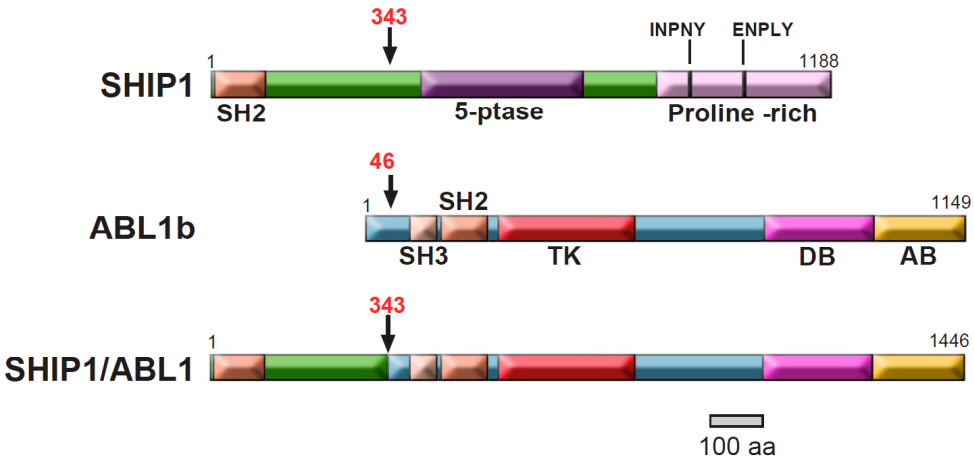


Figure 3.8: Diagram of the SHIP1, ABL1 and SHIP1/ABL1 proteins with their relevant protein domains. SH2: Src homology-2 domain; SH3: Src homology-3 domain; 5-ptase: Inositol 5-phosphatase domain; INPNY and ENPLY: Target sequences for the phospho tyrosine binding domains of other proteins; TK: Tyrosine kinase domain; DB: DNA binding domain; AD: Actin-binding domain. The upper two boxes show the SHIP1 and the ABL1 proteins and the lower box shows the protein domains of SHIP1/ABL1 fusion protein. The arrows indicate the breakpoints in the individual proteins; numbers indicate amino acid positions.

3.4 CLONING OF THE FULL LENGTH SHIP1/ABL1

To characterize and study the function of the SHIP1/ABL1 fusion protein in detail, we cloned the full length SHIP1/ABL1 fusion coding sequence into eukaryotic expression vectors. For this, we first amplified the 5' portion of the

SHIP1/ABL1 fusion from the patient's cDNA with a nested PCR using the primers SHIP-ABL-F20-38 and SHIP-ABL-B1520-1502 for the first PCR and SHIP-ABL1-F38-57 and SHIP-ABL1-B1508-1489 *(2.1.13)* in the second PCR. The amplified fragment contained nucleotides 39 to 1508 of the hypothetical SHIP1/ABL1 sequence *(Figure 3.9)*. At the 5' end of this 1470 bps long fragment, a *Hin*dIII and a *Mun*1 site were introduced by PCR using the primers SHIP-ABL1-HindIII-Mun1-F39-59 and SHIP-ABL1-B1508-1489 *(2.1.13)*. The PCR product was then cloned into the pGEM-Teasy vector. Several recombinant pGEM-Teasy clones were sequenced, and a clone with the correct sequence of the 5' SHIP1/ABL1 fusion, pGEM-Teasy-SA(39-1508)-CLN9, was used in the subsequent cloning steps. The insert sequence of the pGEM-Teasy-SA(39-1508)-CLN9 clone had two single nucleotide substitutions compared to the reference SHIP1/ABL1 sequence: one at position 642 in the SHIP1 and another at position 1236 in the ABL1 portion. Neither one of these substitutions led to a change in the amino acid sequence.

For the cloning of the full length SHIP1/ABL1 into the eukaryotic expression vector, the 5' SHIP1/ABL1 fragment (nucleotides 39-1488 of the SHIP1/ABL1 sequence) was excised from the pGEM-Teasy-SA(39-1508)-CLN9 clone using the 5' *Hin*dIII site, which had been introduced by the primer, and the *Kpn*1 site at position 1488 in the ABL1 portion of the fragment *(Figure 3.9)*. A pcDNA3-BCR/ABL1 construct was linearized with a HindIII and Kpn1 digest. This resulted in the removal of the 5' portion of the BCR/ABL1 fusion (including the whole BCR portion) from the *Hind*III site of the vector back bone until the *Kpn*1 site within the ABL1 sequence. Then the *Hind*III-Mun1-5'SHIP1-ABL1-Kpn1 fragment was inserted into the *Hind*III-pcDNA3-ABL-*Kpn*1 vector to generate a full length SHIP1-ABL1 fusion cDNA in the pcDNA3 vector.

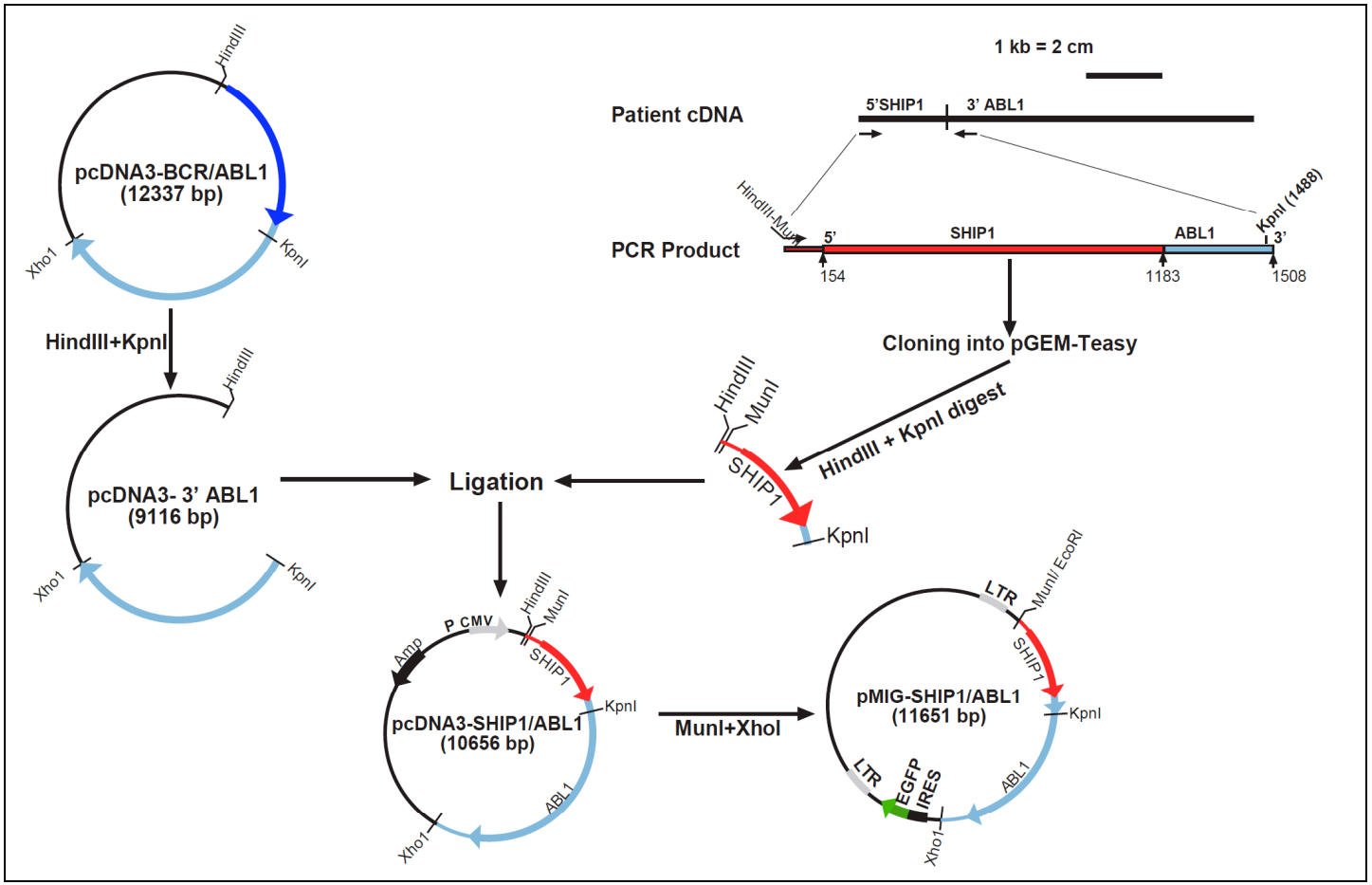


Figure 3.9: Strategy for the cloning of the full length SHIP1/ABL1 fusion into the eukaryotic expression vector pcDNA3 and the retroviral expression vector pMSCV-IRES-GFP.

The full length SHIP1/ABL1 sequence was then cloned into a retroviral expression vector. For this, the whole SHIP1/ABL1 insert was excised from the pcDNA3-SHIP1/ABL1 construct by a *Mun*1/*Xho*1 double digest. The insert was then ligated into *Eco*RI and *Xho*1 sites of the pMSCV-IRES-GFP (pMIG) vector. The resulting pMIG-SHIP1/ABL1 construct would lead to the expression of both a SHIP1/ABL1 and a green fluorescent protein (GFP) because of the internal ribosome entry site (IRES) between the coding region of SHIP1/ABL1 and GFP. The cloning strategy is summarized in *figure 3.9*.

3.5 VERIFICATION OF SHIP1/ABL1 EXPRESSION CONSTRUCTS

The completeness and integrity of the full length SHIP1/ABL1 clones were confirmed by analyzing the expression of the SHIP1/ABL1 protein and by sequencing the complete coding region of SHIP1/ABL1.

3.5.1 Expression of the SHIP1/ABL1 clones in 293T cells

HEK293T cells were transiently transfected *(2.2.3.3)* with the pcDNA3-SHIP1/ABL1 construct and allowed to express the protein for 48 hours. In parallel, the HEK293T cells were also transfected with the pcDNA3-BCR/ABL1 plasmid to serve as a positive control for the efficiency of the anti ABL1 antibody used in the Western analysis. After 48 hours, total cell lysates were prepared *(2.2.4.1)* from the transfected cells and non-transfected HEK293T cells, which were to be used as a control for the endogenous expression of the ABL1 protein. The proteins in the cell lysates were then resolved by SDS-PAGE and analyzed by Western blotting. The blot was probed with an antibody against the ABL1 protein and the expression of the SHIP1/ABL1 (estimated size ~158 kD) and the BCR/ABL1 (p210) proteins could be confirmed *(Figure 3.10)*. The endogenous ABL1 protein was not detected in the non-transfected HEK293T cells.

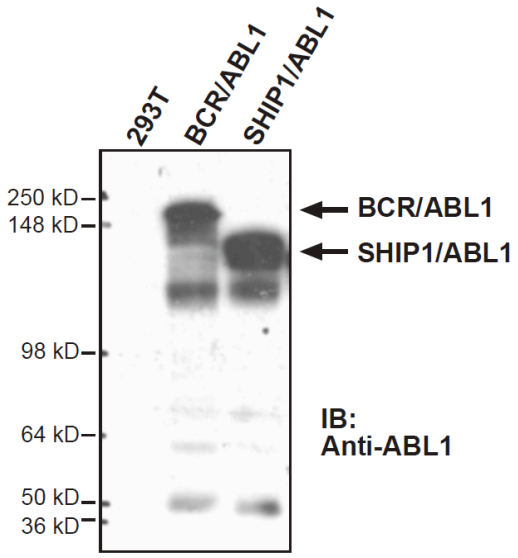


Figure 3.10: **Expression of SHIP1/ABL1 in HEK293T cells detected by an antibody against c-ABL1:** Lane 1: Lysate from non-transfected HEK293T cell lysate; Lane 2: Lysate from BCR/ABL1 transfected cells; Lane2: Lysates from SHIP1/ABL1 transfected cells.

3.5.2 Sequencing of the retroviral SHIP1/ABL1 expression constructs

The complete SHIP1/ABL1 insert in the pMIG-SHIP1/ABL1 construct was sequenced using the "pMIG-5'SHIP/3'ABL1 sequencing primers" listed in section *2.1.13*. The individual sequence reads were assembled into a contig of the complete SHIP1/ABL1 sequence with the help of the program "Sequencher 4.7". The sequence showed a near 100% identity to the sequences of SHIP1 and ABL1 deposited in Genbank with the exception of two silent nucleotide substitutions; one at position 642 in the SHIP1 portion and one at position 1236 in the ABL1 portion of the hypothetical SHIP1/ABL1 sequence. In addition, we found a nucleotide change in the ABL1 portion at position 4297 in the hypothetical SHIP1/ABL1 sequence, which led to a threonine (T) to serine (S) substitution close to the C-terminal end of ABL1. The nucleotide that led to a threonine to serine substitution is at position 3689 of the ABL1 sequence with accession number NM_007313.2. Although this threonine to serine amino acid substitution is not present in any of the ABL1 sequences deposited in Genbank, we chose to ignore it, since the transforming activity of our BCR/ABL1 and SHIP1/ABL1 constructs was not

compromised by this mutation. The positions of the single nucleotide changes are indicated in the sequence of SHIP1/ABL1 fusion *(Appendix I)*.

3.5.3 Expression of SHIP1/ABL1 in Ba/F3 cells

To confirm the expression from the retroviral pMIG-SHIP1/ABL1 construct, Ba/F3 cells were retrovirally transduced with the empty pMIG vector, pMIG-SHIP1/ABL1 or pMIY-BCR/ABL1 and Ba/F3- cell lines stably expressing the green fluorescent protein (GFP) only, GFP and SHIP1/ABL1 or Yellow fluorescence protein (YFP) and BCR/ABL1 were established *(2.2.3.4)*. The Ba/F3 cell line expressing the well studied BCR/ABL1 protein was used for positive control experiments. For negative control experiments (as a control for integration effects of the retroviral vector), the Ba/F3 cell line expressing GFP only was used. Total protein lysates were prepared from all three cell lines and the expression of SHIP1/ABL1 and BCR/ABL1 was confirmed in the appropriate cell lines *(Figure: 3.11)*.

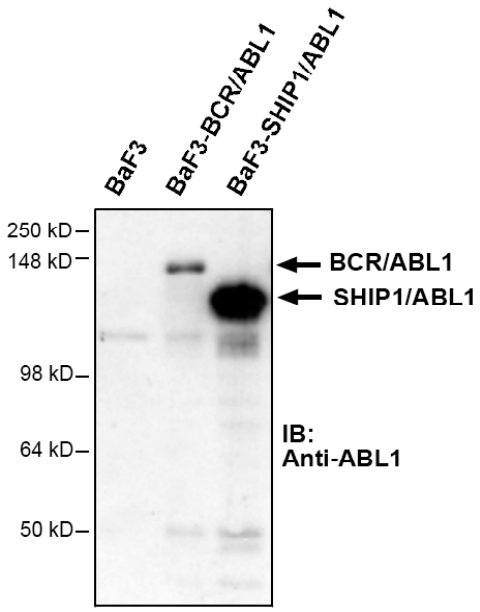


Figure 3.11: Western blot analysis of Ba/F3 cell lines expressing the SHIP1/ABL1 or BCR/ABL1 fusion proteins using an c-ABL1 specific antibody. Lane 1: Lysate from non-transfected Ba/F3 cells; Lane 2: Lysates from BCR/ABL1 transduced Ba/F3 cells; Lane 3: Lysates from SHIP1/ABL1 transduced Ba/F3 cells.

3.6 SHIP1/ABL1 CONFERS GROWTH FACTOR INDEPENDENT GROWTH TO BA/F3 CELLS

The Ba/F3 cell line is an IL3 dependent murine pro B cell line. This cell line is an important biological system for the study of tyrosine kinases. The constitutively activated forms of tyrosine kinases like ABL1, JAK2, PDGFR-alpha, FLT3, and cKIT induce IL3 independent growth in Ba/F3 cells. Therefore we used the Ba/F3 cell line system to study the transformation potential of the SHIP1/ABL1 fusion. We performed cell proliferation assays in presence and absence of IL3 *(2.2.3.6)* using the trypan blue exclusion method *(2.2.3.5.1)* for the assessment of the cell viability. Each experiment was done three times in triplicates resulting in 9 individual data points for each experimental condition. The assays showed that SHIP1/ABL1 induces IL3 independence in Ba/F3 cells, suggesting that the ABL1 tyrosine kinase is constitutively active in the SHIP1/ABL1 fusion. Interestingly, the SHIP1/ABL1 fusion expressing Ba/F3 cells consistently showed a faster growth rate than BA/F3 cells expressing the BCR/ABL1 fusion *(Figure: 3.12)*.

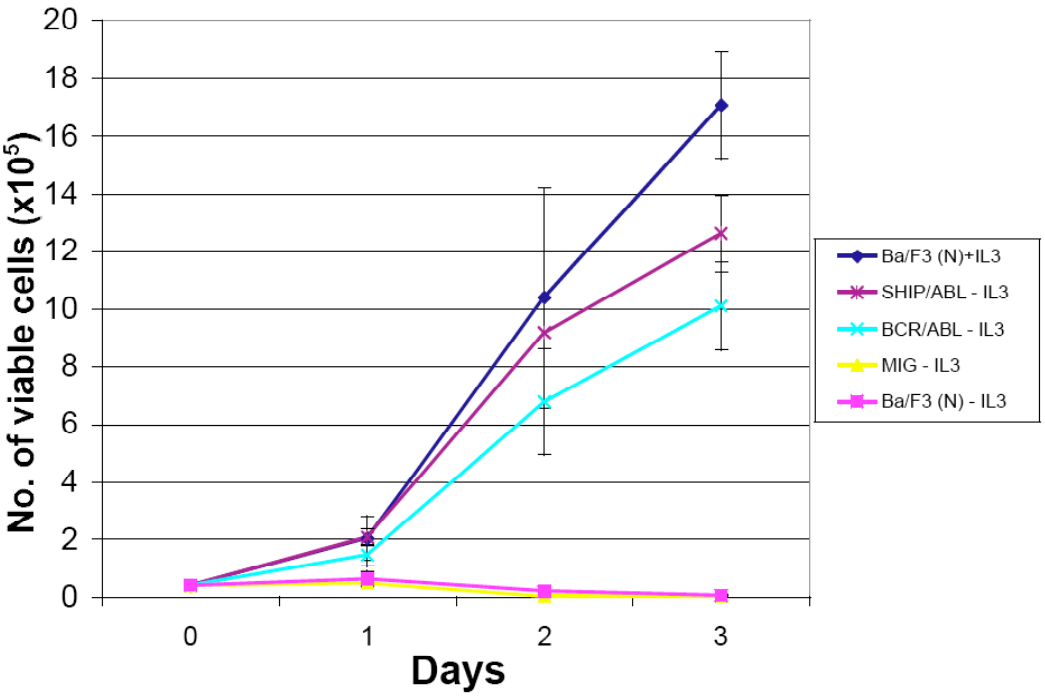


Figure 3.12: **Transformation of Ba/F3 cells to IL3 independent proliferation:** Ba/F3 cells retrovirally transduced with the indicated constructs were grown in the absence or presence of IL3 and their growth was recorded

over a period of 3 days. Proliferation curves were prepared by plotting the mean ± SD values derived from three individual assays.

3.7 THE PROLIFERATIVE EFFECT OF THE SHIP1/ABL1 FUSION PROTEIN CAN BE INHIBITED BY IMATINIB

Imatinib is a competitive inhibitor of the tyrosine kinases ABL1, c-Kit and PDGFRβ. Imatinib is used as a targeted therapeutic agent to treat malignancies which are caused by constitutively active forms of the ABL1, c-Kit or PDGFR tyrosine kinases. To study and compare the Imatinib sensitivity of the SHIP1/ABL1 and the BCR/ABL1 fusion proteins, SHIP1/ABL1 and BCR/ABL1 expressing Ba/F3 cell lines were treated with different concentrations of Imatinib (STI571) in the absence or presence of IL3 *(2.2.3.6)*. The experiments without IL3 were used to determine the effect of Imatinib on the activity of the constitutively activated ABL1 tyrosine kinases. To control for a possible unspecific cytotoxic effect of Imatinib, the same experiments were also performed in the presence of IL3. The cell viability was estimated using the MTT method *(2.2.3.5.2)*. The experiments clearly showed that the Ba/F3 cells expressing the SHIP1/ABL1 fusion are more sensitive to the effects of Imatinib than the BCR/ABL1 expressing Ba/F3 cells *(Figure 3.13)*.

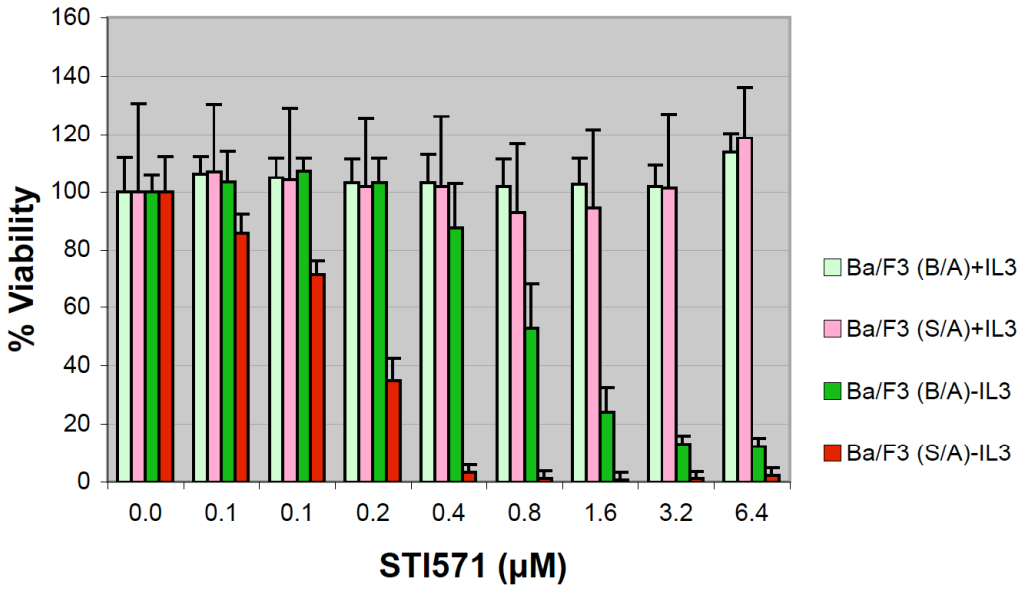


Figure 3.13: Sensitivity of Ba/F3 cells expressing SHIP1/ABL1 to Imatinib: Ba/F3 cells retrovirally transduced with SHIP1/ABL1 or BCR/ABL1 were treated with different concentrations of Imatinib for 3 days and the % viability of the cells was calculated at each concentration with reference to the cell viability of untreated cells at the same time point.

From these experiments we also determined the $IC50^{IMATINIB}$ (the concentration of imatinib at which 50% of the cells are dead) both for the SHIP1/ABL1 and BCR/ABL1 fusion *(Figure 3.14, Table: 3.1)*. The $IC50^{IMATINIB}$ for Ba/F3 cells expressing SHIP1/ABL1 was about 0.15 µM, whereas the $IC50^{IMATINIB}$ for BCR/ABL1 expressing cells was about 0.79 µM. Thus, SHIP1/ABL1 is more than 4 times more sensitive to Imatinib than BCR/ABL1 in our Ba/F3 cell line system.

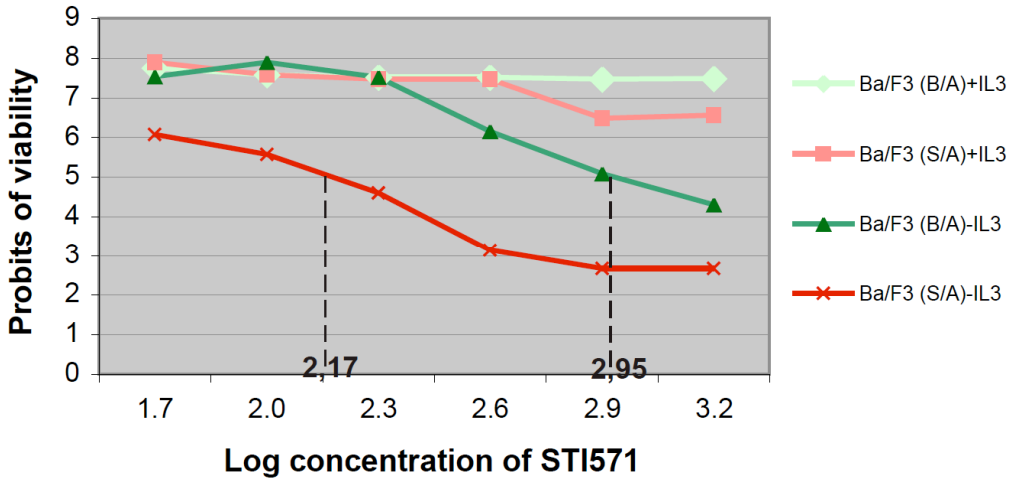


Figure 3.14: Determination of the $IC50^{IMATINIB}$: Probit values corresponding to the % cell viability at different Imatinib concentration were derived using Finney's table (Finney, 1952). The probits were plotted on the Y-axis against the Log_{10} values of the Imatinib (nM) concentrations on the X-axis. For each fusion protein, the concentration at which 50% of the cells were dead was defined as the $IC50^{IMATINIB}$ value.

Corresponding Log concentration "X" for Probit "5" from Finney's table		IC50 (nM)	IC50 (µM)
BCR/ABL1	2.95	891.25094	0.89
SHIP1/ABL1	2.17	147.9108388	0.15

Table 3.1: The $IC50^{IMATINIB}$ of SHIP1/ABL1 and BCR/ABL1 derived from figure 3.14

These results clearly demonstrate that the proliferative effect of the SHIP1/ABL1 fusion protein in Ba/F3 cells is due to the constitutive activation of the ABL1 tyrosine kinase activity in the fusion protein.

