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Tyrosine phosphorylation of the BCR-ABL SH3 domain results in the
recruitment of SH2 domain containing proteins

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Curriculum Vitae

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Summary

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder that is characterized by the presence of the constitutively active BCR-ABL fusion tyrosine kinase. In general, CML is a well-studied disease, but the underlying changes in signal transduction networks and gene expression patterns that lead to oncogenic reprogramming of haematopoietic cells by the expression of BCR-ABL are only incompletely understood. In the present study we are focusing on two different aspects of BCR-ABL signalling. One aim was to assess the consequences of BCR-ABL SH3 domain phosphorylation for the interaction pattern of the fusion protein. In a second approach, we sought to identify microRNAs whose expression profile is dictated by the tyrosine kinase activity of BCR-ABL.

The SH3 domain of BCR-ABL was previously found to be phosphorylated on Tyr134 in CML. We conducted biochemical analyses to investigate if Tyr134 phosphorylation of the BCR-ABL SH3 domain causes the recruitment of phospho-tyrosyl specific, SH2 domain harboring, proteins. We started with a peptide pull-down using two synthetic peptides whose sequence corresponded to the region of interest of the BCR-ABL SH3 domain. One peptide was modified by the covalent attachment of a phosphate group to the tyrosine, while in the other peptide the tyrosine was substituted by a phenylalanine. LS-ESI-MS/MS analysis of the pull-downs yielded two proteins, PLCG1 and SHP2, which both specifically bind to the tyrosine-phosphorylated peptide but not to the control peptide. Both candidates have proto-oncogenic attributes and have already been described to be involved in the manifestation of various malignancies. We subsequently cloned the BCR-ABL SH3 domain as a GST fusion protein into a bacterial expression vector, and after expression in *E. coli* and FPLC-purification we performed additional pull-downs, this time comparing the fully folded SH3 domain with a control construct where the tyrosine residue of interest was mutated to phenylalanine. Finally, overexpression of

various full-length BCR-ABL constructs in HEK-null cells was performed to confirm the phosphotyrosine dependent interaction between the BCR-ABL SH3 domain and the potential interactors in co-immunoprecipitation experiments. To our knowledge this is the first study showing a phosphotyrosine dependent interaction between an SH2 domain and an SH3 domain. Considering the pro-growth nature of PLCG1 and SHP2 it is entirely thinkable that Tyr134 phosphorylation represents a further mechanism by which BCR-ABL exerts its oncogenic function.

MicroRNAs (miRNAs) are a novel class of small noncoding RNAs that modulate the expression of genes at the posttranscriptional level. Aberrant miRNA expression has been described in several human malignancies, including CML.

In the second part of this study we provide an informative profile of the expression of miRNAs that are deregulated upon Dasatinib or Nilotinib treatment of K562 cells. We identified 31 miRNAs that were differentially expressed in the presence of tyrosine kinase activity suppressed BCR-ABL. In line with previous findings, expression of miRNAs belonging to the miR-17-92 family was specifically downregulated by both Dasatinib and Nilotinib treatment. In addition we detected a marked decrease of miR-21 in the drug treated samples, confirming earlier microarray gene expression data. Furthermore, we observed miR-21 downregulation also by a miR-21 specific miR-qRT-PCR assay. Finally, we used the TargetScan platform for the identification of predicted miRNA target genes whose expression levels inversely correlate with any of the 31 miRNA candidates following BCR-ABL inhibition in K562 cells. In summary, the results of this study offer a comprehensive and quantitative profile of BCR-ABL dependent miRNA expression in CML and further implicate the oncogenic miRNAs miR-17-92 and miR-21 in the pathophysiology of the disease.

Zusammenfassung

Bei der Chronischen Myeolischen Leukämie (CML) handelt es sich um eine umfassend charakterisierte myeloproliferative Erkrankung, die aus der anhaltenden Aktivität der Tyrosinkinase BCR-ABL resultiert. Trotz der Vielzahl an bereits gewonnen Erkenntnissen sind weiter Untersuchungen notwendig um die der Krankheit zugrunde liegenden Veränderungen in Signaltransduktion und Genexpression gänzlich zu verstehen. In der vorliegenden Studie wurden zwei Aspekte der BCR-ABL abhängigen Signaltransduktion genauer untersucht. Einen Schwerpunkt legten wir auf die Charakterisierung jener Änderungen im Interaktionsprofil von BCR-ABL, die durch die Phosphorylierung der BCR-ABL eigenen SH3 Domäne verursacht werden. Im zweiten Projekt suchten wir nach mikroRNAs, deren Expressionsprofil von der Aktivität der BCR-ABL Tyrosinkinase bestimmt wird.