3.8 MECHANISM OF TRANSFORMATION USED BY THE SHIP1/ABL1 FUSION

As explained earlier, in all the ABL1 fusions proteins examined so far the constitutive activation the ABL1 tyrosine kinase is due to a dimerization or oligomerization of the fusion protein mediated by a protein domain contributed by the fusion partner. Therefore, we asked the question whether the novel SHIP1/ABL1 fusion protein would also follow the same mechanism of activation: namely dimerization or oligomerization of the SHIP1/ABL1 mediated by the SHIP1 protein domain. To prove homo-dimerization of the SHIP1/ABL1 protein we constructed two SHIP1/ABL1 fusions bearing different 5'-epitope tags in eukaryotic expression vectors and performed co-immunoprecipitation experiments.

3.8.1 Cloning of epitope tagged full length SHIP1/ABL1 into eukaryotic expression vectors

A FLAG or HA epitope tag was introduced in frame at the 5' end of the SHIP1/ABL1 fusion by PCR using the pGEM-Teasy-SA(39-1508)-CLN9 clone as template. Three consecutive PCRs were performed in order to introduce the epitope tags and the necessary restriction enzyme sites at the 5' end of the SHIP1/ABL1(38-1508) fragment. For the generation of Flag-SHIP1/ABL1(154-1508) fragments, the forward primers *Hind3-Mun1-Flag-SHIP1No1* (for PCR no 1)*, Hind3-Mun1-Flag-SHIP1No2* (for PCR no 2) and *Hind3-Mun1-Flag-SHIP1No3* (for PCR no 3) and the reverse primer *SHIP-ABL1-B1508-1489* (PCR no 1, 2 and 3) *(2.1.13)* were used *(Figure 3.15)*. One microliter of a 1:100 dilution of PCR no 1 was used as template for PCR no 2, and similarly 1 µl of a 1:100 dilution of PCR no 2 was used as template in the third PCR. With each PCR, about 15 additional nucleotides were added to the 5' end of the fragment, thus building up the sequence for the Flag epitope and the *Hin*dIII and *Mun*1 restriction sites. The HA-SHIP1/ABL1

fragments were obtained in a similar fashion in three consecutive PCRs by using the forward primers *Hind3-Mun1-HA-SHIP1No1, Hind3-Mun1-HA-SHIP1No2* and *Hind3-Mun1-HA-SHIP1No3* and the reverse primer *SHIP-ABL1-B1508-1489 (2.1.13)*. The epitope-tagged products were cloned into pGEM-Teasy vector and the clones were sequence verified. The sequence-verified clones pGEM-Teasy-Flag-SA(154-1508)-CLN96 and pGEM-Teasy-HA-SA(154-1508)-CLN91 *(Appendix II)* were used for further cloning. The inserts from these pGEM-Teasy clones were excised by a *Hin*dIII/*Kpn*1 double-digest and inserted into the *Hin*dIII and *Kpn*I sites of pcDNA-BCR/ABL replacing the BCR/ABL fusion from the *Hin*dIII site 5' of the BCR ATG till the *Kpn1* site within ABL1 as described in section *3.4*.

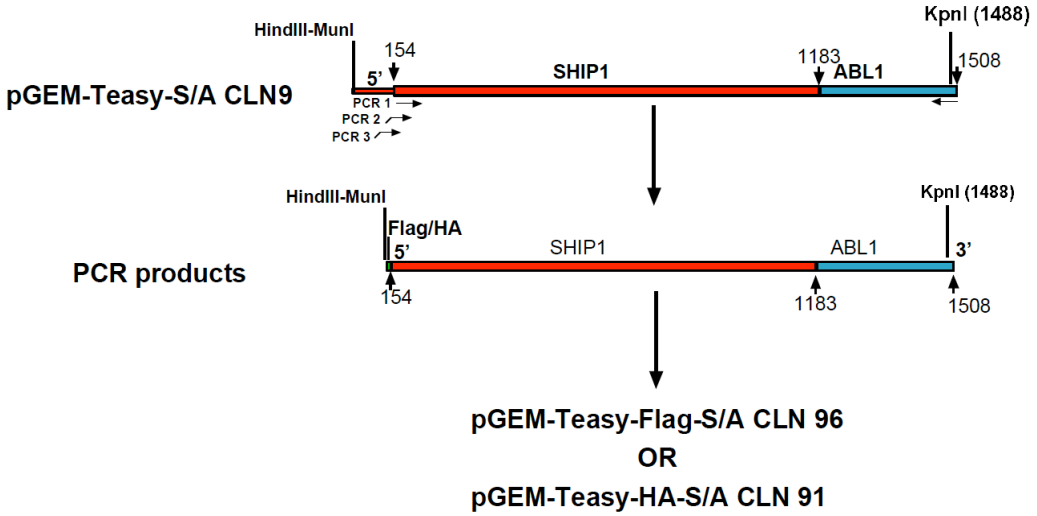


Figure 3.15: Introduction of epitope tags at the 5' end of the SHIP1/ABL1 fusion. In the upper diagram the positions of the primers are shown in the pGEM-Teasy-S/A (39-1508)-CLN9 clone, which was used for the introduction of the Flag or HA epitope at the 5' end of the SHIP1/ABL1 fusion by PCR. The lower diagram shows the resulting PCR products, which were cloned into the pGEM-Teasy vector.

The pcDNA clones with the tagged full-length SHIP1/ABL1 fusion, pcDNA-Flag-SA-96E and pcDNA-HA-SA-91E, were transiently transfected into HEK293T cells. The expression of the epitope-tagged SHIP1/ABL1 proteins was confirmed by Western blot analysis using antibodies against the Flag and HA *(2.1.14)* epitopes as well as against the ABL1 protein *(Figure 3.16)*. As

controls for the anti Flag and anti HA antibodies plasmids expressing Flag-tagged AF10 protein and HA-tagged Ikaros protein were also transiently transfected into HEK293T cells.

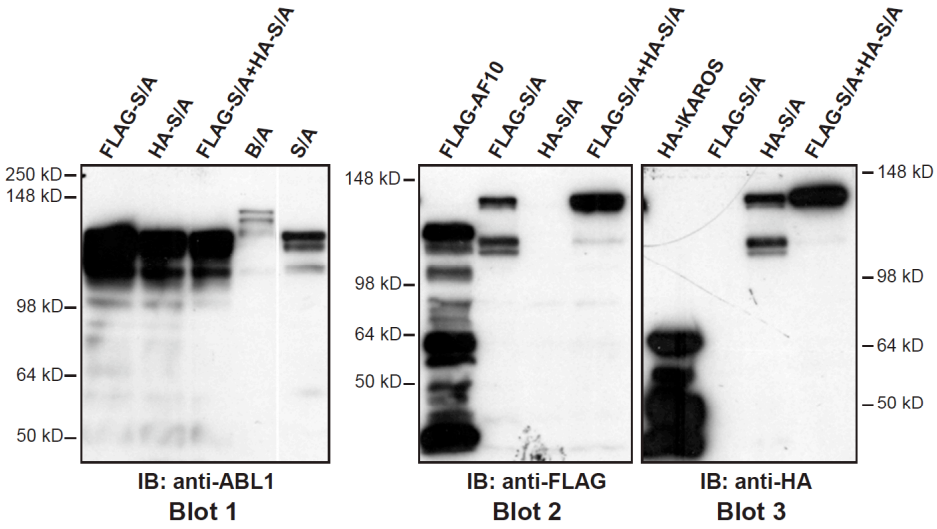


Figure 3.16: Western blot analysis of the total cell lysates of the HEK293T cells transiently over-expressing SHIP1/ABL1, Flag-SHIP1/ABL1 or HA-SHIP1/ABL1. Blot1: Immunoblotting with an antibody against c-ABL1: All the ABL1 fusion proteins are detected; Blot 2: Immunoblotting with an anti-body against the Flag tag: Flag tagged AF10 and SHIP1/ABL1 are detected but HA-SHIP1/ABL1 is not detected; Blot 3: Immunoblotting with an antibody against the HA epitope: HA tagged IKAROS and SHIP1/ABL1 are detected but Flag-SHIP1/ABL1 is not detected.

3.8.2 Homo di- or oligomerization of the SHIP1/ABL1 fusion protein

To demonstrate the dimerization or oligomerization of the SHIP1/ABL1 fusion protein, HEK293T cells were co-transfected *(2.2.3.3)* with the pcDNA-Flag-SA-96E and pcDNA-HA-SA-91E plasmids. For controls, HEK293T cells were also transfected with pcDNA-Flag-SA-CLN96E or pcDNA-HA-SA-CLN91E alone. After 48 hrs, total cell lysates were prepared *(2.2.4.1)* from transfected and non-transfected cultures. The lysates were then subjected to co-immunoprecipitation (CoIP) *(2.2.4.4)*. During the CoIPs, protein complexes were precipitated with an anti–FLAG antibody, followed by immunoblotting with anti-HA antibodies. These CoIP experiments revealed that protein

complexes containing Flag-SHIP1/ABL1 (i.e., complexes precipitated with the anti-FLAG antibody) could also be detected with the anti–HA antibody, demonstrating the interaction between FLAG-tagged SHIP1/ABL1 proteins and HA-tagged SHIP1/ABL1 proteins *(Figure 3.17)*. These results showed that the SHIP1/ABL1 fusion protein is present as a dimer or multimer in the cells, and strongly suggests that the activation of the ABL1 tyrosine kinase activity in SHIP1/ABL1 is mediated via homo di- or oligomerization. Homodimerization or oligomerization has also been demonstrated for the BCR/ABL1, ETV6/ABL1 and EML1/ABL1 fusions (McWhirter et al., 1993; Golub et al., 1996; De Keersmaecker et al., 2005).

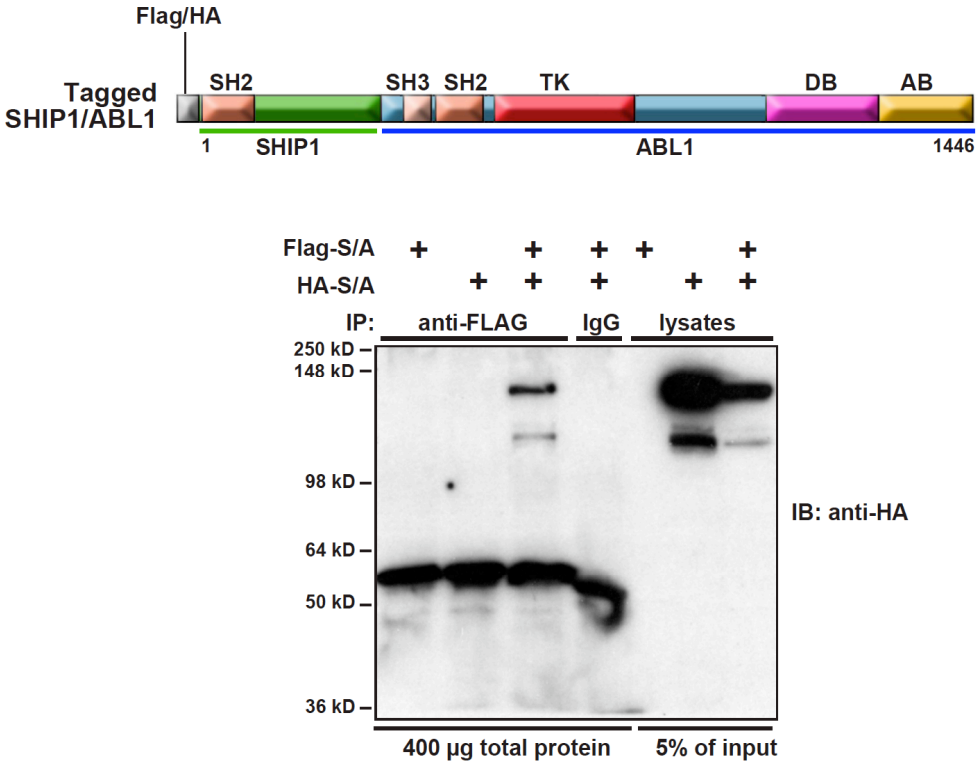


Figure 3.17: Detection of a homotypic interaction of the SHIP1/ABL1 protein: (A) Diagram showing important domains of the SHIP1/ABL1 protein with the two different N-terminal epitope tags used in the co-immunoprecipitation experiments: Flag and HA indicates the Flag and HA epitope tags, SH2: Src homology 2, SH3: Src homology 3, TK: Tyrosine kinase domain, DB: DNA binding domain, AB: Actin binding domain; **(B)** Co-immunoprecipitation of Flag-SHIP1/ABL1 and HA-SHIP1/ABL1: The indicated Flag-tagged SHIP1/ABL1 and HA-tagged SHIP1/ABL1 proteins were transiently expressed in the HEK293T cells and immunoprecipitated with

antibody against the Flag epitope (lane 1-3). As a control, the protein lysates obtained from the Flag-SHIP1/ABL1+HA-SHIP1/ABL1 cotransfected cells, were immunoprecipitated with antibody against normal IGg (lane 4). The immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-HA antibody (lane 1-4). In addition, 5% amount of the total cell lysates were subjected to SDS-PAGE and immunoblotted with anti-HA antibody (lane 5-7).

3.9 IDENTIFICATION OF THE HOMO DIMERIZATION DOMAIN OF SHIP1/ABL1

As mentioned in the Introduction *(1.4)*, in all ABL1 fusion proteins analyzed so far the tyrosine kinase activity of ABL1 is activated via homo di- or oligomerization of the fusion protein. In all of these ABL1 fusion proteins, the protein domain which mediates the homo di or oligomerization is supplied by the fusion partner of ABL1. As observed in the Co-IP experiments *(3.8.2)*, the SHIP1/ABL1 fusion also forms di- or multimers, implying that there should be a di- or oligomerization domain within the SHIP1 portion of the SHIP1/ABL1 fusion. Based on these results, we decided to map the di- or oligomerization domain within the SHIP1 portion of the SHIP1/ABL1 fusion protein. To approach this problem, we decided to divide the amino terminal 343 amino acids of SHIP1 into putative protein domains and to assay their dimerization potential in the Yeast-Two Hybrid (Y2H) system.

3.9.1 Mapping of putative protein domains within the 5' SHIP1 portion

To identify putative protein domains within the amino terminal 343 amino acids of SHIP1 contained in the SHIP1/ABL1 fusion protein, we first examined the already described conserved domains of SHIP1 in Genbank (http://www.ncbi.nlm.nih.gov). The only conserved domain described in the first 343 amino acids of SHIP1 is an SH2 (src-homology 2) domain from aa 5 to 102. Next, we sought to explore whether the remaining 240 amino acids (aa 103 to 343) of the N-terminal SHIP1 portion could be subdivided into structural domains. For this we analyzed the sequence with the "scratch protein predictor" (http://www.ics.uci.edu/~baldig/scratch/) from the University

of California at Irvine. The output of this query predicted two protein domains: the first roughly from aa 100 to 260 and the second from aa 260 to 340. Since these prediction are inherently uncertain, we decided to subdivide the aa 100 to 340 region of SHIP1 into two more equally-sized domain which we named D1 (aa 100 to 220; nt 451-813) and D2 (aa 220 to 340; nt 811-1173) *(Figure 3.18 and 3.19).*

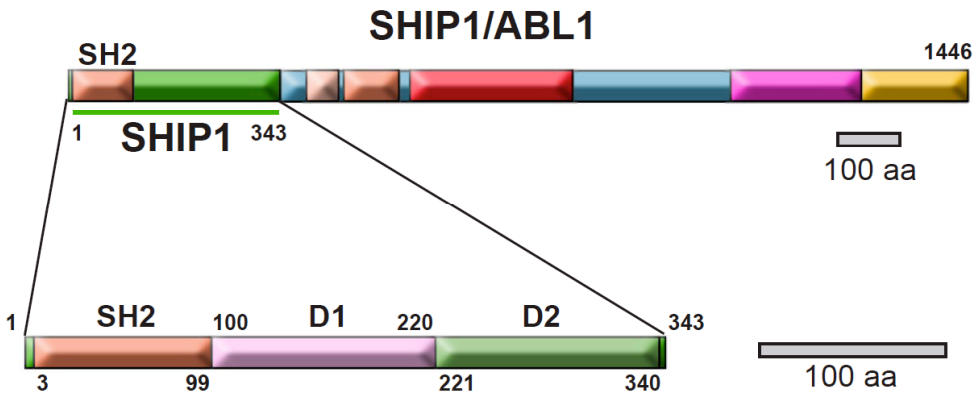


Figure 3.18: Putative protein domains within the N-terminal SHIP1 portion contained in the SHIP1/ABL1 fusion protein: SH2: Src homology-2 domain, D1: putative domain 1, D2: putative domain 2. The amino acids at the domain borders are designated below/above each domain.

```
10         20         30         40         50         60
   AAACAGGAAGTCAGTCAGTTAAGCTGGTGGCAGCAGCCGAGGCCACCAAGAGGCAACGGG

           70         80         90        100        110        120
   CGGCAGGTTGCAGTGGAGGGGCCTCCGCTCCCCTCGGTGGTGTGTGGGTCCTGGGGGTGC

          130        140        150        160        170        180
   CTGCCGGCCCGGCCGAGGAGGCCCACGCCCACCATGGTCCCCTGCTGGAACCATGGCAAC
                                      M  V  P  C  W  N  H  G  N>  10

          190        200        210        220        230        240
   ATCACCCGCTCCAAGGCGGAGGAGCTGCTTTCCAGGACAGGCAAGGACGGGAGCTTCCTC
    I  T  R  S  K  A  E  E  L  L  S  R  T  G  K  D  G  S  F  L>  30

          250        260        270        280        290        300
   GTGCGTGCCAGCGAGTCCATCTCCCGGGCATACGCGCTCTGCGTGCTGTATCGGAATTGC
    V  R  A  S  E  S  I  S  R  A  Y  A  L  C  V  L  Y  R  N  C>  50
```

```
      310       320       330       340       350       360
GTTTACACTTACAGAATTCTGCCCAATGAAGATGATAAATTCACTGTTCAGGCATCCGAA
 V  Y  T  Y  R  I  L  P  N  E  D  D  K  F  T  V  Q  A  S  E>  70

      370       380       390       400       410       420
GGCGTCTCCATGAGGTTCTTCACCAAGCTGGACCAGCTCATCGAGTTTTACAAGAAGGAA
 G  V  S  M  R  F  F  T  K  L  D  Q  L  I  E  F  Y  K  K  E> 90

      430       440       450       460       470       480
AACATGGGGCTGGTGACCCATCTGCAATACCCTGTGCCGCTGGAGGAAGAGGACACAGGC
 N  M  G  L  V  T  H  L  Q  Y  P  V  P  L  E  E  E  D  T  G> 110

      490       500       510       520       530       540
GACGACCCTGAGGAGGACACAGTAGAAAGTGTCGTGTCTCCACCCGAGCTGCCCCCAAGA
 D  D  P  E  E  D  T  V  E  S  V  V  S  P  P  E  L  P  P  R> 130

      550       560       570       580       590       600
AACATCCCGCTGACTGCCAGCTCCTGTGAGGCCAAGGAGGTTCCTTTTTCAAACGAGAAT
 N  I  P  L  T  A  S  S  C  E  A  K  E  V  P  F  S  N  E  N> 150

      610       620       630       640       650       660
CCCCGAGCGACCGAGACCAGCCGGCCGAGCCTCTCCGAGACATTGTTCCAGCGACTGCAA
 P  R  A  T  E  T  S  R  P  S  L  S  E  T  L  F  Q  R  L  Q> 170

      670       680       690       700       710       720
AGCATGGACACCAGTGGGCTTCCAGAAGAGCATCTTAAGGCCATCCAAGATTATTTAAGC
 S  M  D  T  S  G  L  P  E  E  H  L  K  A  I  Q  D  Y  L  S> 190

      730       740       750       760       770       780
ACTCAGCTCGCCCAGGACTCTGAATTTGTGAAGACAGGGTCCAGCAGTCTTCCTCACCTG
 T  Q  L  A  Q  D  S  E  F  V  K  T  G  S  S  S  L  P  H  L> 210

      790       800       810       820       830       840
AAGAAACTGACCACACTGCTCTGCAAGGAGCTCTATGGAGAAGTCATCCGGACCCTCCCA
 K  K  L  T  T  L  L  C  K  E  L  Y  G  E  V  I  R  T  L  P> 230

      850       860       870       880       890       900
TCCCTGGAGTCTCTGCAGAGGTTATTTGACCAGCAGCTCTCCCCGGGCCTCCGTCCACGT
 S  L  E  S  L  Q  R  L  F  D  Q  Q  L  S  P  G  L  R  P  R> 250
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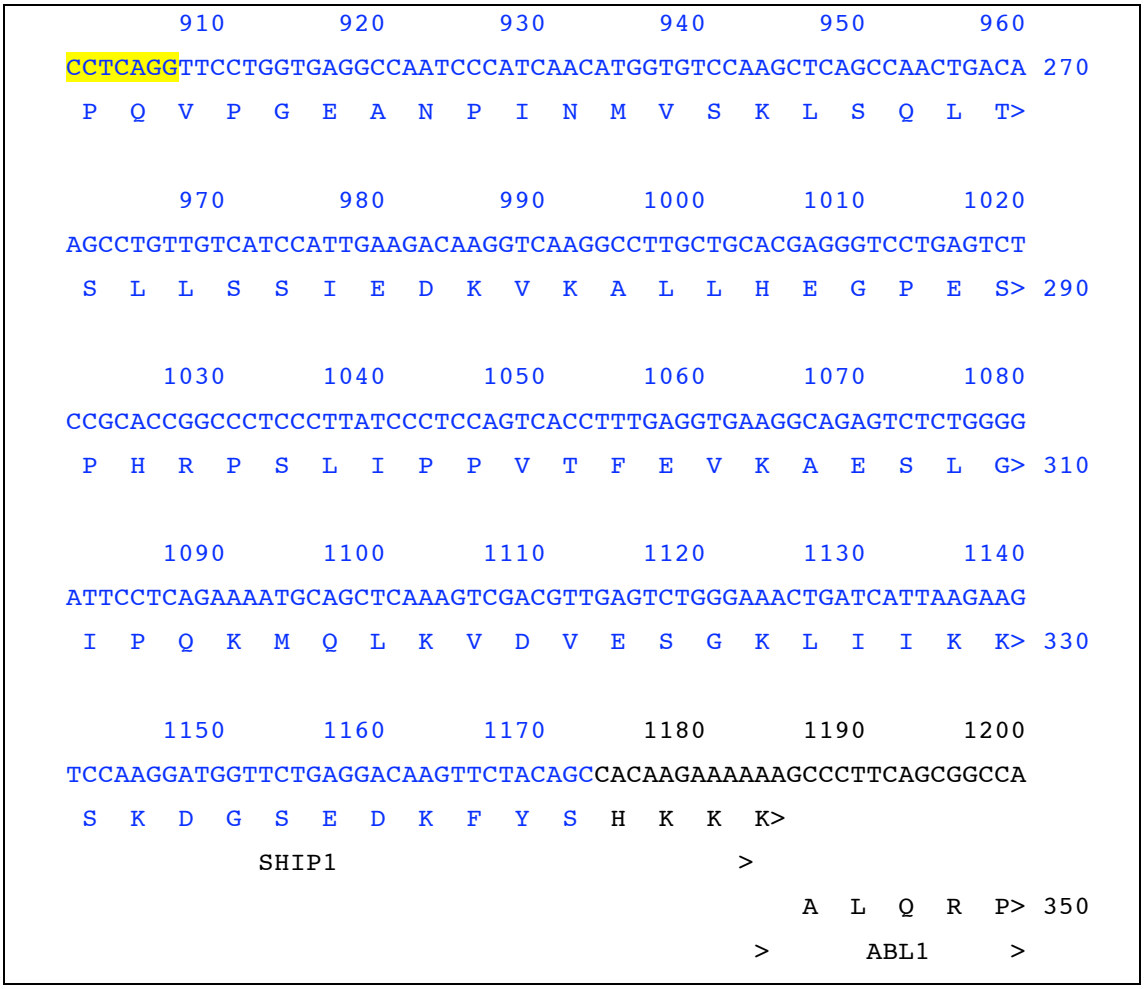


Figure 3.19: Sequence of the 5'SHIP1 portion contained in the SHIP1/ABL1 fusion, showing the putative protein domains: The SH2 domain is indicated by bold letters, domain D1 is indicated by underlined letters and domain D2 is indicated by blue letters. The highlighted sequence shows the Eco81I sites flanking the D1 domain, which were used for the construction of the delta-D1 mutants. The nucleotide numbers are given above the sequence, the amino acids are numbered to the right of the sequence.

3.9.2 Mapping of putative interacting domains within the 5' SHIP1 portion using the Yeast Two Hybrid system

For the mapping of the di- or oligomerization domain within the SHIP1 portion of the SHIP1/ABL1 fusion, the SH2, D1, and the D2 domains were cloned into appropriate bacterial/yeast shuttle vectors (Y2H vectors) and yeast-two-hybrid (Y2H) assays were performed.

3.9.2.1 Cloning of different regions of the 5' SHIP1 portion for Y2H assays

For the Y2H assays, the coding regions of the whole SHIP1 portion of the fusion (5'SHIP) containing the SH2, D1 and D2 domains as well as the coding sequences for the D1, D2 and SH2 domains individually were cloned into the bacterial/yeast shuttle vectors (Y2H vectors) pGBKT7 and pGADT7, to generate GAL4-DBD-fusion and GAL4-AD-fusion proteins, respectively. In addition, a sequence coding for SH2-D2 (also called 5'SHIP-deltaD1) was cloned into both Y2H vectors.

For the cloning of SH2-D1-D2, and the individual domains SH2, D1 and D2 domains, the coding sequences of the these domains were amplified from the pGEM-Teasy-SA(39-1508)-CLN9 plasmid. Along with the amplification, appropriate linkers (restriction enzyme sites) were introduced at the ends of fragments *(Figure 3.20)* by using the appropriate primers listed in section *2.1.13*. The PCR fragments were then cloned into pGEM-Teasy, the sequence of the inserts was verified and the fragments were then cloned into pGBKT7 or into pGADT7 using appropriate restriction enzymes *(Figure 3.20)*. The pGBKT7 and pGADT7 clones are shown diagrammatically in *Figure 3.20*. The pGEM-Teasy, pGBKT7 and pGADT7 clones containing SH2-D1-D2, SH2, D1 and D2 are listed in *Appendix II*

To clone the 5' portion of SHIP1 without the D1 domain (SH2-D2) into the bacterial-yeast shuttle vectors for the Y2H assay, the pGEM-Teasy-BamH1-SH2-D1-D2-Xho1-CLN(6-5) *(also called as pGEM-Teasy-BamH1-5'SHIP1-Xho1-CLN(6-5))* and the pGEM-Teasy-S/A(39-1508)-CLN9 constructs were used *(Appendix II)*. In short, the D1-D2 coding sequence in the pGEM-Teasy-BamH1-SH2-D1-D2-Xho1-CLN(6-5) construct was replaced by D2 coding sequence. For this, the D1-D2 region was removed from the plasmid pGEM-Teasy-BamH1-SH2-D1-D2-Xho1-CLN(6-5) by a *Eco*81I and *Xho*1 double digestion. Then the D2 region of SHIP1 (nt 904-1182) was amplified from the plasmid pGEM-Teasy-S/A(39-1508)-CLN9 along with the introduction of a 5' *Bpu*10I site and a 3' *Xho*1 to obtain the 5'Bpu10I-D2-3'Xho1 fragment using the primers *Bpu10I-SHIP-D2 F(904-921)* and *SHIP-D2-Xho1R(1182-1164) (2.1.13).* This fragment was cloned into the pGEM-Teasy vector. Then the

Bpu10I-D2-Xho1 fragment was excised from a pGEM-Teasy clone with the correct sequence (pGEM-Teasy-Bpu10I-D2-Xho1-CLN(1-5)) and inserted 3' to the SH2 coding sequence into the plasmid pGEM-Teasy-BamH1-SH2-D1-D2-Xho1-CLN(6-5), from which the D1-D2 region had been excised (see above). The construct was named pGEM-Teasy-BamH1-5'SHIP1-deltaD1-Xho1-CLN(6-5). Then using a BamH1-XhoI or a BamH1-Not1 double digest the fragments BamH1-SH2-D2-Xho1 or BamH1-SH2-D2-Not1 were excised from the plasmid pGEM-Teasy-BamH1-5'SHIP1-deltaD1-Xho1-CLN(6-5) and inserted into pGADT7 and into pGBKT7, respectively *(Figure 3.21)*. The *Not1* site used for cloning the fragment into the pGBKT7 plasmid was derived from the pGEM-Teasy backbone. The *Eco81I* recognition sequences in the D1-D2 region of 5'SHIP1 are indicated in Figure 3.19. The *Bpu10I* recognition sequence introduced at the 5' end of the D2 fragment by PCR was compatible with the *Eco81I* site at the 3' end of SH2 region (nt 488) *(Figure 3.19)*. All clones were verified by sequencing. In addition, the expression of the individual GAL4DBD and GALAD fusion proteins with the various SHIP1 domains in the yeast cells was verified by Western analysis (data not shown).

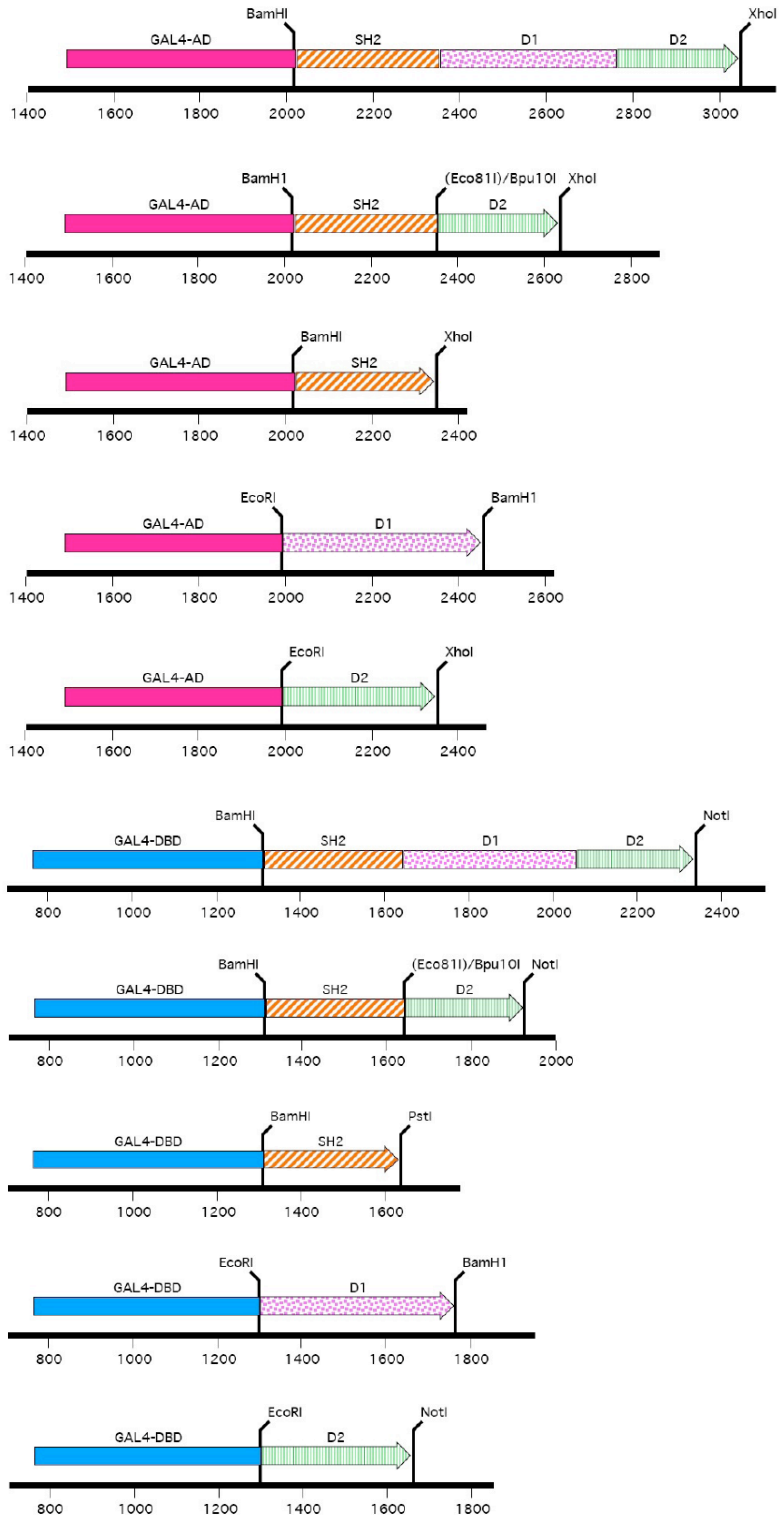


Figure 3.20: Diagrammatic representation of the Y2H clones: The pGADT7 constructs are shown as GAL4-AD (activation domain) fusion proteins and the pGBKT7 constructs are shown as GAL4-DBD (DNA binding domain) fusion proteins. SH2: Src homology-2 domain, D1: domain 1, D2: domain 2. Restriction enzyme sites used for cloning (linkers) are indicated.

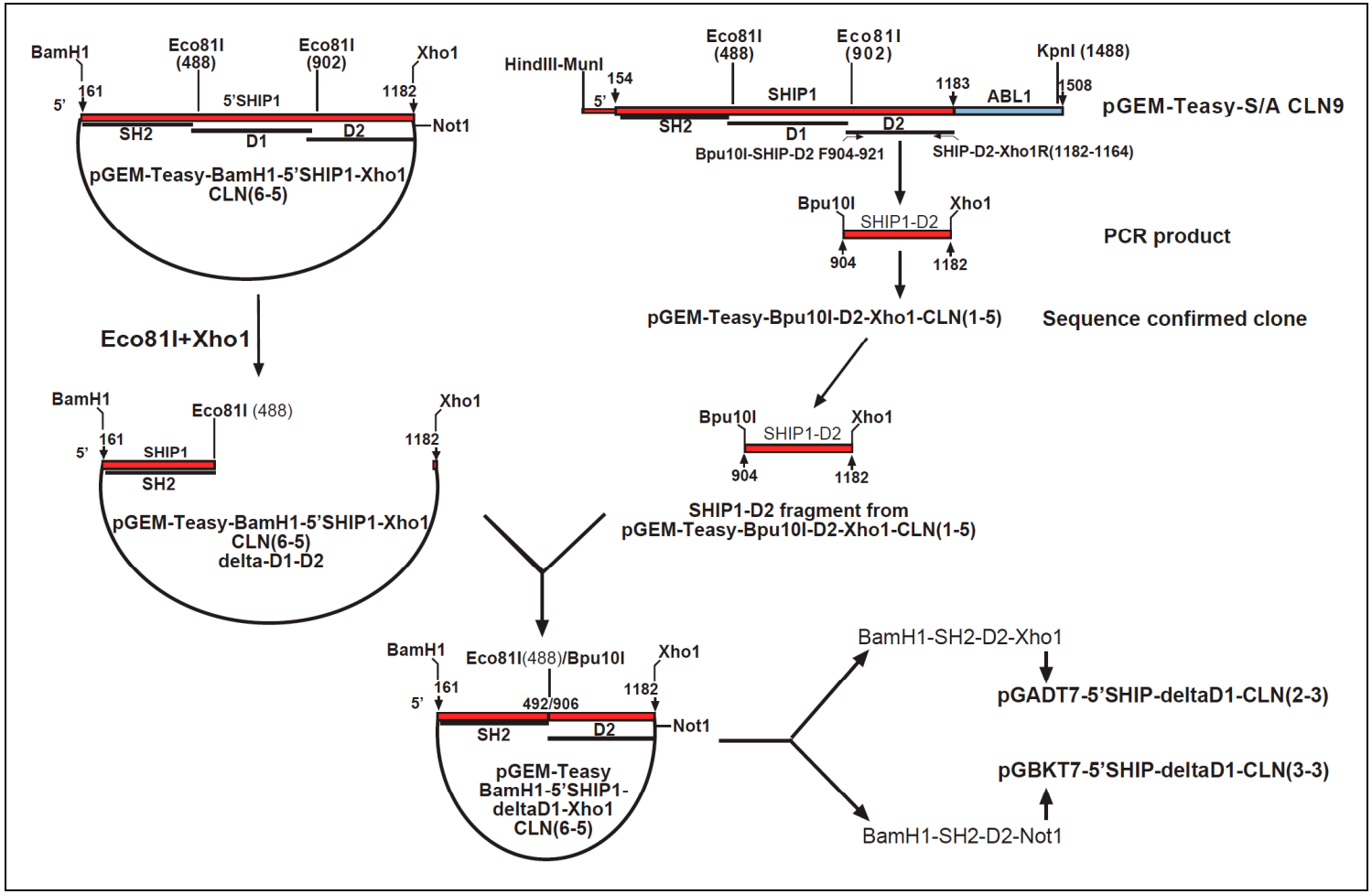
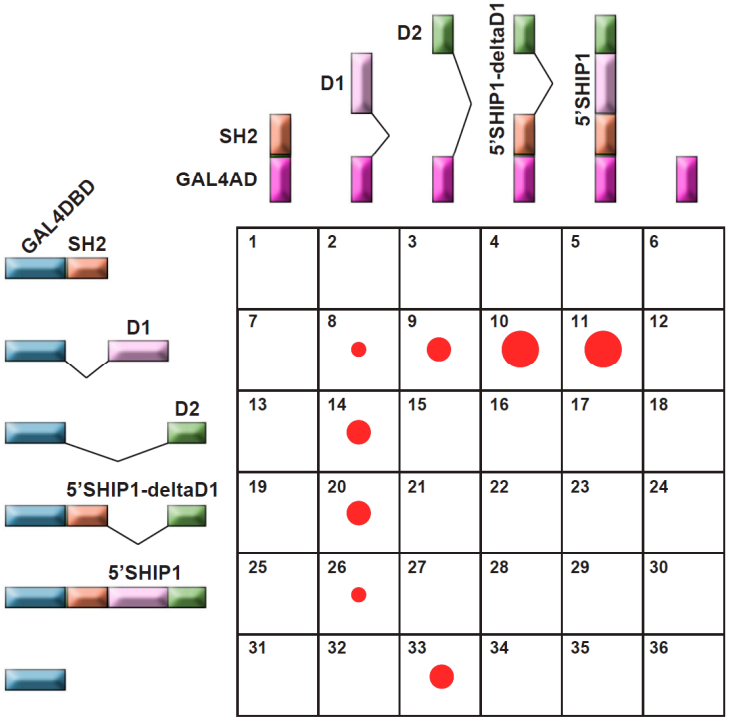


Figure 3.21: Cloning scheme for pGADT7-5'SHIP1-deltaD1 and pGBKT7-5'SHIP1-deltaD1

3.9.2.2 The domains D1 and D2 are critical for SHIP1/ABL1 dimerization

The Yeast-two-hybrid (Y2H) assays were performed *(2.2.2.5.3)* by co-transforming the yeast strain AH109 with the pGBKT7 and the pGADT7 clones with the different domains of 5'SHIP in all possible combinations *(Figure 3.22 A)*. The transformed cells were streaked out on high stringency SD/-Trp, -Leu, -His plates supplemented with 15 mM 3AT *(Figure 3.22 B)* and the growth of the different yeast clones was recorded after 5 days *(Figure 3.22 A)*. We also assayed the interactions on SD/-Trp, -Leu, -His plates supplemented with other concentrations of 3 AT (0, 3, 5, 10 and 30 mM) but found that the most representative read-out was obtained at 15 mM 3AT. The assays showed a strong interaction between D1 and D2 *(Figure 3.22, Experiment 9, 14)* and a weak interaction of D1 with itself *(Figure 3.22, Experiment 8)*. Surprisingly, there was no interaction between the complete 5'SHIP domain (consisting of SH2-D1-D2), which contained all the necessary interaction domains. The assays showed that SH2-D1-D2 can only interact with an isolated D1 domain *(Figure 3.22, Experiment 11, 26)* and not with the D2 domain, regardless of whether D2 is isolated or expressed together with the SH2 domain (SH2-D2) *(Figure 3.22, Experiment 17, 23, 27, 28, 29)*. In contrast, an isolated D1 domain can interact with D2, no matter if D2 is isolated or together with the SH2 domain (as SH2-D2) or part of the whole 5'SHIP domain (SH2-D2-D1) *(Figure 3.22, Experiment 10, 11, 20, 26)*. From these results we conclude that when D1 is flanked by SH2 and D2, it is unable to interact with the D2. One possible explanation could be that D1 engages in an intra-molecular interaction with the neighboring D2 domain, and is unable to form inter-molecular interactions with other D2 domains. Experiment 33 (Figure 3.22) shows a surprising interaction of the GAL4-DBD protein with the D2 domain when fused to the GAL4-AD. We do not have an explanation for this interaction, and we did not pursue it any further.

A.



B.

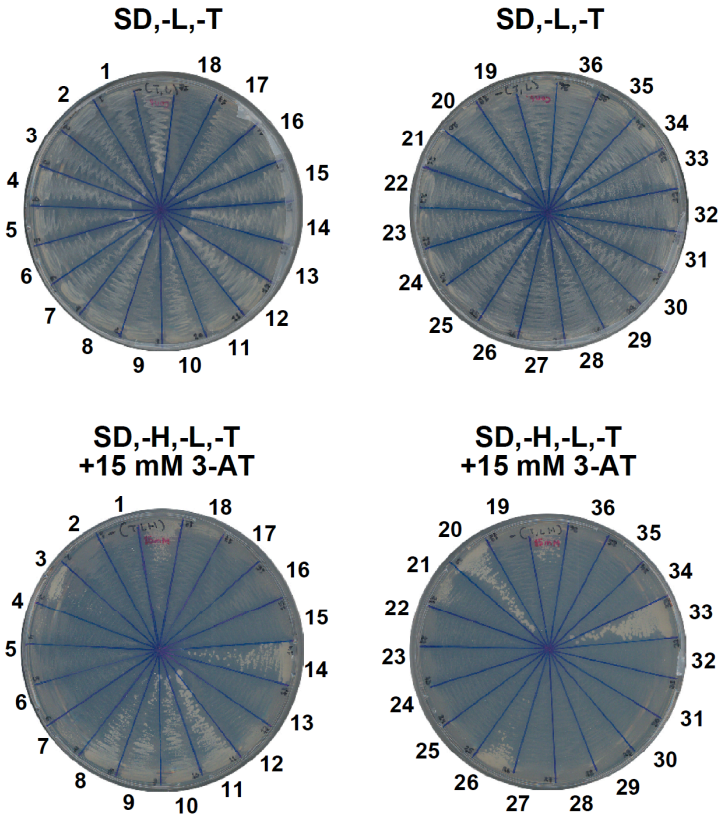


Figure 3.22: Identification of D1 and D2 as putative interacting domains within the SHIP1 portion of the SHIP1/ABL1 fusion protein by Y2H

assays: (A) Overview of the Y2H assays: Each interaction assay is assigned a number from 1 to 36. The size of the red circles corresponds to the strength of the interaction observed on the high stringent SD, -Tr, -Leu, -His, +15mM 3AT selection plates; (B) Plates showing the interaction assays: Yeast strain AH109 was co-transformed with different combinations of pGBKT7 and pGADT7 constructs (see 3.9.2.1). Each combination of co-transformed Y2H constructs (interaction) are described and designated by a number in figure 3.22B. Plates 1 and 2 show the growth of the co-transformants on SD, -Tr, -Leu selection medium. The co-transformants were then streaked out on high stringency SD, -Tr, -Leu, -His, +15mM 3AT selection plates (Plates 3 & 4). The numbers 1-36 in the figure correspond to the number assigned to each interaction assay as described in Figure 3.22A. Visible yeast colonies wre recorded as an interaction between the two corresponding proteins. The strength of the interaction was determined by scoring the density and size of the yeast colonies.

3.9.3 Verification of SHIP1 interaction domains in mammalian cells

As observed in the Y2H assays *(3.9.2.2)*, there was a strong interaction between D1 and D2 and a weak interaction of D1 with itself. However, the yeast assays were performed without the ABL1 portion of the fusion protein. To validate our hypothesis that both the D1 and the D2 domain of the 5'SHIP region are required for SHIP1/ABL1 dimerization in mammalian cells, we performed co-immunoprecipitation experiments using different mutants of the SHIP1/ABL1 fusion protein with deletions in the SHIP1 portion.

3.9.3.1 Cloning of SHIP1/ABL1 deletion mutants in eukaryotic expression vectors for co-immunoprecipitation experiments

For the co-immunoprecipitation experiments we cloned 4 different variants of SHIP1/ABL1 carrying deletion of the SH2, D1, or the D2 domain of SHIP1 into the pcDNA3 vector. These mutant variants are SHIP1-deltaD1/ABL1, D1(SHIP1)/ABL1, D1-D2(SHIP1)/ABL1 and D2(SHIP1)/ABL1.

For the cloning of **SHIP1-deltaD1/ABL1** fusion with and without the FLAG and HA epitope tags, the fragment coding for the D1-D2-ABL1 up to the Kpn1 site of ABL1 (nt 1488) was removed from the plasmid pGEM-Teasy-SA(39-1508)-CLN9 and its tagged derivatives, pGEMTeasy-Flag-SA(154-1508)-CLN96 and pGEMTeasy-HA-SA(154-1508)-CLN91 *(Figure 3.23 A)* by an *Eco*81I and *Kpn*1 double digest to obtain linearized tagged or untagged pGEM-Teasy-SH2, flanked by *Eco*81I and *Kpn*1 sites. A Bpu10I-D2-ABL1 (nt 904-1508) fragment was generated by PCR using the primers *Bpu10I-SHIP-D2 F904-921* and *SHIP-ABL1-B1508-1489 (2.1.13)* and cloned into pGEM-Teasy generating the plasmid pGEM-Teasy-Bpu10I-D2/ABL(904-1508)-CLN(3-4). From this plasmid, the Bpu10I-D2/ABL1-Kpn1 (nt 904-1488) fragment was obtained by a *Bpu*10I and *Kpn*1 double digest and inserted 3' to the SH2 region in the linearized pGEM-Teasy-SH2-Eco81I-Kpn1 vectors (see above), to generate the untagged or tagged versions of SHIP1/ABL1 till nt 1508 lacking the D1 domain. These pGEM-Teasy plasmids were named pGEM-Teasy-S/A-deltaD1-1508-9(5), pGEM-Teasy-Flag-S/A-deltaD1-1508-96(2) and pGEM-Teasy-HA-S/A-deltaD1-1508-91(3) *(Appendix II)*. The pcDNA3 clones of the complete SHIP1-deltaD1/ABL1 with or without tags were obtained following the same strategy as described in section 3.4 *(Figure 3.9)*. The structure of the tagged and untagged SHIP1-deltaD1/ABL1 inserts of the pcDNA3 clones as well as the important restriction enzyme sites are shown in *figure 3.23 B*.

For the cloning of **D1(SHIP1)/ABL1** fusion, the coding sequence of the D1 domain of SHIP1 (nt 460-898 of the SHIP1/ABL1 sequence) and that of ABL1 (nt 1184-1508 of the SHIP1/ABL1 sequence) were amplified separately. During the amplification of the D1 coding sequence, a HindIII-Mun1-Kozak-ATG sequences with or without 5' tags (Flag or HA) were introduced 5' to the D1 sequence and at the 3' end of the D1 sequence a *Bcl*1 linker was introduced using appropriate primers listed in section *2.1.13*. These HindIII-Mun1-D1-Bcl1 fragments were cloned into the pGEM-Teasy vector. A *Bcl*1 linker was also introduced to the 5' end of the ABL1 (1184-1508) fragment by PCR and the fragment was cloned into pGEM-Teasy vector. The primers used to obtain the HindIII-Mun1-D1(460-898)-Bcl1 and Bcl1-ABL1(1184-1508)

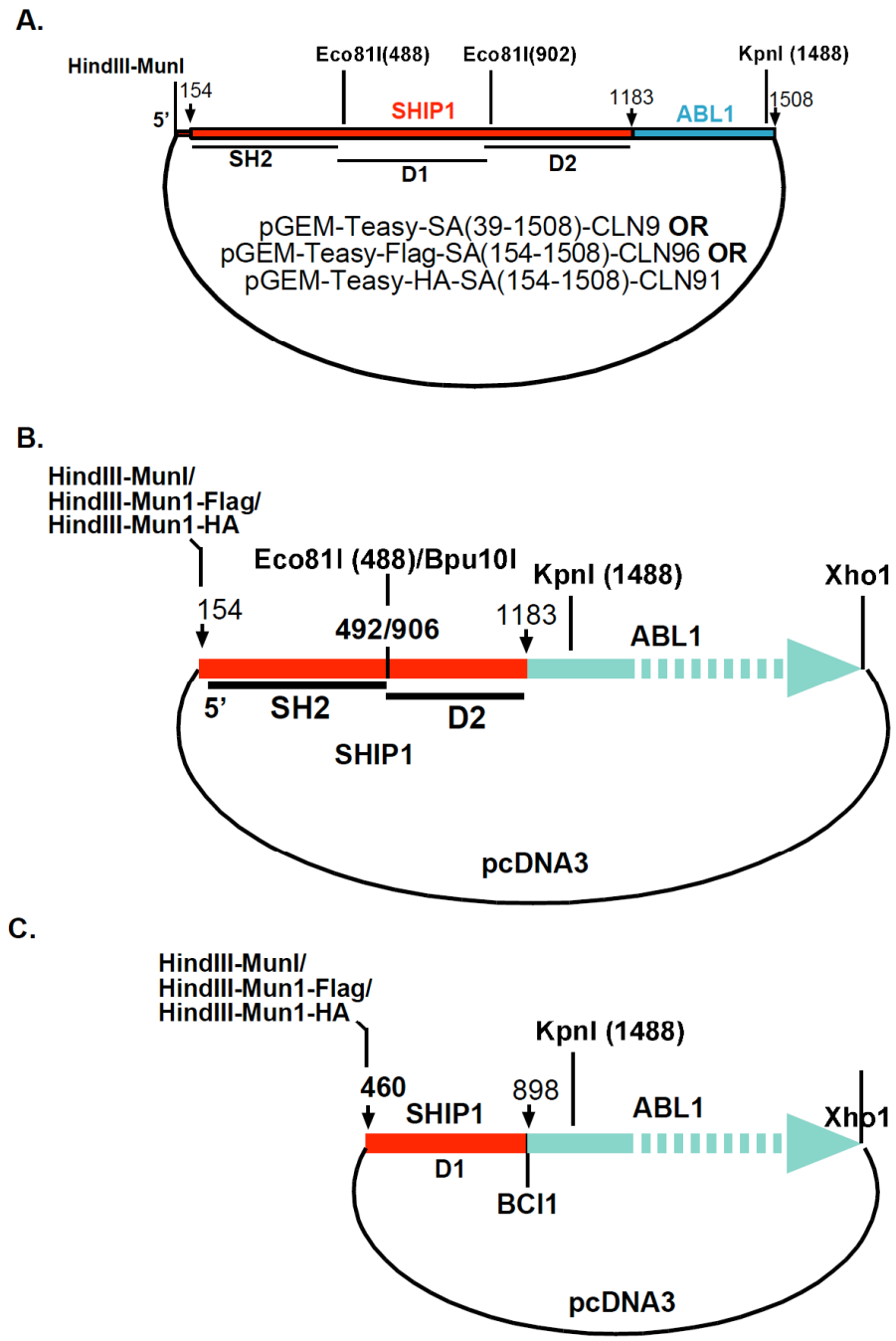


Figure 3.23: **Diagrams of the pcDNA3 constructs of the deletion mutants of SHIP1/ABL1; SHIP1/ABL-delta D1 and the D1/ABL1 (Not to the scale):** (A) Diagram of pGEM–Teasy-SA(39-1508)-CLN9 and its Flag and HA-tagged derivatives; (B) Schematic diagram of the SHIP1-deltaD1/ABL1 insert in the pcDNA3 vector; (C) Schematic diagram of the D1/ABL1 insert in the pcDNA3 vector.

fragments are listed in the section *2.1.13 (Primer no. 18, 19, 21, 22, 49, 50 and 55 for D1 and no. 51, 16 for ABL1 fragment)*. The sequence verified

pGEM-Teasy clones of ABL1 and D1 with or without tags (*Appendix II*) were later used for further cloning step. The Bcl1-ABL1 (1184-1488) *(Appendix II)* fragments were linked 3' to the tagged or untagged D1-Bcl1 fragments in the pGEM-Teasy clones of D1 to generate tagged and untagged versions of incomplete D1/ABL1 fusions (till nt 1508). For this cloning step, in addition to the *Bcl1* site of the insert, either *Apa*1 or *Sal*1 site from the pGEM-Teasy vector were used depending on the direction of the insert. The in-frame fusion of the D1 and ABL1 was confirmed by sequencing. Finally, the full length D1/ABL1 fusions were assembled in the pcDNA3 vector as described in section *3.4 (Figure 3.9)*. The tagged or untagged full length D1/ABL1 inserts of the pcDNA3 clones and the important restriction enzyme sites are shown in *figure 3.23 C*. The pcDNA-SA-deltaD1 and pcDNA-D1/ABL1 constructs were used for co-immunoprecipitation experiments in order to confirm the interactions discovered by Y2H assays *(3.9.3.2)*.

3.9.3.2 Confirmation of the interaction between the D1 and D2 domains of SHIP1 by co-immunoprecipitation

HEK293T cells were co-transfected *(2.2.3.3)* with the plasmids pcDNA-Flag-D1/ABL1-CLN(3-1) and pcDNA-HA-D1/ABL1-CLN(4-1) to analyze the homo-dimerization potential of the D1 domain of SHIP1 in the context of the SHIP1/ABL1 fusion. To confirm the hetero-dimerization of the D1 and the D2 domains of SHIP1 in the context of the SHIP1/ABL1 fusion, HEK293T cells were co-transfected *(2.2.3.3)* with the plasmids pcDNA-Flag-SA-deltaD1-CLN(6-3) and pcDNA-HA-D1/ABL1-CLN(4-1). The structure of the SHIP1-deltaD1/ABL1 and D1/ABL1 proteins are shown in *figure 3.24 A*. Total cell protein lysates from the co-transfected HEK293T cells were subjected to immuno-precipitation using an anti-FLAG antibody, separated on an SDS-PAGE gel and immunoblotted with an anti-HA antibody *(Figure 3.24 B)*. The immunoblots showed that the HA-D1/ABL1 protein was strongly co-precipitated with the Flag-SHIP1-deltaD1/ABL protein. *(Figure: 3.24 A; Blot 1)*. This finding confirmed the strong interaction between the D1 and the D2 domains of SHIP1 in mammalian cells, which we had observed in the Y2H system *(3.9.2.2, Figure: 3.22*). However, this conclusion is only valid under

the assumption that the SH2 domain does not interact with the D1 domain, which was shown in the Y2H assays *(3.9.2.2, Figure: 3.22)*.

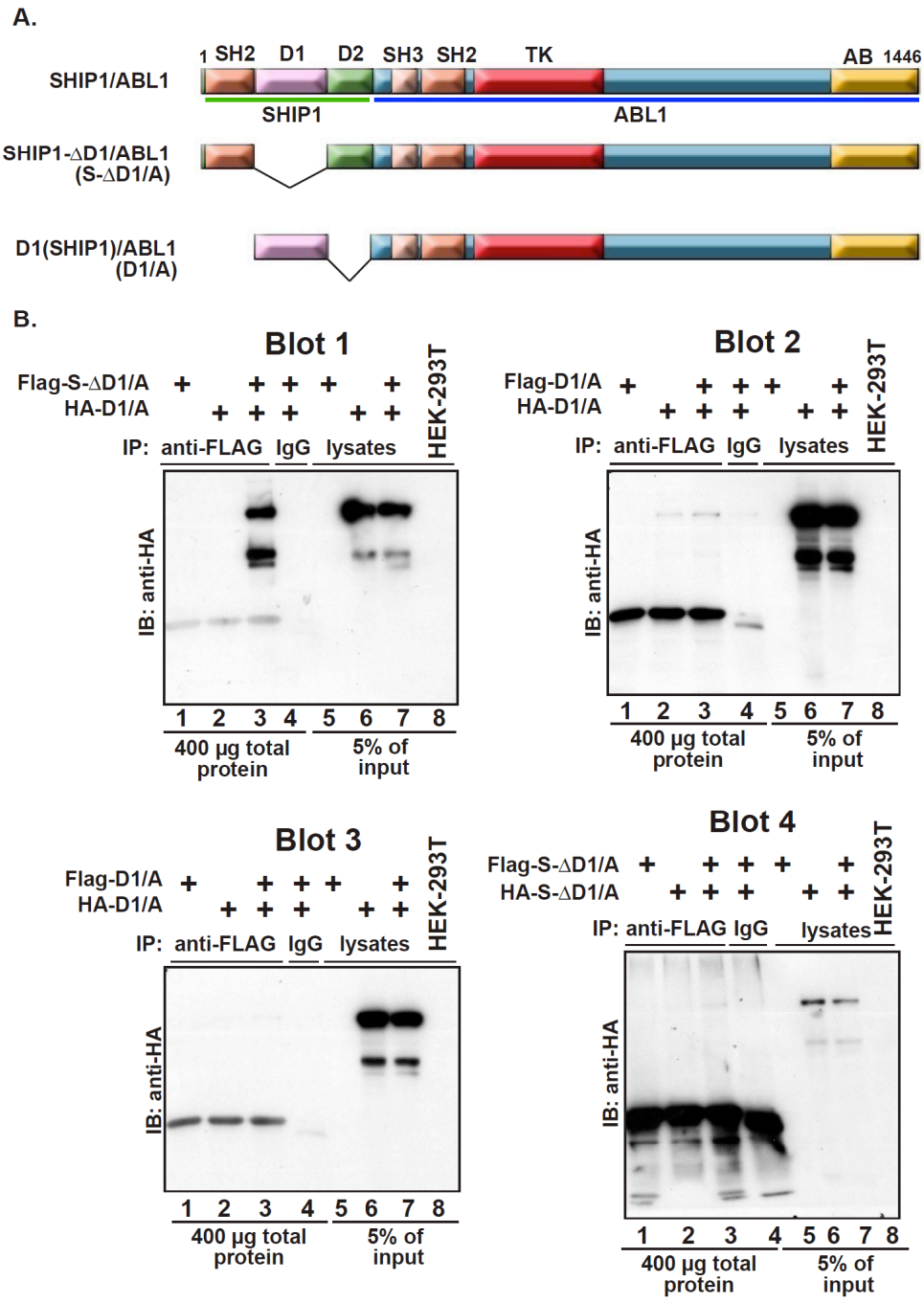


Figure 3.24: Interaction between different deletion mutants of the SHIP1/ABL1 fusion protein assayed by co-immunoprecipitation: (A) Structure of the proteins used in the co-precipitation experiments: SH2: Src homology-2 domain, D1: domain 1, D2: domain 2, SH3: Src homology -3 domain, TK: tyrosine kinase domain, AB: actin binding domain. These proteins

were either Flag or HA tagged. (B) Co-immunoprecipitation experiments showing that domains D1 and D2 in the N-terminal SHIP1 portion interact strongly with each other. Blot 1: Flag-SHIP1-deltaD1/ABL1 and HA-D1/ABL1 can be co-immunoprecipitated; Blot 2: Flag-D1/ABL1 and HA-D1/ABL1 show a very weak interaction visible upon long exposure indacting an interaction between the D1 domains; Blot 3: a shorter exposure of Blot 2; Blot 4: Flag-SHIP1-deltaD1/ABL1 and HA-SHIP1-deltaD1/ABL1 cannot be co-precipitated indicating that the D1 domain is crucial for dimerization. All experiments were performed in transiently transfected HEK293T cells. The protein lysates of the HEK293T cells were subjected to immunoprecipitation with an antibody against the Flag epitope (lanes 1-3). As a control, the protein lysates were also immunoprecipitated with IgG isotype control (lane 4). The immunoprecipitates were separated by SDS-PAGE and immunoblotted with an anti-HA antibody (lanes 1- 4). 5% of the total cell lysates, used for the Co-IPs were also subjected to SDS-PAGE and immunoblotted with anti-HA antibody (lanes 5-7). Protein lysates from untransfected HEK293T cells were used as negative controls (lane 8).

The weak interaction of the D1 domain with itself, which we found in the Y2H assays *(3.9.2.2, Figure: 3.22)*, was also observed in the co-immunoprecipitation experiments, when the immuno-blot was exposed for a longer time *(Figure 3.24 B; Blot 2)*. At shorter exposure times, the band indicating interaction of D1 with itself could hardly be seen *(Figure 3.24 B; Blot 3)*. As positive and negative controls, Co-IPs of Flag-SHIP1/ABL1 with HA-SHIP1/ABL1 *(see Figure 3.17)* and Flag-SHIP1-deltaD1/ABL1 with HA-SHIP1-deltaD1/ABl1 *(Figure 3.24 B; Blot 4)* were performed, respectively. In summary, these results clearly demonstrate that the D1 and D2 domains are the critical domains within the SHIP1 portion of the SHIP1/ABL1 fusion gene which mediate the dimerization or oligomerization of the SHIP1/ABL1 fusion protein and thereby lead to the constitutive activation of the ABL1 tyrosine kinase portion of the SHIP1/ABL1 fusion protein.

3.10 THE PROLIFERATION POTENTIAL OF DIFFERENT SHIP1/ABL1 DELETION MUTANTS

Since we had identified the domains D1 and D2 as strongly interacting protein domains, and the D1 domain as a weakly self-interacting domain, we wanted to determine the role of these domains in inducing factor independent growth in BA/F3 cells. For this, we cloned 4 different SHIP1/ABL1 mutants with deletions of different parts of the SHIP1 portion into the MSCV-IRES-GFP (pMIG) retroviral expression vector.

3.10.1 Cloning of additional SHIP1/ABL1 deletion mutants

In addition to the SHIP1-deltaD1/ABL1 and D1(SHIP1)/ABL1 expressing constructs we cloned **D2(SHIP1)/ABL1** and **D1-D2(SHIP1)/ABL1** expressing plasmids.

To obtain a pcDNA3-D2/ABL1 construct, we generated the D2(SHIP1)/ABL1 fusion (nt 829-1508) *(Appendix I)* with a *Hin*dIII-*Mun*1–KOZAK-ATG sequence at the 5' end of the fragment by PCR. This fragment was amplified from the pGEM-Teasy-SA(39-1508)-CLN9 clone using the primers *HindIII-Mun1-D2-F(829-844)* and *SHIP-ABL1-B1508-1489 (2.1.13)* and cloned into pGEM-Teasy. A sequence verified pGEM-Teasy-D2/ABL(829-1508)-CLN(2-2) clone was used to obtain a full length D2/ABL1 fusion *(Figure: 3.25 A)* in pcDNA3. For this, the same cloning strategy, as explained in section *3.4 (Figure 3.9)* was followed in which the 5' BCR/ABL1 portion of the pcDNA-BCR/ABL1 construct was replaced with the D2(SHIP1)/ABL(829-1508) using the *Hin*dIII and *Kpn*1 sites.

In the same way, a pGEM-Teasy clone for the D1-D2(SHIP1)/ABL1 (nt 463-1508) *(Appendix I)* fragment was obtained from the pGEM-Teasy-SA(39-1508)-CLN9 clone using the primers *HindIII-Mun1-SHIPD1 (463-478)F* and *SHIP-ABL1-B1508-1489 (2.1.13)*. The complete **D1-D2(SHIP1)/ABL1** fusion *(Figure: 2.25 B)* was assembled in the pcDNA3 vector as described in the preceding paragraph using the *Hin*dIII and *Kpn*I sites *(3.4)*. We also cloned the epitope-tagged versions of D1-D2(SHIP1)/ABL1 and D2(SHIP1)/ABL1 into

pcDNA3, but we have not done any further experiments with these two constructs yet.

Finally, to establish the stable Ba/F3 cell lines expressing the SHIP1-deltaD1/ABL1, D1(SHIP1)/ABL1, D1-D2(SHIP1)/ABL1 and D2(SHIP1)/ABL1 fusions, the corresponding coding regions were cloned into the retroviral expression vector pMSCV-IRES-GFP (pMIG) from the pcDNA3 constructs. This was achieved by using the strategy described in section *3.4 (Figure 3.9)*.

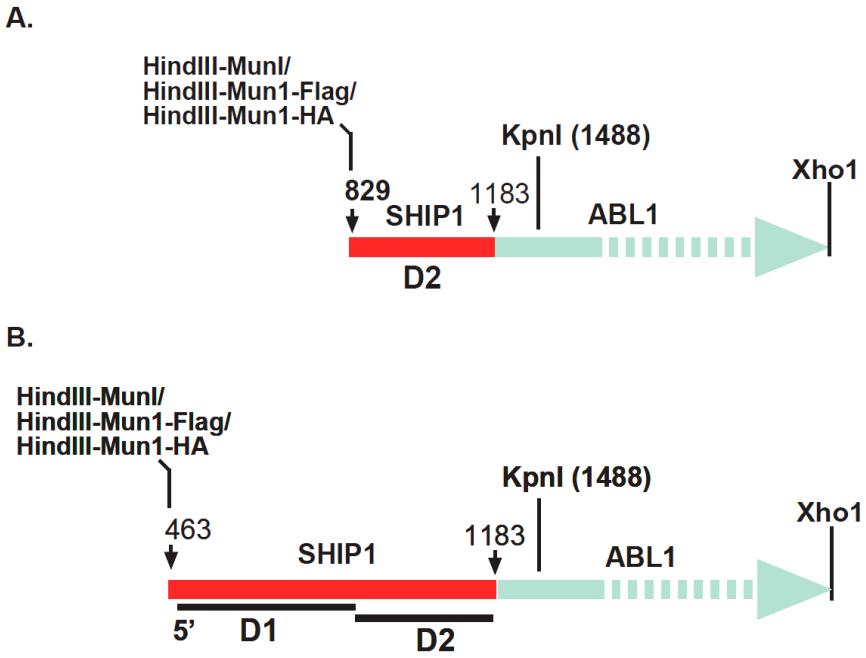


Figure 3.25: Diagrams of the D2/ABL1 (**A**) and D1-D2/ABL1 (**B**) inserts in pcDNA3 with relevant restriction enzymes. Numbers denote nucleotide positions.

3.10.2 Cell proliferation assays for the SHIP1/ABL1 deletion mutants: The D1-D2(SHIP1)/ABL1 fusion induces IL3 independence in Ba/F3 cells

Ba/F3 cells were stably transduced *(2.2.3.4)* with the different pMIG constructs expressing the SHIP1-deltaD1/ABL1, D1(SHIP1)/ABL1, D2(SHIP1)/ABL1 and D1-D2(SHIP1)/ABL1 mutants. To assay growth factor independent growth of Ba/F3 cell lines carrying different mutants of the SHIP1/ABL1, the cell lines were cultured in the presence and absence of IL3 and the number of viable cells was counted on three consecutive days with

the trypan blue exclusion method. In addition, the proliferation potential of the Ba/F3 cells expressing BCR/ABL1 and the SHIP1/ABL1 fusions, in presence and absence of IL3 was also assayed as positive control experiment. As negative controls, non- transduced Ba/F3 cells and the stably transduced Ba/F3 cells with the pMIG empty vectors were used. The assays were performed three times in triplicate for a total of 9 individual assays for each mutant and culture condition.

The assays showed that among all the mutants of SHIP1/ABL1 fusion, only the D1-D2(SHIP1)/ABL1 fusion induces IL3 independent growth of the Ba/F3 cells. However, the proliferation potential of D1-D2(SHIP1)/ABL1 was slightly weaker than that of BCR/ABL1 and the complete SHIP1/ABL1 fusions *(Figure 3.26)*.

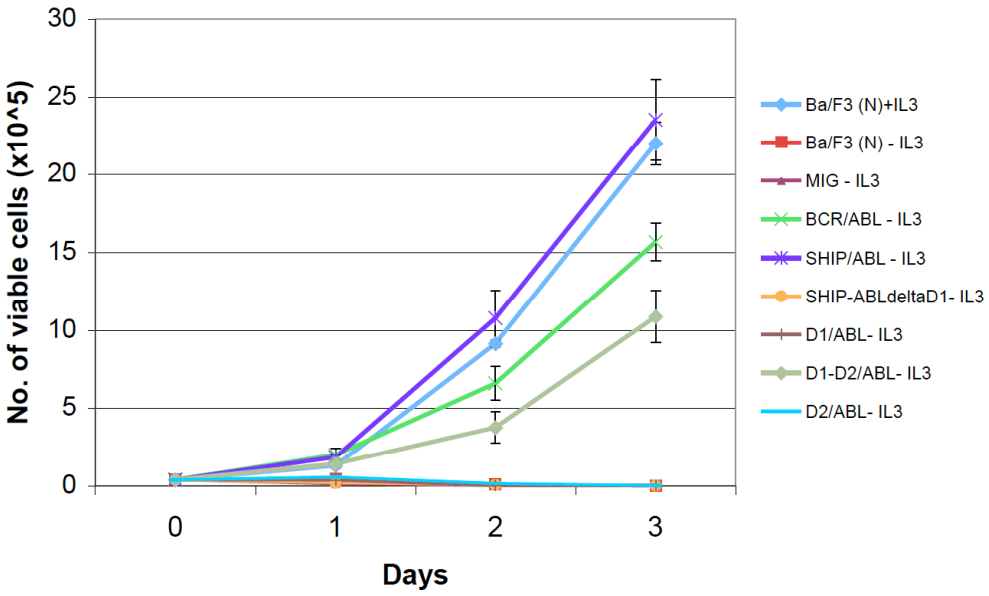


Figure 3.26: Assessment of the proliferation potential of different deletion mutants of the SHIP1/ABL1 fusion protein in Ba/F3 cells. Ba/F3 cells retrovirally transduced with the indicated constructs were grown in the absence or presence of IL3 and their growth was recorded over a period of 3 days. Proliferation curves were prepared by plotting the mean ± SD derived from three individual assays.

3.11 LOCALIZATION OF THE SHIP1/ABL1 FUSION ON METAPHASE CHROMOSOMES BY FISH

In order to analyze the genomic rearrangement that led to the SHIP1/ABL1 fusion, we performed fluorescence in-situ hybridization (FISH) assays. We prepared dual color dual fusion (DCDF)-FISH probes for the detection of the

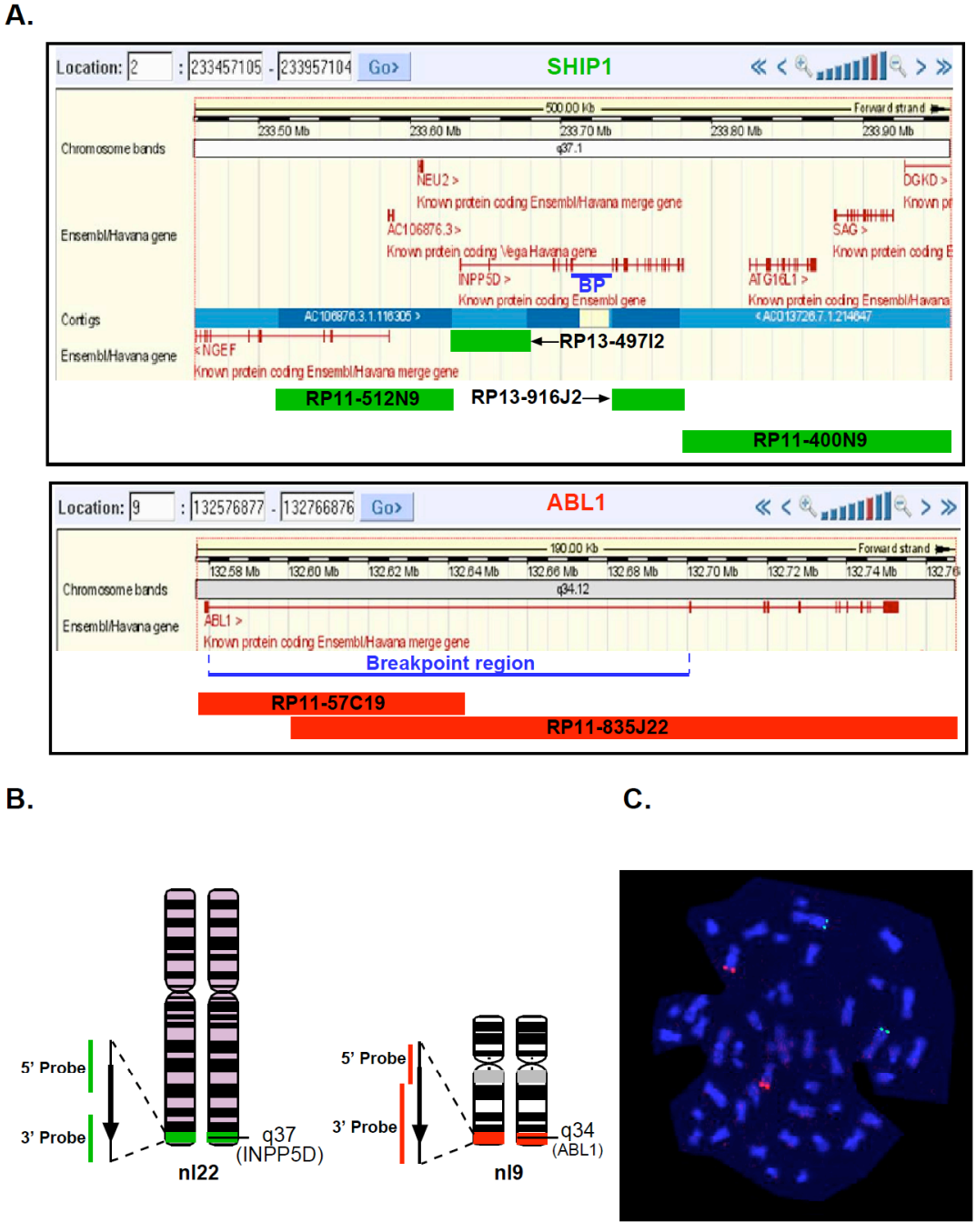


Figure 3.27: Fluorescent in-situ hybridization for the analysis of genomic SHIP1/ABL1 fusion: (A) Position of the selected BAC clones in the genome browser view from www.ensembl.org ; BP is breakpoint region **(B)** SHIP1/ABL1

DCDF FISH probe design for the detection of the SHIP1/ABL1 fusion and the resulting signal pattern in normal cell. **(C)** The SHIP1/ABL1 DCDF probe set hybridized to a metaphase cell of a normal male.

SHIP1/ABL1 fusion from selected bacterial artificial chromosome (BAC) clones corresponding to the genomic locus of the *SHIP1* and *ABL1* genes flanking the breakpoints *(Figure 2.27 A)(2.1.15)*. The SHIP1 specific FISH probes were labeled with Spectrum Green (FITC) and the ABL1 specific FISH probes were labeled with Spectrum Orange (Texas Red, TR) fluorophores. The BAC DNA was amplified by SIA (Sequence independent amplification) before PCR-labelling *(2.2.5.2.1.1)*. The SHIP1/ABL1 DCDF-FISH probe set contained two FITC labelled BACs corresponding to the 5' and the 3' region of the *SHIP1* locus and two TR labelled BACs corresponding to the 5' and the 3' region flanking the breakpoint in the ABL1 locus. With this probe set, normal cells should show 2 green signals on the two *SHIP1* loci on 2q37 and 2 orange signals on the two *ABL1* loci on 9q34 (a so-called 2G2O signal pattern) *(Figure 2.27 B)*. As hypothesized in chapter 3.1.6 *(Figure 3.6),* in our patient sample the insertion of the 3' portion of *SHIP1* into the *ABL1* locus should result in at least one yellow signal on chromosome 9. The specificity and quality of the DCDF-SHIP1/ABL1 FISH probe set was confirmed by hybridizations on the metaphase cells obtained from the peripheral blood sample of a normal male *(Figure 2.27 C)*. Unfortunately, we were unable to achieve good hybridization results with the DCDF-SHIP1/ABL1 FISH probe set in our patient sample. This could be due to the poor quality of the patient sample. We are attempting to improve the hybridization conditions and to optimize the pretreatment of our patient sample, and will hopefully be able to obtain adequate hybridization results in the future.

4 Discussion

4.1 SHIP1/ABL1: A NEW ABL1 FUSION GENE

The study of chromosomal translocations, which lead to the formation of fusion genes has provided us with fundamental insights into the basic mechanisms of malignant transformation not only in leukemias but also in solid tumors. The genes that are found at the translocation breakpoints and are part of the fusion genes occupy central positions in key regulatory pathways in the cells, which control, among other things, proliferation, differentiation and programmed cell death. Despite the cloning of more than a hundred different gene fusions in hematological malignancies and solid tumors, we are still very far from a comprehensive understanding of the cellular pathways that are altered in the many different tumor types. For this reason the identification of each new gene fusions will help us in our quest to understand the formation of tumors.

We identified a new fusion gene involving the *ABL1* tyrosine kinase gene in the leukemic cells of an 18-year old female patient diagnosed with common ALL. The fusion was found through the sequence analysis of an aberrant PCR band from an RT-PCR reaction designed to detect a BCR/ABL fusion transcript. The sequence of this band revealed an in-frame fusion between the *ABL1* (Abelson 1) and the *SHIP1* (SH2 containing Inositol phosphatase-1) gene located on chromosome 2 band q37. Interestingly, the reciprocal ABL1/SHIP1 fusion transcript was not detectable by RT-PCR.

The first *ABL1* gene fusion, the BCR/ABL1 fusion, was the first fusion gene in leukemia characterized at the molecular level in 1985 (Shtivelman et al., 1985), and is the hallmark of Chronic Myeloid Leukemia (CML) (>95% of all CML cases are BCR/ABL positive). The BCR/ABL1 fusion is also the most common known genetic lesion in ALL (25% of adult B-ALL, and 2-4% of childhood B-ALL cases are BCR/ABL1 positive), but is rarely seen in AML (Borowitz and Chan, 2008). Though the constitutive activation of the tyrosine kinase activity of ABL1 through the BCR moiety in the BCR/ABL fusion is such a common event in leukemia, *ABL1* fusion partners other than *BCR* are exceedingly rare. Up to now, only 5 additional *ABL1* fusion partners have

been identified. This includes the *ETV6/ABL1* fusion, which was the second *ABL1* fusion, discovered 10 years after the identification of the BCR/ABL1 fusion (Papadopoulos et al., 1995). The other four *ABL1* fusions, *EML1/ABL1*, *NUP214/ABL1*, *RCSD1/ABL1* and *ZMIZ1/ABL1* were described almost 10 years after the identification of the *ETV6/ABL1* fusion (Graux et al., 2004; De Keersmaecker et al., 2005; De Braekeleer et al., 2007; Soler et al., 2008). It is remarkable that even though *BCR/ABL1* was the first fusion gene found and is the most common fusion gene, up to now only five additional fusion partners of *ABL1* could be identified. This is very different from the situation of several other genes, some of which are much less frequently rearranged than *ABL1*, like *MLL*, *NUP98*, *ETV6* or *ALK*. As an example, even though the first *MLL* fusion genes were characterized 10 years after the identification of the *BCR/ABL1* fusion, there are now over 50 different fusion partners of *MLL* described (Meyer et al., 2006) (http://atlasgeneticsoncology.org/Genes/MLL.html). Thus, comparing the two most common fusion gene subgroups, the *MLL* fusions and the *ABL1* fusions, the identification of a novel *ABL1* fusion partner is a comparatively rare event and should lead to valuable insights into the pathways important in *ABL1*-mediated leukemogenesis. For this reason we found it very important and interesting to characterize the *SHIP1/ABL1* fusion in more detail.

As mentioned above, in contrast to the *BCR/ABL1* fusion, which is the most common genetic aberration in leukemia, all the other *ABL1* fusions are extremely rare. There are only single case reports of the *EML1/ABL1, RCSD1/ABL1* and *ZMIZ1/ABL1* fusions, and just 6 cases of *ETV6/ABL1* have been described (http://atlasgeneticsoncology.org//Indexbychrom/idxa_9.html). The *NUP214/ABL1* fusion is slightly more common and found in 6% of T-ALLs (Graux et al., 2004).

If one compares the different *ABL1* fusion partners, it should be possible to learn more about the requirements for *ABL1*-mediated leukemogenesis. The common denominator of all the *ABL1* fusion partners is that they are all expressed in haematopoietic tissues and they all contribute a di- or oligomerization domain to the fusion protein. The di- or oligomerization domains are required for the constitutive activation of the ABL1 kinase

activity. However, in that respect the *NUP214/ABL1* fusion differs from the other *ABL1* fusion, in that the coiled-coil motifs of NUP214 do not mediate the oligomerization of the NUP214/ABL1 protein, but mediate the interaction of NUP214/ABL1 with other nuclear pore proteins. This NUP214/ABL1 nucleo pore interaction is required to achieve the full NUP214/ABL1 transforming potential (De Keersmaecker et al., 2008). Neverthless, considering these two basic requirements for an *ABL1* fusion partner, one would expect many genes in the cell to fulfil these two requirements. Thus, it is very surprising that there have only been 6 fusion partners of *ABL1* identified till now. This indicates, that the fusion partners of ABL1 are required to have some other unknown important properties. It could be that they have to be able to activate some specific signalling pathways. For example, in addition to the coiled-coil domain, BCR contains a GRB2 binding site (Tyr177), which is required for the maximal transformation potential of BCR/ABL1 by activating the RAS and the GAB2/PI3K/AKT signalling pathways (Pendergast et al., 1993; Puil et al., 1994; Goga et al., 1995; He et al., 2002; Sattler et al., 2002). The ability of NUP214 to localize at the nuclear pore complex via its coiled-coil domains and its interaction with other nuclear pore proteins is a prerequisite to achieve the full transforming potential of NUP214/ABL1 (De Keersmaecker et al., 2008). There is also evidence that some of the fusion partners of *ABL1* have tumor suppressor gene functions. This is known for example for the *ETV6* gene and, as explained below, might also be the case for *SHIP1*.

The *ABL1* breakpoint in the *SHIP1/ABL1* fusion is 5' of exon 2 of *ABL1*. This is the same location as in all the other known *ABL1* fusions like *BCR/ABL1*, *ETV6/ABL1*, or *NUP214/ABL1*. These facts make the *SHIP1/ABL1* fusion a good model to study the requirements for the constitutive activation of *ABL1* and *ABL1*-mediated malignant transformation of hematopoietic cells. It should be noted that ours is the first report that *SHIP1* has been found altered in a malignancy and suggests that the possible tumor suppressor function of SHIP1 might play an important role in leukemogenesis.

4.2 SHIP1: THE NOVEL FUSION PARTNER OF ABL1 IS A PUTATIVE TUMOR SUPPRESSOR GENE

What is known about the normal function of *SHIP1* makes it an extremely attractive target for genetic alterations that would facilitate tumor formation.

SHIP1 (SH2-containing inositol phosphatase-1), which is also known as *INPP5D* (Inositol polyphosphate 5-phosphatase), is widely and specifically expressed in haematopoietic cells. *SHIP1* was cloned in 1996. SHIP1 was originally identified as the major 145 kD tyrosine phosphorylated protein that became associated with SHC (SH2 domain containing transforming protein 1) after cytokine stimulation (Damen et al., 1996). At the same time, SHIP1 was observed as the major tyrosine phosphorylated protein, which bound to the C-terminal SH3 domain of the adaptor protein GRB2 (growth factor receptor-bound protein 2) (Damen et al., 1996; Ware et al., 1996). Thus SHIP1 interacts with both SHC and GRB2, which are adapter proteins known to be involved in cytokine signaling and the activation of the *Ras* pathway (Damen et al., 1996).

SHIP1 contains a central phosphatase domain, an N-terminal SH2 domain and a C-terminal terminal domain containing NPXY (Asn-Pro-Xaa-Tyr) sites, which can be tyrosine phosphorylated, and proline-rich sequences, which can provide the binding sites for the SH3 domains of other proteins. SHIP1 is a phosphatase, which hydrolyzes the 5-phosphates from phosphatidylinositol (3,4,5)–trisphosphate(Ptdins(3,4,5)P_3;PIP_3) and inositol-1,3,4,5 tetrakis-phosphate (Ins(1,3,4,5)P_4) (Damen et al., 1996), thereby negatively regulating the PI3K (phosphoinositide 3-kinase) pathway. The PI3K pathway is part of many important signalling pathways and regulates key cellular functions such as survival, proliferation, cell activation and cell migration (Krystal, 2000; Ward and Cantrell, 2001; Ward, 2006). SHIP1 regulates these important cellular functions by controlling PIP_3 levels and *Ras* activity following cytokine stimulation (Batty et al., 1985; Damen et al., 1996).

A murine *SHIP1* knockout model has provided us with additional information about the function of *SHIP1* in the hematopoietic system. *SHIP1* knockout mice fail to thrive and show an increased number of neutrophils and

monocytes/macrophages due to enhanced survival and proliferation of their progenitors. For this reason, the *SHIP1* knockout mice develop a myeloproliferative-like disease with massive infiltration of myeloid cells in their lungs. The bone marrow progenitor cells of the SHIP1 knockout mice are hypersensitive to hematopoietic growth factors. *SHIP1* knockout mice exhibit a reduction in the number of pre-B and immature B cells in the bone marrow, but an increased number of hypermature B cells in the spleen (Helgason et al., 1998). The B cells from *SHIP1* knockout mice are hypersensitive to B cell receptor stimulation, and less sensitive to apoptotic stimuli (Brauweiler et al., 2000a; Brauweiler et al., 2000b; Helgason et al., 2000). It was shown that *SHIP1* plays a different role in T cells compared to myeloid or B cells (Gloire et al., 2007). *SHIP1* does not seem to inhibit T cell proliferation, but instead its absence creates an anergy to T cell receptor stimulation. Whereas *SHIP1* clearly has pro-apoptotic functions in myeloid and, under certain conditions, in B cells, it appears to play an anti-apoptotic role in T cells (Gloire et al., 2007). In cell line models it could be shown that SHIP1 negatively regulates cellular growth, differentiation or migration, and that it may play an important role in apoptosis (Lioubin et al., 1996; Ono et al., 1996; Liu et al., 1997; Sattler et al., 2001).

Interestingly, it has been observed that *SHIP1* is reversibly down regulated in BCR/ABL transformed Ba/F3 cells (Sattler et al., 1999). In this murine pro-B cell line, SHIP1 was found to form a signaling complex with BCR/ABL that includes DOK1 (Downstream of tyrosine kinase 1), phosphatidylinositol 3-kinase (PI3K) and CRKL (v-crk sarcoma virus CT10 oncogene homolog (avian)-like), each of of these proteins is known to regulate cell migration. SHIP1 interacts directly with the PTB (phosphotryrosine binding) domain of DOK1 and with the SH2 domain of CRKL. The association of PI3K with this complex is thought to occur via CRKL (Sattler et al., 2001).

Thus, SHIP1 is found to be involved in multiple important hematopoietic signaling pathways in myeloid cells and in B cells but also in T cells. Most importantly, considering the phenotype of the *SHIP1* knockout mice, *SHIP1* is an excellent candidate for a tumor suppressor gene.

4.3 THE GENOMIC REARRANGEMENT LEADING TO THE SHIP1/ABL1 FUSION

Interestingly, the cytogenetic analysis of the leukemic cells from the patient with the *SHIP1/ABL1* fusion showed a normal karyotype. At first sight, this finding is not surprising considering the location and direction of transcription of the SHIP1 and ABL1 genes. Both genes located close to the telomeres of their chromosomes and are transcribed 5' to 3', centromere to telomere. Thus a SHIP1/ABL1 fusion gene can be generated by a simple balanced t(2;9)(q37;q34) translocation *(Figure 3.4 A)*. Given the sub-telomeric genomic locations of *SHIP1* and *ABL1*, this translocation could be easily overlooked by conventional cytogenetics, but should be easily detectable upon FISH analysis using a BCR/ABL dual color dual fusion (DCDF) FISH probe on metaphase chromosomes *(Figure 3.5)*. In this analysis one would expect to see two signals for the *BCR* loci on chromosome 22, and three *ABL1* signals: one signal on the normal chromosome 9, a smaller signal (5'ABL1) on the derivative chromosome 9, and a third *ABL1* signal (3'ABL1) on the derivative chromosome 2 *(Figure 3.4 B)*. But unexpectedly, we observed only two *ABL1* signals, both on chromosome 9 and no *ABL1* signal on chromosome 2. However, in about 50% of the interphase nuclei, we saw a peculiar *ABL1* signal pattern with four *ABL1* signals in a pair wise arrangement with a small gap equivalent to the size of 1-2 signals separating the signals of each pair. These observations led us to speculate that there might be an insertion of the 5'SHIP1 portion into the *ABL1* genomic locus, forming a 5'SHIP1-3'ABL1 fusion on chromososme 9. The explanation for the observation of the signal pairs of the *ABL1* loci in the interphase nuclei but not on metaphase chromosomes, is the decondensed chromatin in the interphase nuclei. In the metaphase chromosomes, the chromatin is so condensed that the gap between the 5' and the 3' ABL1 signals cannot be resolved. It is interesting to note that, even though the 3' portion of *ABL1* can easily form a fusion with 5'SHIP1 by a simple translocation, apparently a more complex rearrangement took place.

4.4 GENOMIC CONSEQUENCES OF THE SHIP1/ABL1 FUSION

The FISH analysis on interphase nuclei revealed rearrangements involving both *ABL1* loci, resulting in the absence of a normal *ABL1* allele. The possible mechanism for the generation of the two identically aberrant *ABL1* loci can be explained as follows. Initially, an insertion of a 5'SHIP1 fragment within one of the *ABL1* loci occurred. Then, possibly through a mitotic non-disjuntion event, this derivative chromosome 9 was duplicated while the normal copy of chromosome 9 was lost from the cell. Thus, it can be speculated that this rearrangement should have led to a LOH (Loss Of Heterozygosity) covering all of chromosome 9.

4.5 ASSAYING THE TRANSFORMATION POTENTIAL OF ONCOGENES

One of the key questions that arise during the study of leukemia-associated rearrangements and especially during the analysis of fusion genes is whether the fusion gene is capable of causing leukemia. The question whether a given (fusion) oncogene is actually able to transform hematopoietic cells is not easy to answer. The transforming potential of a fusion gene can be analyzed in cell line systems or in animal models.

The murine pro-B cell line Ba/F3 and fibroblast cell lines like NIH-3T3 or Rat-1 are some of the cellular systems used for the study of the oncogenic potential of fusion genes or mutated genes, especially in the case of constitutively activated tyrosine kinases. Ba/F3 cells are interleukin-3 (IL3) dependent and can only proliferate in the presence of IL3. The fibroblast cell lines (eg. NIH3T3) are anchorage dependent and need to attach to the surface of the culture flask to proliferate. As an indication of its transformation potential the expression of a putative oncogene can induce IL3 independent proliferation of Ba/F3 cells or anchorage independent growth of the fibroblasts in soft agar.

As mentioned above, these two cellular systems are especially suitable to test the activity of activated tyrosine kinases. However, these cell line systems are not sufficient to accurately measure the transformation potential of all types of fusion genes or oncogenes, and they can not recapitulate the complexity of tumor development in the organism. To obtain a more complete

understanding of the true tumorogenic potential of a given oncogene whole animal models like bone marrow transplantation (BMT) models, transgenic animals and knock-in models are available. Bone marrow transplantation models, in which the oncogene is retrovirally transduced into primary bone marrow cells which are then transplanted into lethally irradiated syngeneic mice, are especially useful to assay the potential of an oncogene to induce tumors in the hematopoietic systems.

It should be kept in mind that in many cases the read outs obtained in the cell line systems do not match the read outs obtained from the animal model systems. For example, the transformation potentials of the *BCR/ABL1* and the *ETV6/ABL1* fusiongenes are more or less similar in the Ba/F3 cell line system, but in the bone marrow transplant (BMT) mouse models these two *ABL1* fusions induce different diseases with different latency periods. The recipients (Balb-c mice) of *BCR/ABL1* transduced bone marrow develop a CML-like disease, whereas the recipients of *ETV6/ABL1* expressing bone marrow cells did not develop leukemia but rather a myeloproliferative syndrome or a small bowel syndrome (Golub et al., 1996; Million et al., 2002). These differences were also observed for the *NUP214/ABL1* fusion. The *NUP214/ABL1* fusion induces very weak IL3 independent growth of the Ba/F3 cells compared to the *BCR/ABL1* fusion. However, in the BM transplanted mice a strong ALL phenotype is seen (De Keersmaecker et al., 2008).

Thus, for the study of the oncogenic potential of particular genetic lesion, animal models are more realistic. However, these model systems also have their limitations. For example, in BMT models, the gene, which is retrovirally transduced in the bone marrow cells, randomly integrates in the genome of these cells. These random integrations may disrupt or activate neighboring tumor suppressor gene or oncogene, respectively, thereby altering the phenotype. In addition, animal models are very expensive and time consuming to establish and to evaluate. This is especially true for the transgenic and knock-in models.

4.5.1 Behavior of SHIP1/ABL1 in Ba/F3 cells: SHIP1/ABL1 induces IL3 independent proliferation in a murine pro-B

cell line, which is inhibited by the tyrosine kinase inhibitor Imatinib

To obtain an initial cellular read out of the oncogenic potential of the *SHIP1/ABL1* fusion, we used the IL3-dependent Ba/F3 cell line system. We expressed the *SHIP1/ABL1* and the *BCR/ABL1* fusion in IL3 dependent Ba/F3 cells. The expression of *SHIP1/ABL1* induced IL3 independent proliferation of Ba/F3 cells, which was comparable with that induced by the BCR/ABL1 fusion. The IL3 independent growth induced by the *SHIP1/ABL1* fusion and the fact that the SHIP1/ABL1 protein contains the same portion of ABL1 as the other ABL1 fusions, suggested that the ABL1 tyrosine kinase is constitutively activated in the SHIP1/ABL1 fusion protein. To assess this possibility, we analyzed the sensitivity of the *SHIP1/ABL1* fusion to the tyrosine kinase inhibitor STI571 (Imatinib).

STI571, also known as Imatinib, is an *ABL1* specific tyrosine kinase inhibitor as described in the introduction. All *ABL1* fusions are sensitive to Imatinib. We assessed the sensitivity of SHIP1/ABL1 and BCR/ABL1 by proliferation assays using the Ba/F3 cell line. As expected, the IL3-independent proliferation of *SHIP1/ABL1* expressing Ba/F3 cells could be inhibited by Imatinib treatment. Interestingly, SHIP1/ABL1 expressing Ba/F3 cells were significantly more sensitive to Imatinib than BCR/ABL1 expressing cells. The rate of proliferation was much lower in the SHIP1/ABL1 expressing Ba/F3 cells compared to that in the Ba/F3 cells expressing BCR/ABL1 at a particular concentration of Imatinib. The Imatinib concentrations required to inhibit the proliferation of SHIP1/ABL1 expressing Ba/F3 cells by 50% (inhibitory concentration 50, IC50) was less than one-fourth of that required in case of the BCR/ABL1-transduced Ba/F3 cells. The $IC50^{IMATNIB}$ for SHIP1/ABL1 was 0.16 µM and that for BCR/ABL1 was 0.71 µM. These results clearly demonstrated that the *ABL1* tyrosine kinase is constitutively active in the *SHIP1/ABL1* fusion.

This higher sensitivity of SHIP1/ABL1 to Imatinib compared to that of BCR/ABL1 is surprising and difficult to explain. One explanation could be that, because of the strong tetramerization domain of BCR, the tyrosine activity of

the BCR/ABL1 fusion protein is more strongly activated than that of the SHIP1/ABL1 fusion protein.

To better understand these results and the leukemogenic mechanisms used by the *SHIP1/ABL1* fusion, it might be helpful to look at the mechanism of *ABL1* tyrosine kinase activation in the other *ABL1* fusions and to examine more closely the protein domains in the SHIP1 portion of the *SHIP1/ABL1* fusion.

4.6 THE N-TERMINAL PORTION OF SHIP1 CONTAINS TWO PROTEIN INTERACTION DOMAINS

The common mechanism of the activation of the ABL1 tyrosine kinase used by almost all the other ABL1 fusions is oligo or dimerization of the fusion protein. The oligo- or dimerization of the fusion protein is mediated by protein domains in the *ABL1* fusion partner. As expected, we could see a clear interaction between the full-length SHIP1/ABL1 fusion proteins in co-immunoprecipitation experiments using Flag and HA-tagged versions of the SHIP1/ABL1 fusion protein. This suggested the presence of an oligo- or dimerization domain in the SHIP1 portion of the fusion protein.

To dissect the protein interaction domain within the N-terminal portion of SHIP1, we first performed an *in silico* protein domain homology search. The SH2 domain (aa 5-102) of SHIP1 was the only conserved domain that was detected in this region. Based on the calculations of a protein structure prediction program, we defined two additional putative protein domains (roughly aa 100-220 and aa 220-340) in the N-terminal portion of SHIP1. We called these two domains domain 1 (D1) and domain 2 (D2). To further characterize the protein interaction domains, we used the Yeast-two-hybrid system (Y2H).

In the Y2H assays, we discovered a strong heterotypic interaction between domain D1 and domain D2, when these domains were fused individually to the GAL4-DNA-binding protein and to the GAL4-activation domain. But unexpectedly, we could not detect any interaction between the complete N-terminal portions (SH2-D1-D2) of SHIP1 in the Y2H assays, even though the D1 and the D2 domain were contained in these proteins. There was also no

interaction between an isolated D2 domain and the N-terminal portion (SH2-D1-D2) of SHIP1. This was very surprising, because in the co-immunoprecipitation experiments with the full-length SHIP1/ABL1 fusion proteins a strong homotypic interaction between the SHIP1/ABL1 fusion proteins was detected. The Y2H assays suggested that when the D1 domain is flanked by the SH2 and D2 domains, it can not interact with an isolated D2 domain or with a D2 domain flanked by a D1 and an SH2 domain. The possible explanation for this observation is the inhibition of intramolecular interactions by intermolecular interactions combined with differences in the posttranslational modifications in different species.

4.6.1 Inhibition of intermolecular interaction by intramolecular interaction in the Y2H system

As shown in the Y2H assays, D1 and D2 interact strongly with each other. This interaction might also occur within the SH2-D1-D2 protein as an intramolecular interaction. The intramolecular interaction between D1 and D2 in the SH2-D1-D2 protein would result in a closed conformation of this protein. In this closed conformation of the SH2-D1-D2 protein, the protein interaction sites of D1 and D2 are occupied and embedded within the SH2-D1-D2 protein *(Figure 4.1 A)*. This model can explain why the intermolecular interaction between the SH2-D1-D2 proteins could not be observed in the Y2H assays.

4.6.2 Difference in the post-translational modifications between yeast and mammals

The difference between the Y2H and the co-immunoprecipitation results can be explained by differences in the post-translational modifications (PTM) between yeast and mammalian cells. It could be that a specific PTM, like a phosphorylation or acetylation, of the N-terminal SHIP1 portion (SH2-D1-D2) is required to prevent this protein region from forming the intramolecular interaction between the D1 and D2 domains *(Figure 4.1B, C).* It is conceivable that the enzymes required for this PTM are not present in the yeast and thus the SH2-D1-D2 region would mainly be in its closed conformation in yeast.

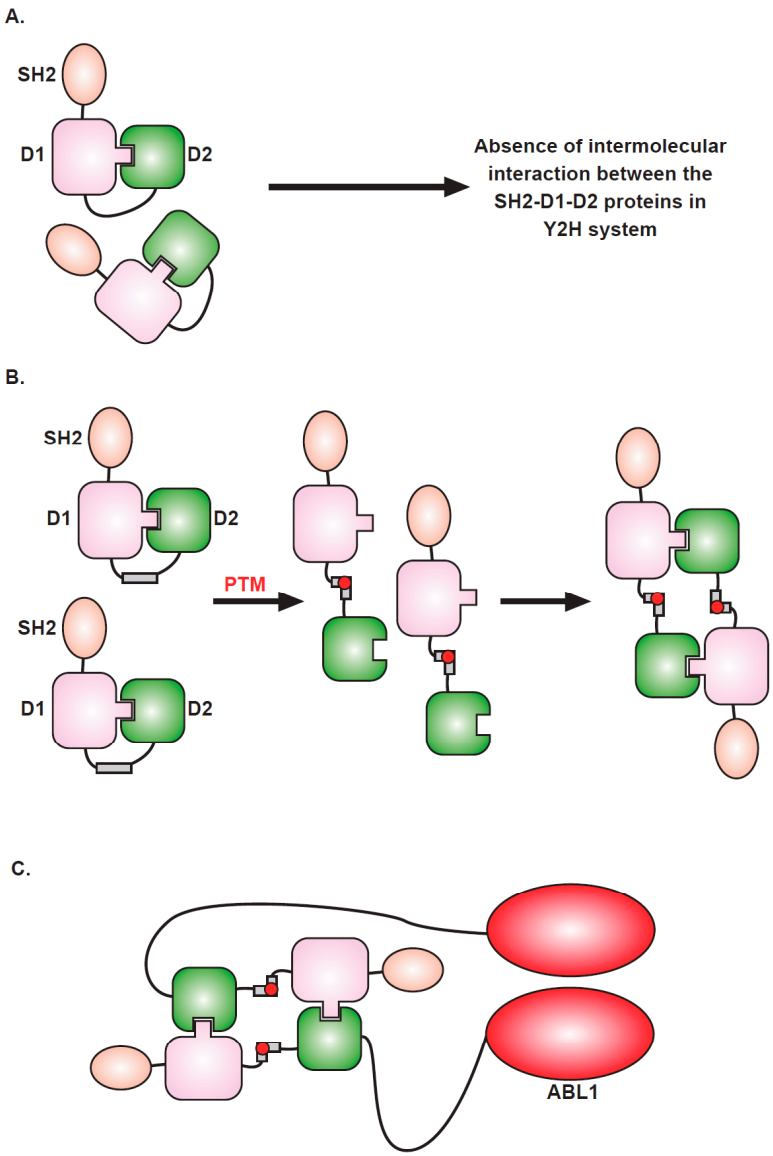


Figure 4.1: Protein interaction model explaining the difference between the Y2H and co-immunoprecipitation results: (A) model showing the intramolecular interaction within the isolated 5'SHIP1 protein, which would explain the absence of intermolecular interaction between the 5'SHIP1(SH2-D1-D2) proteins observed in the Y2H assays **(B)** Schematic representation of a possible post translational modification (PTM) of the N- terminal SHIP1 portion (SH2-D1-D2) regulating the intermolecular interaction between the full length SHIP1/ABL1 proteins in the mammalian system. **(C)** A model showing the interaction between the full-length SHIP1/ABL1 proteins, as observed in the co-immunoprecipitation experiments.

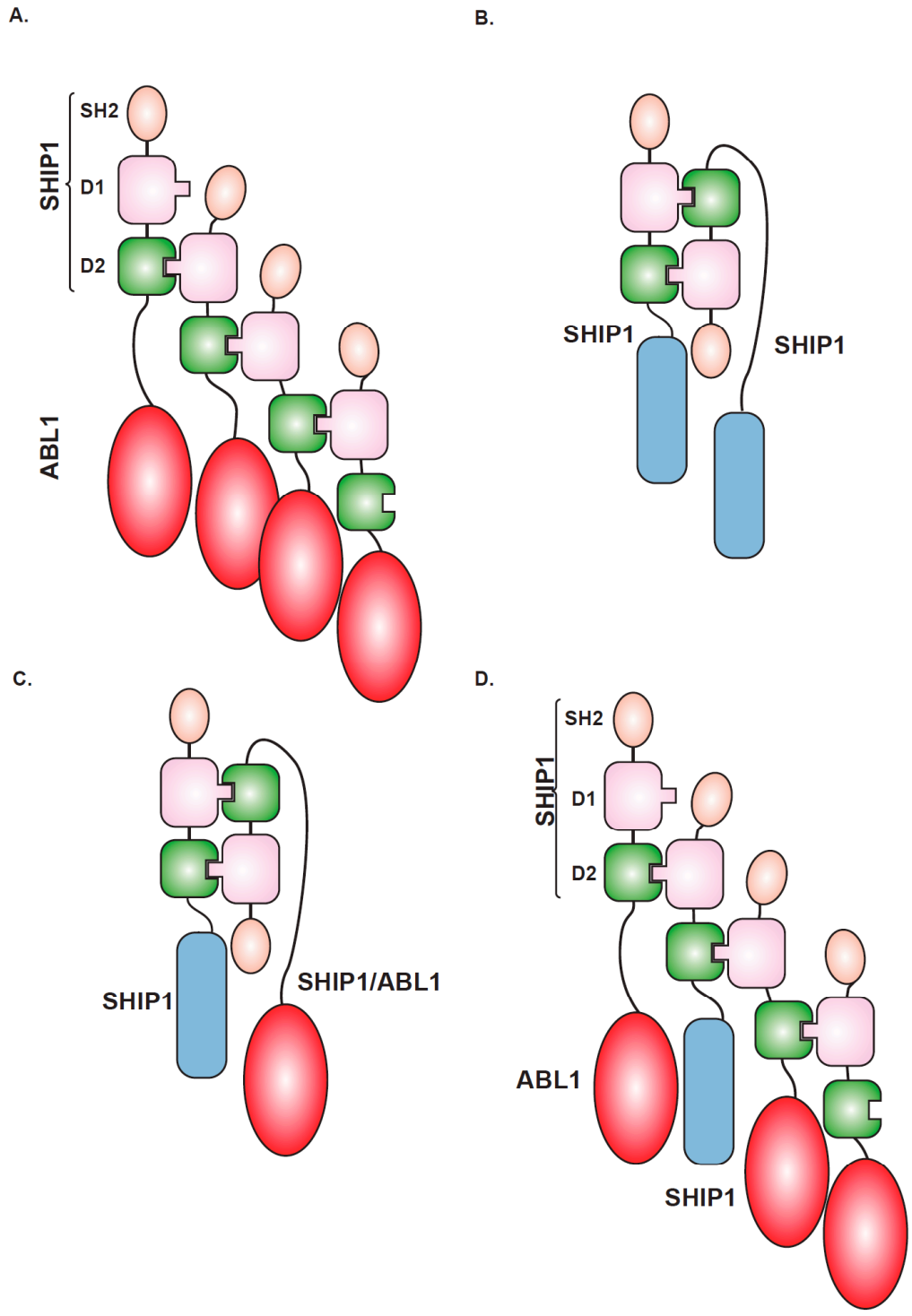


Figure 4.2: Models explaining possible protein interactions lead by the D1 and D2 domains within the N-terminal SHIP1 portion. Models showing (A) the homo-oligomerisation of the SHIP1/ABL1 fusion proteins, (B) the homo-dimerisation of SHIP1, (C) the hetero dimerisation and (D) the hetero-oligomerisation of the SHIP1-SHIP1/ABL1 proteins.

The interaction between the D1 and the D2 domains of SHIP1 was confirmed by co-immunoprecipitations in mammalian cells by co-expressing the Flag-tagged SHIP1-deltaD1/ABL1 and the HA-tagged SHIP1(D1)/ABL1 proteins ***(3.9.3.2, Figure 3.24 B)***. Thus, we demonstrated that, in addition to the SH2 domain, the SHIP1 portion of the SHIP1/ABL1 protein contains two other domains, which interact with each other and which are crucial for the dimerization/oligomerization of the SHIP1/ABL1 fusion protein.

The two protein domains D1 and D2 in the N-terminal SHIP1 portion would not only allow for the dimerization of the fusion protein *(Figure 4.1 C)* but also for an oligo- or multimerization *(Figure 4.2)*.

In addition to the formation of the homo-dimers of the SHIP1/ABL1 fusion proteins (Figure 4.1 C), the two protein domains D1 and D2 in the N-terminal SHIP1 portion might also allow for an oligo- or multimerization of the SHIP1/ABL1 protein (Figure 4.2 A), homodimerisation of the wild type SHIP1 (Figure 4.2 B) and hetero di- or oligomers of the wild type SHIP1 and SHIP1/ABL1 proteins (Figure 4.2 C, D).

4.7 D1 AND D2 ARE REQUIRED FOR IL3 INDEPENDENT PROLIFERATION OF BA/F3 CELLS

The importance of the domain 1 (D1) and the domain 2 (D2) was also demonstrated by *in vivo* assays in Ba/F3 cells using different deletion mutants of the SHIP1/ABL1 fusion protein. This included the deletion mutants lacking D1 or D2 or the SH2 domain. Among all the deletion mutants, only the SHIP1(D1-D2)/ABL1 fusion lacking the SH2 domain of SHIP1 was able to induce IL3 independent proliferation of Ba/F3 cells. Whereas Ba/F3 cells carrying the SHIP1(D1)/ABL1 or SHIP1(D2)/ABL1 fusions died after IL3 withdrawal. However, the SHIP1(D1-D2)/ABL1 showed lower proliferation potential compared to the BCR/ABL1 and SHIP1/ABL1 fusions. These results indicate that the SH2 domain does not contribute to the activation of the ABL1 kinase activity and that the domains D1 and D2 are necessary for the activation of the ABL1 kinase activity. However, we cannot exclude the possibility that the SH2 domain of SHIP1 also contributes in a certain way to factor independent growth in Ba/F3 cells.

In addition to the importance of D1 and D2 in the activation of the tyrosine kinase activity of ABL1, the interaction between the domains D1 and D2 in the N-terminal portion of SHIP1 suggests two interesting hypotheses for additional leukemogenic effects of the SHIP1/ABL1 fusion protein.

4.7.1 Hypothesis I: The SHIP1/ABL1 fusion acts as a dominant negative regulator of normal SHIP1 function

As discussed earlier, *SHIP1* functions as a negative regulator of myeloid cell proliferation and differentiation. The formation of the *SHIP1/ABL1* fusion gene, disrupts one of the *SHIP1* alleles resulting in a condition similar to *SHIP1* heterozygosity (+/-). The mouse models of *SHIP1* have shown that *SHIP1* heterozygosity is not associated with a disease phenotype. However, mice with a homozygous loss of *SHIP1* (-/-) failed to thrive and developed a myeloproliferative syndrome-like condition. As we demonstrate here that SHIP1 contains two protein interaction domains, it can be speculated that the interaction between different SHIP1 molecules in the form of di- or oligomers might be necessary for the regulation of SHIP1 function itself. On the other hand, the domains D1 and D2 are also important for the dimerization/oligomerization of the SHIP1/ABL1 fusion protein and thereby for the constitutive activation of the ABL1 kinase. Based on this background, it can be speculated that in addition to the formation of the SHIP1/ABL1 homo-dimers or homo-oligomers *(Figure 4.1 C, 4.2 A)* and SHIP1 homo-dimers *(Figure 4.2 B)* or homo-oligomers, the D1-D2 interaction could also result in the formation of the SHIP1/ABL1 - SHIP1 hetero-dimers or hetero-oligomers *(Figure 4.2 C and D)*. The formation of the SHIP1/ABL1 - SHIP1 hetero di- or oligodimers might interfere with the proper function of the SHIP1 protein *(Figure 4.3)*. Thus, the formation of the SHIP1/ABL1 fusion, in addition to the induction of the factor independent growth in the hematopoietic cells, disrupts not only one of the *SHIP1* alleles, but may also interfere with the activity of the normal SHIP1 protein through a dominant negative mechanism. Therefore, it is tempting to speculate that the *SHIP1/ABL1* fusion has two leukemogenic consequences: (1) activation of the ABL1 tyrosine kinase and (2) down regulation of normal SHIP1 function *(Figure 4.3)*.

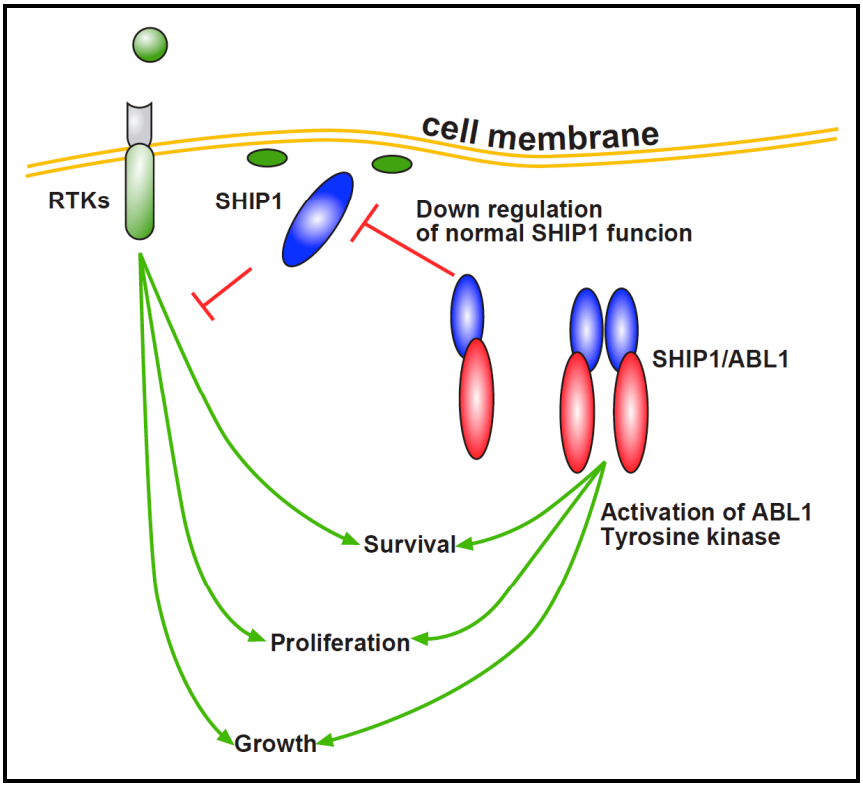


Figure 4.3: Pathways affected by the SHIP1/ABL1 fusion protein. Activation of an oncogene and interference with a tumor suppressor gene function by the SHIP1/ABL1 fusion.

4.7.2 Hypothesis II: D1 and D2 mediated inhibition of the ABL1 kinase activity of SHIP1/ABL1 by normal SHIP1

Considering the above hypothesis proposing a dominant negative effect of SHIP1/ABL1 over SHIP1, the inverse mechanism might also be possible. The wild-type SHIP1 protein might have a negative effect on the function of the SHIP1/ABL1 fusion protein. In this scenario, the normal SHIP1 protein would interfere with the formation of SHIP1/ABL1 di- or oligomers which are necessary for the activation of the tyrosine kinase function of the fusion protein. In this situation, the loss of the remaining non-rearranged *SHIP1* allele might provide the cells with an additional growth advantage.

This situation is reminiscent of the cases with *ETV6/ABL1* or *ETV6/AML1* fusion genes. In these cases, in addition to the disruption of one *ETV6* allele by the generation of the fusion gene, the other *ETV6* allele was found to be deleted, resulting in the loss of wild-type ETV6 function in the majority of cases with these fusion genes (Golub et al., 1995; Romana et al., 1995;

Golub et al., 1996). In addition, in the T-ALL cell line MT-ALL, which carries an *ETV6/ABL2* fusion, the non-rearranged *ETV6* allele has a point mutation in the DNA-binding domain (Griesinger et al., 2002). It should be noted however, that in the cases of ETV6/ABL1, ETV6/ABL2 and ETV6/AML1, the reason for the loss or inactivation of the non-rearranged ETV6 allele is believed to be the inactivation of the tumor suppressor function of ETV6 and not the interference of the wild-type ETV6 protein with the function of the ETV6 fusion proteins. But as noted earlier: there is even more evidence from the phenotype of the SHIP1 knockout mice than from the ETV6 knock-out mice that SHIP1 is a good candidate tumor suppressor gene.

To support the hypothesis of the dominant negative effect of SHIP1 over SHIP1/ABL1 or the dominant negative effect of SHIP1/ABL1 over SHIP1, further experiments are required. The second hypothesis would be supported by the detection of a deletion of the *SHIP1* locus in our patients. For this we performed FISH assays using SHIP1/ABL1-DCDF probes. The specificity of the FISH probes was confirmed by the hybridization on metaphase chromosomes from a normal male, but unfortunately, we could not obtain satisfactory hybridization results from the patient sample.

4.8 THE SHIP1/ABL1 FUSION IN CONTEXT OF OTHER GENETIC ABERRATIONS IN ALL

It is well known that almost always several genetic alterations are required to transform a normal cell into a cancer cell. There are, to our knowledge, no mutational events described that will transform on their own a normal cell into a cancer cell. Constitutively activated tyrosine kinases can confer a proliferative and survival advantage to hematopoietic progenitors and are sufficient to induce myeloproliferative disease, but usually require the co-operative effect of other mutations to induce acute leukemia (Kelly and Gilliland, 2002; Kelly et al., 2002). There are several well-studied recurring genetic lesions, like chromosomal translocations that lead to fusion genes or gene deregulation but also gene specific mutations, described in ALL. In B lineage ALL, which is the more common form of ALL, these include alteration of transcription factors such *ETV6*, *AML1*, *EBF1*, *PAX5*, *E2A* and *Ikaros*,

translocations affecting the *MLL* gene or tyrosine kinases like the activation of *ABL1* in the BCR/ABL1 fusion protein. In T lineage ALL these recurring alterations include gain-of-function mutations of *NOTCH1*, which is a transmembrane receptor playing an essential role in T cell development, rearrangements of the *TAL1* gene (Teitell and Pandolfi, 2008), or the *NUP214/ABL1* fusion (Graux et al., 2004). It is very obvious that several, if not all of the genes we find altered in ALL play important roles in lineage determination, differentiation, and growth of lymphoid cells. These genes are thus part of critical cellular pathways in lymphoid development. The establishment of murine models of leukemia with these alteration has also taught us, as mentioned above, that a single mutation (eg. like the ETV6/AML1 fusion) is frequently inadequate to initiate a full-blown leukemia, but rather that additional, so-called cooperating mutations are required (Teitell and Pandolfi, 2008). In this context it is important to note, that more than 80% of B-ALL with a BCR/ABL fusion have a deletion within the *IKZF1(IKAROS)* genes (Mullighan et al., 2008). However, cooperating mutations are not easy to find. It is therefore tempting to speculate that rare mutational events, like the rare SHIP1/ABL1 fusion, give important hints to pathways that are critical for leukemogenesis.

The available evidence indicates that activating mutations in the tyrosine kinases confer proliferative advantage to hematopoietic progenitors and cooperate with loss-of-function mutations in hematopoietic transcription factors to cause acute leukemia which is characterized by proliferation and impaired differentiation.

Let us take a closer look at the other known *ABL1* fusion in B cell ALL. About 20% of B-ALLs have the *BCR/ABL1* fusion gene. However, it is well known that additional mutations are required to synergize with the *BCR/ABL1* for ALL to develop. For example, deletions of *CDKN2A (INK4A-ARF, p16)*, a tumorsuppressor gene at 9p21, are found in 30% of the BCR-ABL1 positive ALLs (Heerema et al., 2004; Primo et al., 2005). Another frequent additional lesion in *BCR/ABL1* positive ALL are affecting the *IKZF1* locus, which encodes the nuclear protein IKAROS (Mullighan et al., 2008). It has been shown that haploinsufficiency, homozygous loss, or expression of a dominant

negative IKAROS isoform occurs in about 84% of the pediatric BCR-ABL1 positive ALL. Both, *CDKN2* and *IKAROS* are tumor suppressor genes. Mice with reduced IKAROS expression have a partial block at the pro-B cell stage in B cell development (Kirstetter et al., 2002).

It is quite obvious that the activation of the tyrosine kinase ABL1 in early B cells is not sufficient to cause an acute leukemia phenotype.

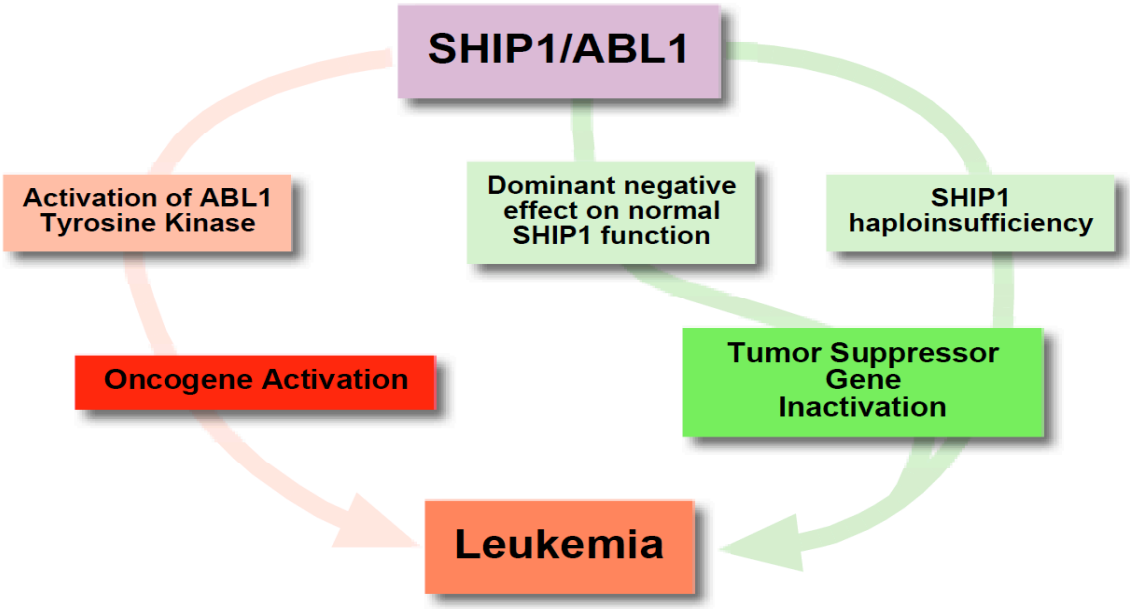


Figure 4.4: Summary of the potential mechanism of the SHIP1/ABL1 fusion to promote leukemogenesis.

While the activation of the ABL1 tyrosine kinase activity in the *SHIP1/ABL1* fusion, as explained earlier, induces IL3 independent growth in BaF3 cells, this alone is, with all likelihood, not sufficient to cause a full-blown leukemia. In this context, the interference with the tumor suppressor properties of *SHIP1* might play a crucial role in collaborating with tyrosine kinase activation of *ABL1*. As explained earlier, the formation of the *SHIP1/ABL1* fusion results in the inactivation of one *SHIP1* allele through the genomic rearrangement. Considering the down regulation of SHIP1 expression by BCR/ABL (Sattler et al., 1999), which was described in Ba/F3 cells, one can also speculate that it is advantages for a BCR/ABL1 transformed cell to reduce SHIP1 expression. In addition, it is very likely that SHIP1/ABL1 can also reduce the expression of

SHIP1. Thus taking into account the potential dominant negative effects of the SHIP1/ABL1 fusion protein on the normal SHIP1 protein and the loss of one SHIP1 allele during the formation of the SHIP1/ABL1 fusion gene, it can be hypothesized that the formation of the SHIP1/ABL1 fusion might not only activate important signaling pathways downstream of ABL1, but would also reduce the negative regulation which is normally exerted by SHIP1 on multiple cytokine and growth factor signaling pathways. The combination of both effects could be an extremely powerful force to cause leukemia *(Figure 4.4)*.

It is important to stress that with the SHIP1/ABL1 fusion an alteration of the SHIP1 gene has been reported for the first time in a malignancy. We believe that the tumor suppressor gene function of SHIP1 in B cell malignancy has not been recognized fully up to now.

5 Summary

In the past four decades, the study of chromosomal translocations in leukemia has given us tremendous insight into the mechanisms responsible for leukemogenesis. One of the most dramatic consequences of a chromosomal translocation is the formation of a fusion gene. *BCR/ABL1* is the most common fusion gene observed in leukemia. The *BCR/ABL1* fusion is the hallmark of Chronic Myeloid Leukemia (CML) and is frequently found in B-lineage Acute Lymphoblastic Leukemia (ALL). As a consequence of the *BCR/ABL1* fusion, the ABL1 tyrosine kinase is constitutively activated through the oligomerization of the fusion protein mediated by the coiled-coil domain of BCR. While the *BCR* gene is by far the most common fusion partner of ABL, there are 5 other, very rare fusion partners of ABL1: *ETV6*, *RCSD1*, *EML1*, *NUP214* and *ZMIZ1*. *ETV6*, *EML1* and *NUP214*, like *BCR*, contribute a dimerization/oligomerization domain for the constitutive activation of the ABL1 tyrosine kinase of their respective fusion proteins.

In this doctoral thesis, we describe a novel fusion of *ABL1* to the Inositol Polyphosphate-5 Phosphatase gene (*INPP5D* or *SHIP1* for SH2 containing inositol phosphatase), which we discovered in the leukemic cells of an 18-year-old woman with c-ALL. This SHIP1/ABL1 fusion was detected as an unexpected PCR amplification product during a routine RT-PCR diagnostic screening for *BCR/ABL* fusion transcripts. Sequence analysis of the amplification product revealed an in-frame fusion of the first 343 amino acids of *SHIP1* to the second exon of the *ABL1*. The presence of the *SHIP1/ABL1* fusion transcript in the patient's bone marrow cells was confirmed by RT-PCR using primers specific for the *SHIP1/ABL1* fusion transcript. The putative SHIP1/ABL1 fusion protein is predicted to contain an SH2 domain in the SHIP1 portion (amino acids 5 to 102) and an SH3, SH2, tyrosine kinase and an actin binding domain in the ABL1 portion. Of note, the SHIP1/ABL1 fusion has the same protein domains of ABL1 like the other ABL1 fusions.

In order to anlyze this novel fusion in more detail, we cloned the full length *SHIP1/ABL1* fusion open reading frame without tag or with HA or FLAG epitope tags into eukaryotic expression vectors. For this, the 5' portion of

SHIP1/ABL1 fusion till the *Kpn*1 site in ABL1 was amplified from the patient sample and then insertd into a pcDNA3-BCR/ABL construct replacing the *BCR/ABL* fusion up to the the *Kpn*1 site of *ABL1*. The expression of the SHIP1/ABL1 fusion protein was confirmed in transiently transfected HEK293T cells by Western blotting. To analyze the dimerization/oligomerization of the SHIP1/ABL1 fusion protein, HA-tagged and Flag-tagged versions of the SHIP1/ABL1 fusion protein were co-expressed in HEK293T cells. We were able to coimmunoprecipitate the HA-tagged SHIP1/ABL1 protein with the Flag-tagged SHIP1/ABL1 protein, confirming the dimerization/oligomerization kinase activation mechanism used by the SHIP1/ABL1 fusion. In addition, like the BCR/ABL1 fusion, the SHIP1/ABL1 fusion protein induces IL3 independent proliferation in a stably transduced murine pro-B cell line (Ba/F3). The IL3 independent growth induced in the Ba/F3 cells expressing the *SHIP1/ABL1* fusion was inhibited by the ABL1-specific tyrosine kinase inhibitor Imatinib in a dose dependent fashion, confirming that the ABL tyrosine kinase is activated in the SHIP/ABL1 fusion. Furthermore, we were able to map two protein domains (domain D1: aa 100 to 220 and domain D2: aa 220 to 343) in SHIP1 which are critical for the dimerization of the fusion protein and for the induction of the IL3 independent proliferation in the Ba/F3 cells.

As the *SHIP1* gene is located on 2q37 and transcribed from the centromere to the telomere like the *ABL1* gene on 9q34, we predicted that a simple balanced translocation generated the *SHIP1/ABL1* fusion gene on a derivative chromosome 2. But surprisingly, FISH analysis using a commercial BCR/ABL dual color dual fusion probe on metaphase chromosomes did not show an *ABL1* signal on chromosome 2. However, the same FISH probe, when hybridized to interphase nuclei, revealed two pairs of closely spaced *ABL1* signals. These findings suggest that an insertion of the 5' portion of *SHIP1* into both *ABL1* loci generated the *SHIP1/ABL1* fusion gene, possibly disrupting both *SHIP1* alleles. However, since we were unable to achieve adequate FISH signals with a SHIP1 probe, the true nature of the genomic rearrangement leading to the *SHIP1/ABL1* fusion still needs to be elucidated

SHIP1 removes the 5' phosphate from phosphatidylinositol (3,4,5)-trisphosphate and inositol-1,3,4,5-tetrakisphosphate, thereby negatively regulating multiple cellular signaling pathways. Interestingly, the expression of *SHIP1* is restricted to hematopoietic cells and it functions among other things as a negative regulator of myeloid cell proliferation and survival. *SHIP1* knock-out mice develop a myeloproliferative syndrome-like disease. Thus, it is tempting to speculate that the formation of the *SHIP1/ABL1* fusion, in addition to the activation of the *ABL1* tyrosine kinase function, also results in a haplo-insufficiency of *SHIP1*, which might contribute to cellular transformation by relieving proliferative pathways from negative regulation. Thus the *SHIP1/ABL1* fusion would not only generate an oncogene but would also compromise the function of the putative tumor suppressor gene *SHIP1*. Since our protein interaction studies have a revealed a previously unknown strong dimerization or oligomerization domain in SHIP1, it is also conceivable that the SHIP1/ABL1 fusion protein can function as a dominant negative protein for SHIP1. Of note, this is the first time that a mutation or alteration of the SHIP1 gene has been described in a malignancy.

The discovery of the *SHIP1/ABL1* fusion gene will enable us to dissect the pathways that lead to B-cell acute lymphoblastic leukemia in greater detail and will also give us the opportunity to understand better the mechanisms that underlie *BCR/ABL1* mediated leukemogenesis.

6 Zusammenfassung

In den letzten vier Jahrzehnten haben wir durch das Studium von Chromosomentranslokationen große Erkenntnisse über die Mechanismen gewonnen, die für die Leukämogeneses verantwortlich sind. Eine der dramatischsten Folgen einer Chromosomentranslokation ist die Bildung eines Fusionsgens. BCR/ABL ist das häufigste Fusionsgen, das bei Leukämien beobachtet wird. Die BCR/ABL1-Fusion ist typisch für the Chronisch Myeloische Leukämie (CML) und wird auch sehr häufig bei der Akuten B-Zell lymphoblastischen Leukämie (ALL) beobachtet. Als Folge der BCR/ABL1-Fusion kommte es zu einer konstitutiven Aktivierung der ABL1 Tyrosinekinaseaktivität durch die Oligomerisierung des Fusionsproteins, die durch die Coiled-coil Domäne von BCR bewirkt wird. BCR ist der häufigste Fusionspartner von ABL1. Es gibt jedoch noch 5 andere, sehr seltene Fusionspartner von *ABL1*: *ETV6*, *RCSD1*, *EML1*, *NUP214* und *ZMIZ1. ETV6*, *EML1* und *NUP214* tragen, wie BCR, durch eine Dimerisierungs- oder Oligomerisierungsdomäne zur konstitutiven Aktivierung der ABL1 Tyrosinkinaseaktivität in den jeweiligen Fusionsproteinen bei.

In dieser Doktorarbeit beschreiben wir eine neue *ABL1*-Fusion zum Inositol Polyphospate-5 Phosphastase Gen (*INPP5D* oder *SHIP1* für SH2-Domäne enthaltende Inositol Phosphatase), die wir in den Leukämiezellen einer 18 Jahre alten Frau mit c-ALL entdeckten. Diese *SHIP1/ABL1*-Fusion wurde bei einer Routine RT-PCR-Untersuchung für *BCR/ABL1*-Fusionstranskripte als unerwartetes Amplifikationsprodukt entdeckt. Die Sequenzierung dieses Amplifikationsprodukt zeigte eine im-Leserahmen Fusion der ersten 343 Aminosäuren von *SHIP1* mit dem zweiten Exon von *ABL1.* Das Vorhandensein des *SHIP1/ABL1* Fusionstranskripts in den Knochenmarkszellen der Patientin konnte durch eine RT-PCR mit für *SHIP1/ABL1* spezifischen Primern bestätigt werden. Das putative SHIP1/ABL1-Fusionsprotein enthält eine SH2-Domäne im SHIP1-Anteil (Aminosäuren (As) 5 bis 102) sowie eine SH3-, eine SH2-, eine Tyrosinkinase- und eine Actinbindungsdomäne im ABL1-Anteil. Es ist zu

beachten, dass das SHIP1/ABL1-Fusionsprotein dieselben Proteindomänen im ABL1-Anteil hat wie die anderen ABL1-Fusionen.

Um die neue Fusion im Detail zu untersuchen, klonierten wir den gesamten offenen Leserahmen von sowohl mit als auch ohne HA- oder Flag-Epitoptags in eukaryontische Expressionsvektoren. Hierfür wurde der 5'-Anteil von *SHIP1/ABL1* bis zur *Kpn*1 Schnittstelle in *ABL1* aus der Patientenprobe amplifiziert und dann in ein pcDNA1-BCR/ABL Konstrukt inseriert, wobei es die *BCR/ABL*-Fusion bis zur *Kpn*1 Schnittstelle in *ABL1* ersetzte. Die Proteinexpression der SHIP1/ABL1-Fusion wurde mittels Westernblotanalyse in transient transfizierten HEK293T Zellen bestätigt. Um die Dimerisierung bzw. Oligmerisierung des SHIP1/ABL1-Fusionsproteins zu analysieren, wurde die HA- und die Flag-markierten Versionen des SHIP1/ABL1-Fusionsproteins zusammen in HEK293T Zellen exprimiert. Wir waren dann in der Lage das HA-markierte SHIP1/ABL1-Fusionsprotein zusammen mit dem Flag-markierten SHIP1/ABL1-Fusionsprotein zu präzipitieren. Dieses Experiment bestätigte, dass auch bei *SHIP1/ABL1*-Fusion die Kinase durch einen Dimerisierungs- bzw. Oligomerisierungsmechanisms aktiviert wird. Weiterhin konnten wir zeigen, dass, genauso wie die *BCR/ABL1*-Fusion, die *SHIP1/ABL1*-Fusion IL3-unabhängiges Wachstum in einer stabil transduzierten murinen Pro-B Zelllinie (Ba/F3) induziert. Das IL3-unabhängige Wachstum, das in den SHIP1/ABL1 exprimierenden Ba/F3-Zellen induziert wurde, konnte durch den ABL1-spezifischen Tyrosinkinaseinhibitor Imatinib in einer Dosis-abhängigen Weise gehemmt werden. Zusätzlich waren wir in der Lage zwei Proteindomänen in SHIP1 zu kartieren (Domäne D1: As 100 bis 220 und Domäne D2: As 220 bis 343), die essentiell für die Dimerisierung des Fusionsprotein und für die Induktion von IL3-unabhängigem Wachstum sind.

Da das *SHIP1*-Gen in der Chromosomenbande 2q37 liegt und genauso wie das *ABL1*-Gen auf 9q34 vom Zentromer zum Telomer abgelesen wird, gingen wir davon aus, dass eine einfache balanzierte Translokation zur Bildung des *SHIP1/ABL1*-Fusionsgen auf dem derivativen Chromosom 2 führte. Überraschenderweise zeigte sich jedoch bei der FISH-Analyse auf Metaphasechromosomen mit einer kommerziell erhältlichen BCR/ABL-Dual Color Dual Fusion-Sonde kein -Signal auf einem Chromosom 2. Jedoch

ergab dieselbe FISH-Sonde, als sie auf Interphasekerne hybridisiert wurde, zwei Paare von dicht beeinanderliegenden *ABL1*-Signalen. Diese Ergebnisse legten den Schluss nahe, dass das *SHIP1/ABL1*-Fusionsgen durch eine Insertion des 5'-Anteils des *SHIP1*-Gens in beide *ABL1*-Loci zustande gekommen war, was möglicherweise zu einer Veränderung oder Inaktivierung beider *SHIP1*-Loci geführt hat. Da wir jedoch nicht in der Lage waren adäquate FISH-Signale mit eine *SHIP1*-Sonde bei den Zellen unserer Patientin zu erzeugen, muß die genaue Art des genomischen Rearrangements, die zur *SHIP1/ABL1*-Fusion führte noch genauer untersucht werden.

Dadurch dass das SHIP1-Enzym 5' Phosphatreste von Phoshpoinositol(3,4,5)-triphosphat und Inositol-(1,2,4,5)-tetrakisphosphate entfernt, reguliert es negative viele zelluläre Signaltransduktionswege. Interessanterweise ist die Expression von *SHIP1* auf hämatopoetisches Gewebe beschränkt. Hier fungiert SHIP1 unter anderem als ein negativer Regulator der Proliferation und der Überlebens von myeloischen Zellen. *SHIP1* knock-out Mäuse enwickeln eine Erkrankung, die an ein myeloproliferatives Syndrom erinnert. Es ist somit naheliegend anzunehmen, dass die Bildung der *SHIP1/ABL1*-Fusion zusätzlich zur konstitutiven Aktivierung der Tyrosinkinaseaktivität von ABL1, auch zu einer Haploinsuffizienz von *SHIP1* führt, die ebenfalls zur malignen Transformation durch eine verminderte Hemmung von proliferativen Signalwegen beiträgt. In diesem Sinne würde die *SHIP1/ABL1*-Fusion nicht nur zur Bildung eines Onkogens führen, sondern würde zusätzlich die Funktion des mutmaßlichen Tumorsuppressorgens *SHIP1* beeinträchtigen. Da unsere Proteininteraktionsstudien bisher unbekannte starke Dimerisierungs- oder Oligomerisierungsdomänen in SHIP1 selbst identifizierten, ist es ebenfalls denkbar, dass das SHIP1/ABL1-Fusionprotein zusätzlich als dominant-negativer Regulator der normalen SHIP1-Funktion fungiert. Es muß betont werden, dass unsere Arbeit zum ersten Mal eine Mutation oder Veränderung des *SHIP1*-Gens bei einer Tumorerkrankung beschreibt.

Die Entdeckung des *SHIP1/ABL1*-Fusionsgens wird es uns nicht nur ermöglichen, die Mechanismen, die zur Entstehung der B-Zell ALL führen

detailliert zu analysieren, sondern wird uns zusätzlich die Möglichkeit eröffnen, die BCR/ABL-mediierte Leukämogenese besser zu verstehen.

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APPENDIX I: Sequence of the SHIP1/ABL1 fusion

The nucleotides highlighted in yellow denote the (genbank sequence) and that highlighted in blue denote the sequence used in this work (as per the sequencing of the pMIG-SA-9A6a) at the place of nucleotide differences. The green highlight denotes the **Kpn1(1488)** site. The red highlight indicated the single amino acid change found in the ABL1 sequence. The breakpoint is indicated by an arrow. nt no.: nucleotide position; nt seq:nucleotide sequence; aa: amino acids; '*': STOP codon

```
         10         20         30         40         50         60 ← nt no.
AAACAGGAAG TCAGTCAGTT AAGCTGGTGG CAGCAGCCGA GGCCACCAAG AGGCAACGGG ← nt seq

         70         80         90        100        110        120
CGGCAGGTTG CAGTGGAGGG GCCTCCGCTC CCCTCGGTGG TGTGTGGGTC CTGGGGGTGC

        130        140        150          160        170
CTGCCGGCCC GGCCGAGGAG GCCCACGCCC ACC ATG GTC CCC TGC TGG AAC CAT
                                      M   V   P   C   W   N   H> ← aa

    180          190          200          210          220
GGC AAC ATC ACC CGC TCC AAG GCG GAG GAG CTG CTT TCC AGG ACA GGC
 G   N   I   T   R   S   K   A   E   E   L   L   S   R   T   G>

        230          240          250          260          270
AAG GAC GGG AGC TTC CTC GTG CGT GCC AGC GAG TCC ATC TCC CGG GCA
 K   D   G   S   F   L   V   R   A   S   E   S   I   S   R   A>

          280          290          300          310
TAC GCG CTC TGC GTG CTG TAT CGG AAT TGC GTT TAC ACT TAC AGA ATT
 Y   A   L   C   V   L   Y   R   N   C   V   Y   T   Y   R   I>

320          330          340          350          360
CTG CCC AAT GAA GAT GAT AAA TTC ACT GTT CAG GCA TCC GAA GGC GTC
 L   P   N   E   D   D   K   F   T   V   Q   A   S   E   G   V>

  370          380          390          400          410
TCC ATG AGG TTC TTC ACC AAG CTG GAC CAG CTC ATC GAG TTT TAC AAG
 S   M   R   F   F   T   K   L   D   Q   L   I   E   F   Y   K>

    420          430          440          450          460
AAG GAA AAC ATG GGG CTG GTG ACC CAT CTG CAA TAC CCT GTG CCG CTG
 K   E   N   M   G   L   V   T   H   L   Q   Y   P   V   P   L>

        470          480          490          500          510
GAG GAA GAG GAC ACA GGC GAC GAC CCT GAG GAG GAC ACA GTA GAA AGT
 E   E   E   D   T   G   D   D   P   E   E   D   T   V   E   S>

          520          530          540          550
GTC GTG TCT CCA CCC GAG CTG CCC CCA AGA AAC ATC CCG CTG ACT GCC
 V   V   S   P   P   E   L   P   P   R   N   I   P   L   T   A>
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560          570          580          590          600
 AGC TCC TGT GAG GCC AAG GAG GTT CCT TTT TCA AAC GAG AAT CCC CGA
  S   S   C   E   A   K   E   V   P   F   S   N   E   N   P   R>

                                                  G
   610          620          630          640          650
 GCG ACC GAG ACC AGC CGG CCG AGC CTC TCC GAG ACA TTG TTC CAG CGA
  A   T   E   T   S   R   P   S   L   S   E   T   L   F   Q   R>

     660          670          680          690          700
 CTG CAA AGC ATG GAC ACC AGT GGG CTT CCA GAA GAG CAT CTT AAG GCC
  L   Q   S   M   D   T   S   G   L   P   E   E   H   L   K   A>

        710          720          730          740          750
 ATC CAA GAT TAT TTA AGC ACT CAG CTC GCC CAG GAC TCT GAA TTT GTG
  I   Q   D   Y   L   S   T   Q   L   A   Q   D   S   E   F   V>

           760          770          780          790
 AAG ACA GGG TCC AGC AGT CTT CCT CAC CTG AAG AAA CTG ACC ACA CTG
  K   T   G   S   S   S   L   P   H   L   K   K   L   T   T   L>

800          810          820          830          840
 CTC TGC AAG GAG CTC TAT GGA GAA GTC ATC CGG ACC CTC CCA TCC CTG
  L   C   K   E   L   Y   G   E   V   I   R   T   L   P   S   L>

   850          860          870          880          890
 GAG TCT CTG CAG AGG TTA TTT GAC CAG CAG CTC TCC CCG GGC CTC CGT
  E   S   L   Q   R   L   F   D   Q   Q   L   S   P   G   L   R>

     900          910          920          930          940
 CCA CGT CCT CAG GTT CCT GGT GAG GCC AAT CCC ATC AAC ATG GTG TCC
  P   R   P   Q   V   P   G   E   A   N   P   I   N   M   V   S>

        950          960          970          980          990
 AAG CTC AGC CAA CTG ACA AGC CTG TTG TCA TCC ATT GAA GAC AAG GTC
  K   L   S   Q   L   T   S   L   L   S   S   I   E   D   K   V>

          1000         1010         1020         1030
 AAG GCC TTG CTG CAC GAG GGT CCT GAG TCT CCG CAC CGG CCC TCC CTT
  K   A   L   L   H   E   G   P   E   S   P   H   R   P   S   L>

1040         1050         1060         1070         1080
 ATC CCT CCA GTC ACC TTT GAG GTG AAG GCA GAG TCT CTG GGG ATT CCT
  I   P   P   V   T   F   E   V   K   A   E   S   L   G   I   P>

  1090         1100         1110         1120         1130
 CAG AAA ATG CAG CTC AAA GTC GAC GTT GAG TCT GGG AAA CTG ATC ATT
  Q   K   M   Q   L   K   V   D   V   E   S   G   K   L   I   I>
1140         1150         1160         1170         1180
 AAG AAG TCC AAG GAT GGT TCT GAG GAC AAG TTC TAC AGC CAC AAG AAA
  K   K   S   K   D   G   S   E   D   K   F   Y   S   H   K   K>
 <____________1 to 1183 of SHIP1 (NM001017915)_________________>
```

Breakpoint

```
 1183      1190         1200          1210         1220         1230
 A AA GCC CTT CAG CGG CCA GTA GCA TCT GAC TTT GAG CCT CAG GGT CTG
  K>
      A   L   Q   R   P   V   A   S   D   F   E   P   Q   G   L>
   ___________576 to 5881 of ABL1B  (NM_0073132)_________________>
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          G
          1240         1250         1260         1270
  AGT GAA GCC GCT CGT TGG AAC TCC AAG GAA AAC CTT CTC GCT GGA CCC
   S   E   A   A   R   W   N   S   K   E   N   L   L   A   G   P>

1280         1290         1300         1310         1320
  AGT GAA AAT GAC CCC AAC CTT TTC GTT GCA CTG TAT GAT TTT GTG GCC
   S   E   N   D   P   N   L   F   V   A   L   Y   D   F   V   A>

  1330         1340         1350         1360         1370
  AGT GGA GAT AAC ACT CTA AGC ATA ACT AAA GGT GAA AAG CTC CGG GTC
   S   G   D   N   T   L   S   I   T   K   G   E   K   L   R   V>

    1380         1390         1400         1410         1420
  TTA GGC TAT AAT CAC AAT GGG GAA TGG TGT GAA GCC CAA ACC AAA AAT
   L   G   Y   N   H   N   G   E   W   C   E   A   Q   T   K   N>

      1430         1440         1450         1460         1470
  GGC CAA GGC TGG GTC CCA AGC AAC TAC ATC ACG CCA GTC AAC AGT CTG
   G   Q   G   W   V   P   S   N   Y   I   T   P   V   N   S   L>

          1480         1490         1500         1510
  GAG AAA CAC TCC TGG TAC CAT GGG CCT GTG TCC CGC AAT GCC GCT GAG
   E   K   H   S   W   Y   H   G   P   V   S   R   N   A   A   E>

1520         1530         1540         1550         1560
  TAT CTG CTG AGC AGC GGG ATC AAT GGC AGC TTC TTG GTG CGT GAG AGT
   Y   L   L   S   S   G   I   N   G   S   F   L   V   R   E   S>

  1570         1580         1590         1600         1610
  GAG AGC AGT CCT GGC CAG AGG TCC ATC TCG CTG AGA TAC GAA GGG AGG
   E   S   S   P   G   Q   R   S   I   S   L   R   Y   E   G   R>

    1620         1630         1640         1650         1660
  GTG TAC CAT TAC AGG ATC AAC ACT GCT TCT GAT GGC AAG CTC TAC GTC
   V   Y   H   Y   R   I   N   T   A   S   D   G   K   L   Y   V>

      1670         1680         1690         1700         1710
  TCC TCC GAG AGC CGC TTC AAC ACC CTG GCC GAG TTG GTT CAT CAT CAT
   S   S   E   S   R   F   N   T   L   A   E   L   V   H   H   H>

1720         1730         1740         1750
  TCA ACG GTG GCC GAC GGG CTC ATC ACC ACG CTC CAT TAT CCA GCC CCA
   S   T   V   A   D   G   L   I   T   T   L   H   Y   P   A   P>

1760         1770         1780         1790         1800
  AAG CGC AAC AAG CCC ACT GTC TAT GGT GTG TCC CCC AAC TAC GAC AAG
   K   R   N   K   P   T   V   Y   G   V   S   P   N   Y   D   K>

  1810         1820         1830         1840         1850
  TGG GAG ATG GAA CGC ACG GAC ATC ACC ATG AAG CAC AAG CTG GGC GGG
   W   E   M   E   R   T   D   I   T   M   K   H   K   L   G   G>

    1860         1870         1880         1890         1900
  GGC CAG TAC GGG GAG GTG TAC GAG GGC GTG TGG AAG AAA TAC AGC CTG
   G   Q   Y   G   E   V   Y   E   G   V   W   K   K   Y   S   L>

      1910         1920         1930         1940         1950
  ACG GTG GCC GTG AAG ACC TTG AAG GAG GAC ACC ATG GAG GTG GAA GAG
   T   V   A   V   K   T   L   K   E   D   T   M   E   V   E   E>
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          1960         1970         1980         1990
  TTC TTG AAA GAA GCT GCA GTC ATG AAA GAG ATC AAA CAC CCT AAC CTG
   F   L   K   E   A   A   V   M   K   E   I   K   H   P   N   L>

2000         2010         2020         2030         2040
  GTG CAG CTC CTT GGG GTC TGC ACC CGG GAG CCC CCG TTC TAT ATC ATC
   V   Q   L   L   G   V   C   T   R   E   P   P   F   Y   I   I>

  2050         2060         2070         2080         2090
  ACT GAG TTC ATG ACC TAC GGG AAC CTC CTG GAC TAC CTG AGG GAG TGC
   T   E   F   M   T   Y   G   N   L   L   D   Y   L   R   E   C>

    2100         2110         2120         2130         2140
  AAC CGG CAG GAG GTG AAC GCC GTG GTG CTG CTG TAC ATG GCC ACT CAG
   N   R   Q   E   V   N   A   V   V   L   L   Y   M   A   T   Q>

      2150         2160         2170         2180         2190
  ATC TCG TCA GCC ATG GAG TAC CTG GAG AAG AAA AAC TTC ATC CAC AGA
   I   S   S   A   M   E   Y   L   E   K   K   N   F   I   H   R>

         2200         2210         2220         2230
  GAT CTT GCT GCC CGA AAC TGC CTG GTA GGG GAG AAC CAC TTG GTG AAG
   D   L   A   A   R   N   C   L   V   G   E   N   H   L   V   K>

2240         2250         2260         2270         2280
  GTA GCT GAT TTT GGC CTG AGC AGG TTG ATG ACA GGG GAC ACC TAC ACA
   V   A   D   F   G   L   S   R   L   M   T   G   D   T   Y   T>

  2290         2300         2310         2320         2330
  GCC CAT GCT GGA GCC AAG TTC CCC ATC AAA TGG ACT GCA CCC GAG AGC
   A   H   A   G   A   K   F   P   I   K   W   T   A   P   E   S>

    2340         2350         2360         2370         2380
  CTG GCC TAC AAC AAG TTC TCC ATC AAG TCC GAC GTC TGG GCA TTT GGA
   L   A   Y   N   K   F   S   I   K   S   D   V   W   A   F   G>

      2390         2400         2410         2420         2430
  GTA TTG CTT TGG GAA ATT GCT ACC TAT GGC ATG TCC CCT TAC CCG GGA
   V   L   L   W   E   I   A   T   Y   G   M   S   P   Y   P   G>

         2440         2450         2460         2470
  ATT GAC CTG TCC CAG GTG TAT GAG CTG CTA GAG AAG GAC TAC CGC ATG
   I   D   L   S   Q   V   Y   E   L   L   E   K   D   Y   R   M>

2480         2490         2500         2510         2520
  GAG CGC CCA GAA GGC TGC CCA GAG AAG GTC TAT GAA CTC ATG CGA GCA
   E   R   P   E   G   C   P   E   K   V   Y   E   L   M   R   A>

  2530         2540         2550         2560         2570
  TGT TGG CAG TGG AAT CCC TCT GAC CGG CCC TCC TTT GCT GAA ATC CAC
   C   W   Q   W   N   P   S   D   R   P   S   F   A   E   I   H>

    2580         2590         2600         2610         2620
  CAA GCC TTT GAA ACA ATG TTC CAG GAA TCC AGT ATC TCA GAC GAA GTG
   Q   A   F   E   T   M   F   Q   E   S   S   I   S   D   E   V>

      2630         2640         2650         2660         2670
  GAA AAG GAG CTG GGG AAA CAA GGC GTC CGT GGG GCT GTG AGT ACC TTG
   E   K   E   L   G   K   Q   G   V   R   G   A   V   S   T   L>
```

```
          2680         2690         2700         2710
  CTG CAG GCC CCA GAG CTG CCC ACC AAG ACG AGG ACC TCC AGG AGA GCT
   L   Q   A   P   E   L   P   T   K   T   R   T   S   R   R   A>

2720         2730         2740         2750         2760
  GCA GAG CAC AGA GAC ACC ACT GAC GTG CCT GAG ATG CCT CAC TCC AAG
   A   E   H   R   D   T   T   D   V   P   E   M   P   H   S   K>

   2770         2780         2790         2800         2810
  GGC CAG GGA GAG AGC GAT CCT CTG GAC CAT GAG CCT GCC GTG TCT CCA
   G   Q   G   E   S   D   P   L   D   H   E   P   A   V   S   P>

     2820         2830         2840         2850         2860
  TTG CTC CCT CGA AAA GAG CGA GGT CCC CCG GAG GGC GGC CTG AAT GAA
   L   L   P   R   K   E   R   G   P   P   E   G   G   L   N   E>

       2870         2880         2890         2900         2910
  GAT GAG CGC CTT CTC CCC AAA GAC AAA AAG ACC AAC TTG TTC AGC GCC
   D   E   R   L   L   P   K   D   K   K   T   N   L   F   S   A>

          2920         2930         2940         2950
  TTG ATC AAG AAG AAG AAG AAG ACA GCC CCA ACC CCT CCC AAA CGC AGC
   L   I   K   K   K   K   K   T   A   P   T   P   P   K   R   S>

2960         2970         2980         2990         3000
  AGC TCC TTC CGG GAG ATG GAC GGC CAG CCG GAG CGC AGA GGG GCC GGC
   S   S   F   R   E   M   D   G   Q   P   E   R   R   G   A   G>

   3010         3020         3030         3040         3050
  GAG GAA GAG GGC CGA GAC ATC AGC AAC GGG GCA CTG GCT TTC ACC CCC
   E   E   E   G   R   D   I   S   N   G   A   L   A   F   T   P>

     3060         3070         3080         3090         3100
  TTG GAC ACA GCT GAC CCA GCC AAG TCC CCA AAG CCC AGC AAT GGG GCT
   L   D   T   A   D   P   A   K   S   P   K   P   S   N   G   A>

       3110         3120         3130         3140         3150
  GGG GTC CCC AAT GGA GCC CTC CGG GAG TCC GGG GGC TCA GGC TTC CGG
   G   V   P   N   G   A   L   R   E   S   G   G   S   G   F   R>

          3160         3170         3180         3190
  TCT CCC CAC CTG TGG AAG AAG TCC AGC ACG CTG ACC AGC AGC CGC CTA
   S   P   H   L   W   K   K   S   S   T   L   T   S   S   R   L>

3200         3210         3220         3230         3240
  GCC ACC GGC GAG GAG GAG GGC GGT GGC AGC TCC AGC AAG CGC TTC CTG
   A   T   G   E   E   E   G   G   G   S   S   S   K   R   F   L>

   3250         3260         3270         3280         3290
  CGC TCT TGC TCC GCC TCC TGC GTT CCC CAT GGG GCC AAG GAC ACG GAG
   R   S   C   S   A   S   C   V   P   H   G   A   K   D   T   E>

     3300         3310         3320         3330         3340
  TGG AGG TCA GTC ACG CTG CCT CGG GAC TTG CAG TCC ACG GGA AGA CAG
   W   R   S   V   T   L   P   R   D   L   Q   S   T   G   R   Q>

       3350         3360         3370         3380         3390
  TTT GAC TCG TCC ACA TTT GGA GGG CAC AAA AGT GAG AAG CCG GCT CTG
   F   D   S   S   T   F   G   G   H   K   S   E   K   P   A   L>
```

```
          3400         3410         3420         3430
  CCT CGG AAG AGG GCA GGG GAG AAC AGG TCT GAC CAG GTG ACC CGA GGC
    P   R   K   R   A   G   E   N   R   S   D   Q   V   T   R   G>

3440         3450         3460         3470         3480
  ACA GTA ACG CCT CCC CCC AGG CTG GTG AAA AAG AAT GAG GAA GCT GCT
    T   V   T   P   P   P   R   L   V   K   K   N   E   E   A   A>

   3490         3500         3510         3520         3530
  GAT GAG GTC TTC AAA GAC ATC ATG GAG TCC AGC CCG GGC TCC AGC CCG
    D   E   V   F   K   D   I   M   E   S   S   P   G   S   S   P>

     3540         3550         3560         3570         3580
  CCC AAC CTG ACT CCA AAA CCC CTC CGG CGG CAG GTC ACC GTG GCC CCT
    P   N   L   T   P   K   P   L   R   R   Q   V   T   V   A   P>

       3590         3600         3610         3620         3630
  GCC TCG GGC CTC CCC CAC AAG GAA GAA GCT GGA AAG GGC AGT GCC TTA
    A   S   G   L   P   H   K   E   E   A   G   K   G   S   A   L>

          3640         3650         3660         3670
  GGG ACC CCT GCT GCA GCT GAG CCA GTG ACC CCC ACC AGC AAA GCA GGC
    G   T   P   A   A   A   E   P   V   T   P   T   S   K   A   G>

3680         3690         3700         3710         3720
  TCA GGT GCA CCA GGG GGC ACC AGC AAG GGC CCC GCC GAG GAG TCC AGA
    S   G   A   P   G   G   T   S   K   G   P   A   E   E   S   R>

   3730         3740         3750         3760         3770
  GTG AGG AGG CAC AAG CAC TCC TCT GAG TCG CCA GGG AGG GAC AAG GGG
    V   R   R   H   K   H   S   S   E   S   P   G   R   D   K   G>

     3780         3790         3800         3810         3820
  AAA TTG TCC AGG CTC AAA CCT GCC CCG CCG CCC CCA CCA GCA GCC TCT
    K   L   S   R   L   K   P   A   P   P   P   P   P   A   A   S>

       3830         3840         3850         3860         3870
  GCA GGG AAG GCT GGA GGA AAG CCC TCG CAG AGC CCG AGC CAG GAG GCG
    A   G   K   A   G   G   K   P   S   Q   S   P   S   Q   E   A>

          3880         3890         3900         3910
  GCC GGG GAG GCA GTC CTG GGC GCA AAG ACA AAA GCC ACG AGT CTG GTT
    A   G   E   A   V   L   G   A   K   T   K   A   T   S   L   V>

3920         3930         3940         3950         3960
  GAT GCT GTG AAC AGT GAC GCT GCC AAG CCC AGC CAG CCG GGA GAG GGC
    D   A   V   N   S   D   A   A   K   P   S   Q   P   G   E   G>

   3970         3980         3990         4000         4010
  CTC AAA AAG CCC GTG CTC CCG GCC ACT CCA AAG CCA CAG TCC GCC AAG
    L   K   K   P   V   L   P   A   T   P   K   P   Q   S   A   K>

     4020         4030         4040         4050         4060
  CCG TCG GGG ACC CCC ATC AGC CCA GCC CCC GTT CCC TCC ACG TTG CCA
    P   S   G   T   P   I   S   P   A   P   V   P   S   T   L   P>

       4070         4080         4090         4100         4110
  TCA GCA TCC TCG GCC CTG GCA GGG GAC CAG CCG TCT TCC ACC GCC TTC
    S   A   S   S   A   L   A   G   D   Q   P   S   S   T   A   F>
```

```
          4120         4130         4140         4150
  ATC CCT CTC ATA TCA ACC CGA GTG TCT CTT CGG AAA ACC CGC CAG CCT
   I   P   L   I   S   T   R   V   S   L   R   K   T   R   Q   P>

4160         4170         4180         4190         4200
  CCA GAG CGG ATC GCC AGC GGC GCC ATC ACC AAG GGC GTG GTC CTG GAC
   P   E   R   I   A   S   G   A   I   T   K   G   V   V   L   D>

  4210         4220         4230         4240         4250
  AGC ACC GAG GCG CTG TGC CTC GCC ATC TCT AGG AAC TCC GAG CAG ATG
   S   T   E   A   L   C   L   A   I   S   R   N   S   E   Q   M>

                                                          T
    4260         4270         4280         4290         4300
  GCC AGC CAC AGC GCA GTG CTG GAG GCC GGC AAA AAC CTC TAC ACG TTC
   A   S   H   S   A   V   L   E   A   G   K   N   L   Y   T   F>
                                                          ↓
                                                          S

      4310         4320         4330         4340         4350
  TGC GTG AGC TAT GTG GAT TCC ATC CAG CAA ATG AGG AAC AAG TTT GCC
   C   V   S   Y   V   D   S   I   Q   Q   M   R   N   K   F   A>

          4360         4370         4380         4390
  TTC CGA GAG GCC ATC AAC AAA CTG GAG AAT AAT CTC CGG GAG CTT CAG
   F   R   E   A   I   N   K   L   E   N   N   L   R   E   L   Q>

4400         4410         4420         4430         4440
  ATC TGC CCG GCG ACA GCA GGC AGT GGT CCA GCG GCC ACT CAG GAC TTC
   I   C   P   A   T   A   G   S   G   P   A   A   T   Q   D   F>

  4450         4460         4470         4480         4490
  AGC AAG CTC CTC AGT TCG GTG AAG GAA ATC AGT GAC ATA GTG CAG AGG
   S   K   L   L   S   S   V   K   E   I   S   D   I   V   Q   R>

    4500       4510       4520       4530       4540       4550
  TAG CAG CAGTCAGGGG TCAGGTGTCA GGCCCGTCGG AGCTGCCTGC AGCACATGCG
   *>

      4560       4570       4580       4590       4600       4610
  GGCTCGCCCA TACCCGTGAC AGTGGCTGAC AAGGGACTAG TGAGTCAGCA CCTTGGCCCA

      4620       4630       4640       4650       4660       4670
  GGAGCTCTGC GCCAGGCAGA GCTGAGGGCC CTGTGGAGTC CAGCTCTACT ACCTACGTTT

      4680       4690       4700       4710       4720       4730
  GCACCGCCTG CCCTCCCGCA CCTTCCTCCT CCCCGCTCCG TCTCTGTCCT CGAATTTTAT

      4740       4750       4760       4770       4780       4790
  CTGTGGAGTT CCTGCTCCGT GGACTGCAGT CGGCATGCCA GGACCCGCCA GCCCCGCTCC

      4800       4810       4820       4830       4840       4850
  CACCTAGTGC CCCAGACTGA GCTCTCCAGG CCAGGTGGGA ACGGCTGATG TGGACTGTCT

      4860       4870       4880       4890       4900       4910
  TTTTCATTTT TTTCTCTCTG GAGCCCCTCC TCCCCCGGCT GGGCCTCCTT CTTCCACTTC

      4920       4930       4940       4950       4960       4970
  TCCAAGAATG GAAGCCTGAA CTGAGGCCTT GTGTGTCAGG CCCTCTGCCT GCACTCCCTG
```

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      4980       4990       5000       5010       5020       5030
GCCTTGCCCG TCGTGTGCTG AAGACATGTT TCAAGAACCG CATTTCGGGA AGGGCATGCA

      5040       5050       5060       5070       5080       5090
CGGGCATGCA CACGGCTGGT CACTCTGCCC TCTGCTGCTG CCCGGGGTGG GGTGCACTCG

      5100       5110       5120       5130       5140       5150
CCATTTCCTC ACGTGCAGGA CAGCTCTTGA TTTGGGTGGA AAACAGGGTG CTAAAGCCAA

      5160       5170       5180       5190       5200       5210
CCAGCCTTTG GGTCCTGGGC AGGTGGGAGC TGAAAAGGAT CGAGGCATGG GGCATGTCCT

      5220       5230       5240       5250       5260       5270
TTCCATCTGT CCACATCCCC AGAGCCCAGC TCTTGCTCTC TTGTGACGTG CACTGTGAAT

      5280       5290       5300       5310       5320       5330
CCTGGCAAGA AAGCTTGAGT CTCAAGGGTG GCAGGTCACT GTCACTGCCG ACATCCCTCC

      5340       5350       5360       5370       5380       5390
CCCAGCAGAA TGGAGGCAGG GGACAAGGGA GGCAGTGGCT AGTGGGGTGA ACAGCTGGTG

      5400       5410       5420       5430       5440       5450
CCAAATAGCC CCAGACTGGG CCCAGGCAGG TCTGCAAGGG CCCAGAGTGA ACCGTCCTTT

      5460       5470       5480       5490       5500       5510
CACACATCTG GGTGCCCTGA AAGGGCCCTT CCCCTCCCCC ACTCCTCTAA GACAAAGTAG

      5520       5530       5540       5550       5560       5570
ATTCTTACAA GGCCCTTTCC TTTGGAACAA GACAGCCTTC ACTTTTCTGA GTTCTTGAAG

      5580       5590       5600       5610       5620       5630
CATTTCAAAG CCCTGCCTCT GTGTAGCCGC CCTGAGAGAG AATAGAGCTG CCACTGGGCA

      5640       5650       5660       5670       5680       5690
CCTGCGCACA GGTGGGAGGA AAGGGCCTGG CCAGTCCTGG TCCTGGCTGC ACTCTTGAAC

      5700       5710       5720       5730       5740       5750
TGGGCGAATG TCTTATTTAA TTACCGTGAG TGACATAGCC TCATGTTCTG TGGGGGTCAT

      5760       5770       5780       5790       5800       5810
CAGGGAGGGT TAGGAAAACC ACAAACGGAG CCCCTGAAAG CCTCACGTAT TTCACAGAGC

      5820       5830       5840       5850       5860       5870
ACGCCTGCCA TCTTCTCCCC GAGGCTGCCC CAGGCCGGAG CCCAGATACG GGGGCTGTGA

      5880       5890       5900       5910       5920       5930
CTCTGGGCAG GGACCCGGGG TCTCCTGGAC CTTGACAGAG CAGCTAACTC CGAGAGCAGT

      5940       5950       5960       5970       5980       5990
GGGCAGGTGG CCGCCCCTGA GGCTTCACGC CGGGAGAAGC CACCTTCCCA CCCCTTCATA

      6000       6010       6020       6030       6040       6050
CCGCCTCGTG CCAGCAGCCT CGCACAGGCC CTAGCTTTAC GCTCATCACC TAAACTTGTA

      6060       6070       6080       6090       6100       6110
CTTTATTTTT CTGATAGAAA TGGTTTCCTC TGGATCGTTT TATGCGGTTC TTACAGCACA

      6120       6130       6140       6150       6160       6170
TCACCTCTTT GCCCCCGACG GCTGTGACGC AGCCGGAGGG AGGCACTAGT CACCGACAGC
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      6180        6190        6200        6210        6220        6230
GGCCTTGAAG ACAGAGCAAA GCGCCCACCC AGGTCCCCCG ACTGCCTGTC TCCATGAGGT

      6240        6250        6260        6270        6280        6290
ACTGGTCCCT TCCTTTTGTT AACGTGATGT GCCACTATAT TTTACACGTA TCTCTTGGTA

      6300        6310        6320        6330        6340        6350
TGCATCTTTT ATAGACGCTC TTTTCTAAGT GGCGTGTGCA TAGCGTCCTG CCCTGCCCCC

      6360        6370        6380        6390        6400        6410
TCGGGGGCCT GTGGTGGCTC CCCCTCTGCT TCTCGGGGTC CAGTGCATTT TGTTTCTGTA

      6420        6430        6440        6450        6460        6470
TATGATTCTC TGTGGTTTTT TTTGAATCCA AATCTGTCCT CTGTAGTATT TTTTAAATAA

      6480
ATCAGTGTTT ACATTAGAA
___576 TO 5881 ____>
```

APPENDIX II: List of clones

Clones prepared during the cloning of the full length SHIP/ABL1 fusion

1 pGEM-Teasy-SA(39-1508)-CLN9

2 pcDNA3-SA-9A

3 pMIG-SA-9A6a

Clones prepared to obtain the full length SHIP/ABL1 fusion with 5' epitope tags

4 pGEM-Teasy-Flag-SA(154-1508)-CLN96

5 pGEM-Teasy-HA-SA(154-1508)-CLN91

6 pcDNAFlag-SA-96E

7 pcDNA-HA-SA-91E

8 pMIG-FLAG-SA-96E1

9 pMIG-HA-SA-91E2

Constructs prepared during the cloning of different regions of 5'SHIP1 into the bacterial-yeast shuttle vectors for the Yeast-two-hybrid (Y2H) experiments

For the deletion mutants of 5'SHIP1

10 pGEM-T-easy-BamH1-SH2-Xho1(3-3)

11 pGEM-T-easy-EcoR1-D1-BamH1*(2-3)*

12 pGEM-T-easy-EcoR1-D2-Not1(4-2)

13 pGEM-T-easy-EcoR1-D2-Xho1(5-1)

14 pGBKT7-BamH1-SH2-Pst1 (1-1)

15 pGBKT7-EcoR1-D1-BamH1*(2-1)*

16 pGBKT7-EcoR1-D2-Not1(3-1)

17 pGADT7-BamH1-SH2-Xho1(5-1)

18 pGADT7-EcoR1-D1-BamH1*(6-1)*

19 pGADT7-EcoR1-D2-Xho1(7-1)

For the complete 5'SHIP1 portion

20 pGEM-T-easy-BamH1-5'SHIP-Not1(7-7)

21 pGEM-T-easy-BamH1-SH2-D1-D2-Xho1(6-5) *OR*
pGEM-T-easy-BamH1-5'SHIP-Xho1(6-5)

22 pGBKT7-BamH1-5'SHIP-Not1(6-2)

23 pGADT7-BamH1-5'SHIP-Xho1(5-2)

For the 5'SHIP1-deltaD1 fragment

24 pGEM-T-easy-Bpu10I-D2-Not1(2-1)

25 pGEM-T-easy-Bpu10I-D2-Xho1(1-3)

26 pGEM-T-easy-BamH1-5'SHIP-deltaD1-Xho1-CLN(6-5)

27 pGBKT7-BamH1-5'SHIPdeltaD1-Not1(3-3)

28 pGADT7-BamH1-5'SHIPdeltaD1-Xho1(2-3)

Clones prepared during the construction of the deletion mutants of the SHIP1/ABL1 fusion

For SHIP1-deltaD1 mutants

29 pGEM-T-easy-Bpu10I-D2/ABL(904-1508)-CLN(3-4)

30 pGEM-T-easy-SA-deltaD1-1508-9(5)

31 pGEM-T-easy-Flag-SA-deltaD1-1508-96(2)

32 pGEM-T-easy-HA-SA-deltaD1-1508-91(3)

33 pcDNA3-SA-deltaD1(5-2)

34 pcDNA3-Flag-SA-deltaD1-CLN(6-3)

35 pcDNA3-HA-SA-deltaD1(7-2)

36 pMIG-SA-deltaD1(3-1)

For SHIP1(D1)/ABL1 fusion

37 pGEM-T-easy-D1-Bcl1-Sal1-9(1)

38 pGEM-T-easy-Flag-D1-Bcl1-Apa1-9N2

39 pGEM-T-easy-HA-D1-Bcl1-Apa1-9(6)

40 pGEM-T-easy-Bcl1-ABL(1184-1508)-Apa1(4-1)

41 pGEM-T-easy-Bcl1-ABL-(1184-1508)-Sal1(1-9)

42 pGEM-T-easy-D1/ABL (3-4)

43 pGEM-T-easy-Flag-D1/ABL (1-4)

44 pGEM-T-easy-HA-D1/ABL (2-1)

45 pcDNA3-D1/ABL(2-5)

46 pcDNA-Flag-D1/ABL-CLN(3-1)

47 pcDNA-HA-D1/ABL-CLN(4-1)

48 pMIG-D1/ABL(2-3)

For SHIP1(D1-D2)/ABL1 fusion

49 pGEM-T-easy-D1-D2-ABL(1-2)

50 pGEM-T-easy-Flag-D1-D2-ABL(3-5)

51 pGEM-T-easy-HA-D1-D2-ABL(4-1)

52 pcDNA-D1-D2-ABL(1-4)

53 pcDNA-Flag-D1-D2-ABL(3-1)

54 pcDNA-HA-D1-D2-ABL(4-1)

55 pMIG-D1-D2-ABL(1-1)

For SHIP1(D2)/ABL1 fusion

56 pGEM-Teasy-D2/ABL(829-1508)-CLN(2-2)

57 pGEM-T-easy-Flag-D2/ABL(5-4)

58 pGEM-T-easy-HA-D2/ABL(6-2)

59 pcDNA-D2/ABL(2-4)

60 pcDNA-Flag-D2/ABL(3-2)

61 pcDNA-HA-D2/ABL(4-1)

62 pMIG-D2/ABL(2-1)

ABBREVIATIONS

aa	amino acids
ab	antibody
ALL	acute lymphoblastic leukemia
AML	acute myeloblastic leukemia
Amp	ampicillin
approx.	approximately
APS	ammonium persulfate
BAC	bacterial artificial chromosome
B-ALL	B-cell acute lymphoblastic leukemia
bp	base pairs
BSA	bovine serum albumine
cALL	common accute lymphoblastic leukemia
cDNA	complementary DNA
CoIP	co-immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DCDF	dual color dual fusion
ddH_2O	double distilled water
DIG	digoxigenin
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
e.g.	example
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbant assay
EST	expressed sequence tags
Ex	Exon
FAB	French-American-British classification system for acute leukemia
FACS	fluorescence activated cell sorting
FISH	Fluorescent In situ hybridization
g	gram
GAL4-AD	GAL4 transcriptional activation domain

GAL4-DBD	GAL4 DNA binding domain
GAL4-UAS	GAL4 upstream activating sequence
GFP	green fluorescent protein
HBS	Hank's balanced salt
hr(s)	hour(s)
HRP	horse radish peroxidase
i.e.,	example
Intr	intron
IPTG	isopropyl beta-D-1-thiogalactopyranoside
IRES	Internal ribosome entry site
kb	kilobases
kD	kilodalton
l	liter
LacZ	*E. coli* gene encoding beta-galactosidase
LB	Luria Bertani medium
LiAc	lithium acetate
LSI	Locus specific indentifier
M	Molar
m-	milli (1×10^{-3})
MCS	multi cloning site
MIC	Morphologic-Immunologic-Cytogenetic
min	minute(s)
MOPS	3-N-morpholino-propanesulfonic acid
mRNA	messenger RNA
n	nano (1×10^{-9})
NaAC	sodium acetate
NaOH	sodium hydroxide
nt	nucleotide
O/N	overnight
ºC	degree Celsius
OD	optical density
p	pico (1×10^{-12})
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEG	polyethylenglycol

PFA	paraformaldehyde
PtdIns(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
RNA	ribonucleic acid
RNAse	A ribonuclease A
rpm	revolutions per minute
RT	room temperature
RT-PCR	Reverse transcriptase PCR
SD	synthetic drop out
SDS	sodium dodecylsulfate
sec	second(s)
SSC	saline sodium citrate buffer
TBE	Tris-borate-EDTA
TBS	tris-buffered saline
TCA	Tris-chloro-acetate
TE	tris-EDTA buffer
Tm	melting temperature
U	unit
V	volts
VCM	Viral conditioned medium
vol.	volume
w/v	weight per volume
WB	western blot
WCP	whole chromosome paint
x g	gravity (9.81 m/s^2)
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
Y2H	yeast two hybrid
YFP	yellow fluorescent protein
YPD	yeast extract, peptone, dextrose
µ-	micro (1 x 10^{-6})

SINGLE LETTER CODES FOR AMINO ACIDS

A (Ala)	Alanine
M (Met)	Methionine
B	Asparagine or Aspartic acid
N (Asn)	Asparagine
C (Cys)	Cysteine
P (Pro)	Proline
D (Asp)	Aspartic acid
Q (Glu)	Glutamine
E (Glu)	Glutamic acid
R (Arg)	Arginine
F (Phe)	Phenylalanine S (Ser) Serine
G (Gly)	Glycine
T (Thr)	Threonine
H (His)	Histidine
V (Val)	Valine
I (Ile)	Isoleucine
W (Trp)	Tryptophan
K (Lys)	Lysine
Y (Tyr)	Tyrosine
L (Leu)	Leucine
Z	Glutamine or Glutamic acid

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CURRICULUM VITAE

Purvi M. Kakadiya

Date of birth: 22.08.1976

Place of birth: Mumbai, India

Residential address:
Heiglhofstr. 55, Apt. 2-35
81377 München
Email: purvikakadia@yahoo.com
Phone ++49-17620938879 (M)
++49-8933094581 (R)

Work address:
Medizinische Klinik und Poliklinik III
Labor für Leukämiediagnostik
Klinikum Grosshadern
Marchioninistr. 15
81377 München
Phone ++49-7095-4973

EDUCATION

PhD Thesis **Title: "**First description and analysis of the novel SHIP1/ABL1 fusion in acute lymphoblastic leukemia (ALL)"
University of Munich, Department of Medicine III, Klinikum Grosshadern, Munich, Germany
2005-2009

Master's Degree M.Sc. Zoology (Cell Biology & Cytogenetics) 1998
Gujarat University, Ahmedabad, India.

Bachelor's Degree Education, (B.Ed. Biology & Mathematics) 2000
Gujarat University, Ahmedabad, India.

B. Sc. Zoology 1996
Gujarat University, Ahmedabad, India.

RESEARCH EXPERIENCE

Research Fellow, PhD Student **July 2005- till date**
Medizinische Klinik und Poliklinik III, Labor für Leukämiediagnostik, Klinikum Grosshadern, Munich, Germany.
Leukaemia Cytogenetics: Clinical Research.

PhD project: Discovery and analysis of the novel SHIP1/ABL1 fusion in acute lymphoblastic leukemia (ALL)

Research Fellow **June 2002 - June 2005**

Cell-Biology Division, Gujarat Cancer & Research Institute, Ahmedabad, Gujarat, India.

Project Title: *Study Of Inheritance Of Chromosomal Markers In Pediatric Leukemia Patients.*

Junior Research Fellow **December 2000-May 2001**

Cell-Biology Division, Gujarat Cancer & Research Institute, Ahmedabad, Gujarat, India.

Project Title: *Study of radio sensitivity in Head and Neck cancer patients using cytogenetic end points: predictive assay for response to radiotherapy.*

TRAINING

Observer **June 2001- July 2001**

Cell-Biology Division, Gujarat Cancer & Research Institute, Ahmedabad, Gujarat, India.

Leukemia cytogeneticist **October - December 2005**

Under the supervision of Prof. Dr. Harald Rieder, Institute of Human Genetics, University of Düsseldorf, Germany.

PUBLICATIONS

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PRESENTATIONS

Poster presentation

"AML-M2 with a novel der(18) chromosome in addition to t(8;21) and del(9q)."
Indian Association of Cancer Research (IACR), Gujarat chapter at the Gujarat Cancer & Research Institute
Ahmedabad ***August, 2003***

Oral presentation

"Loss of a sex chromosome in Acute Myeloid Leukemia patients"
29th Annual meeting of Indian Society of Human Genetics (ISHG)
Bangalore ***January, 2004***

Poster presentation

"Familial Supernumerary Marker Chromosome: candidate for prenatal assessment?"

7th National Conference of Indian Society of Prenatal Diagnosis and Therapy (ISPAT).

Ahmedabad ***January, 2004***

Oral presentation

"Acute Myeloid Leukemia Cytogenetics: Probing with Bioinformatics"

30th Annual Conference of Indian Society of Human at the Center for cellular and Molecular biology (CCMB)

Hyderabad ***February, 2005.***

LANGUAGES

Gujarati	Mother tongue
Hindi	Fluent
English:	Fluent
German	Working knowledge