Bereits in einer früheren Studie wurde gezeigt, dass bei CML die SH3 Domäne von BCR-ABL am Tyr134 phosphoryliert wird. Wir führten verschiedene biochemische Analysen durch, mit dem Ziel Proteine zu identifizieren die über eine etwaige SH2 Domäne mit BCR-ABL über eben diesen phosphorylierten Tyrosinrest interagieren. Wir behelfen uns dabei eines sogenannten Peptid Pull-downs. Kurze synthetische Peptide wurde designt deren Sequenz dem Tyr134 umgebenden Bereich der SH3 Domäne entspricht. Eines der beiden Peptide wurde durch das kovalente Anhängen einer Phosphatgruppe and Tyr134 modifiziert. Im zweiten Peptid, dem Kontrollpeptid, wurde das Tyrosin durch einen Phenylalaninrest ersetzt. Die massenspektrometrische Analyse beider Pull-downs führte zur Identifizierung von PLCG1 und SHP2, zwei Proteine die spezifisch das Tyrosin-phosphorylierte Peptid binden, jedoch nicht an das Kontrollpeptid. Beiden Kandidaten wurden bereits in frühern Studien eine Rolle in der Entwicklung verschiedener Tumorarten zugeschrieben. In einem nächsten Schritt klonierten wir die SH3 Domäne BCR-ABLs

als GST-Fusionsprotein in einen bakteriellen Expressionsvektor. Nach der Aufreinigung der in *E. coli* exprimierten SH3 Domäne mittels FPLC führten wir weiter Pull-down Experimente durch. Diesmal verglichen wir die vollständige SH3 Domäne mit einem Kontrollkonstrukt bei dem wiederum Tyr134 durch ein Phenylalanin substituiert wurde. Die abschließende Verifizierung erfolgte über einen Co-Immunoprecipitationsansatz, bei welchem wir verschiedene BCR-ABL Konstrukte hinsichtlich einer spezifischen Interaktion zwischen PLCG1 und SHP2 und der Tyrosin phosphorylierten SH3 Domäne untersuchten. Es dürfte sich hierbei um die erste Studie handeln, die eine Phosphotyrosin abhängige Interaktion zwischen einer SH2 Domäne und einer SH3 Domäne zeigt. In Anbetracht der wachstumsbegünstigenden Natur von sowohl PLCG1 als auch SHP2 ist es nicht auszuschließen, dass die Phosphorylierung von Tyr134 einen weiteren Mechanismus der BCR-ABL vermittelten Onkogenese darstellt.

MikroRNAs (miRNAs) sind neuartige, kurze nicht kodierende, RNAs, die die Expression von Genen auf posttranskriptioneller Ebene modulieren. Die aberrante Expression von miRNAs wurde in Verbindung zu zahlreichen bösartigen zellulären Veränderungen, einschließlich CML gebracht.

Im zweiten Teil unserer Studie wurden 31 miRNAs identifiziert deren Expressionprofile durch die Inkubation von K562 Zellen mit Dasatinib und Nilotinib signifikant verändert wurden. Wir konnten in Übereinstimmung mit früheren Studien zeigen, dass die Expression von miRNAs der miR-17-92 Familie durch Inhibierung der BCR-ABL Aktivität negativ reguliert wird. Ferner beobachteten wir eine Reduktion der miR-21 Expression durch sowohl Dasatinib als auch Nilotinib, und verifizierten dadurch das Ergebnis einer schon länger zurückliegenden Mikroarray basierenden Genexpressionsanalyse. Eine weitere Bestätigung der Runterregulierung von miR-21 wurde durch eine miR-21 spezifische miR-qRT-PCR Analyse erzielt. Anhand der TargetScan Plattform bestimmten wir abschließend die Zielgene der identifizierten miRNAs und konzentrierten uns dabei auf jene Gene deren Expressionslevel umgekehrt proportional zu den Expressionsleveln der jeweiligen miRNAs verlaufen. In der vorliegenden Studie wird eine umfassende Analyse der BCR-ABL abhängigen miRNA Expression durchgeführt. Außerdem liefern wir weitere Hinweise dafür, dass die onkogene miRNAs miR-17-92 und miR-21 an der Pathophysiologie von CML beteiligt sind.

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1. Introduction

1.1. Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a clonal disorder caused by the neoplastic transformation of hematopoietic stem cells (HSCs). A common distinctive feature of all CML patients is the presence of a consistent chromosomal abnormality – the Philadelphia (Ph) chromosome – a reciprocal translocation involving the long arms of chromosomes 9 and 22, $t(9;22)(q34;q11)$. This translocation leads to the juxtaposition of the 3' end of the Abelson kinase proto-oncogene (*ABL1*) on chromosome 9 with the 5' end of breakpoint cluster region (*BCR*) on chromosome 22 forming a *BCR-ABL* fusion gene¹⁻³. The new gene encodes the oncoprotein p210_{BCR/ABL}, a constitutively active tyrosine kinase which promotes cell survival and proliferation through several intracellular signal transduction pathways^{4,5}. In a series of studies it was shown that expression of *BCR-ABL* in mouse bone-marrow cells using retroviral transduction and bone-marrow transplantation methods is sufficient for the induction of a myeloproliferative disorder that has all the major hallmarks of CML proving the direct causal relationship to CML⁶⁻⁸.

CML is characterized by distinct clinical phases and evolves through a multi-step pathogenetic process (Fig. 1.1). In the initial, chronic phase of the disease (CP) expression of the *BCR-ABL* fusion gene in self-renewing HSCs causes a massive expansion of the mature, granulocytic cell lineage^{9,10}. After 3-4 years, the disease progresses into the accelerated phase (AP) which typically lasts 4-6 months and is characterized by an increase in the amount of progenitor cells. Finally patients enter the blast phase (BP) in which hematopoietic differentiation has become arrested and a rapid expansion of lymphoid and myeloid progenitor cells (blast cells) in bone marrow, peripheral blood and extramedullary tissues occurs^{11,12}.

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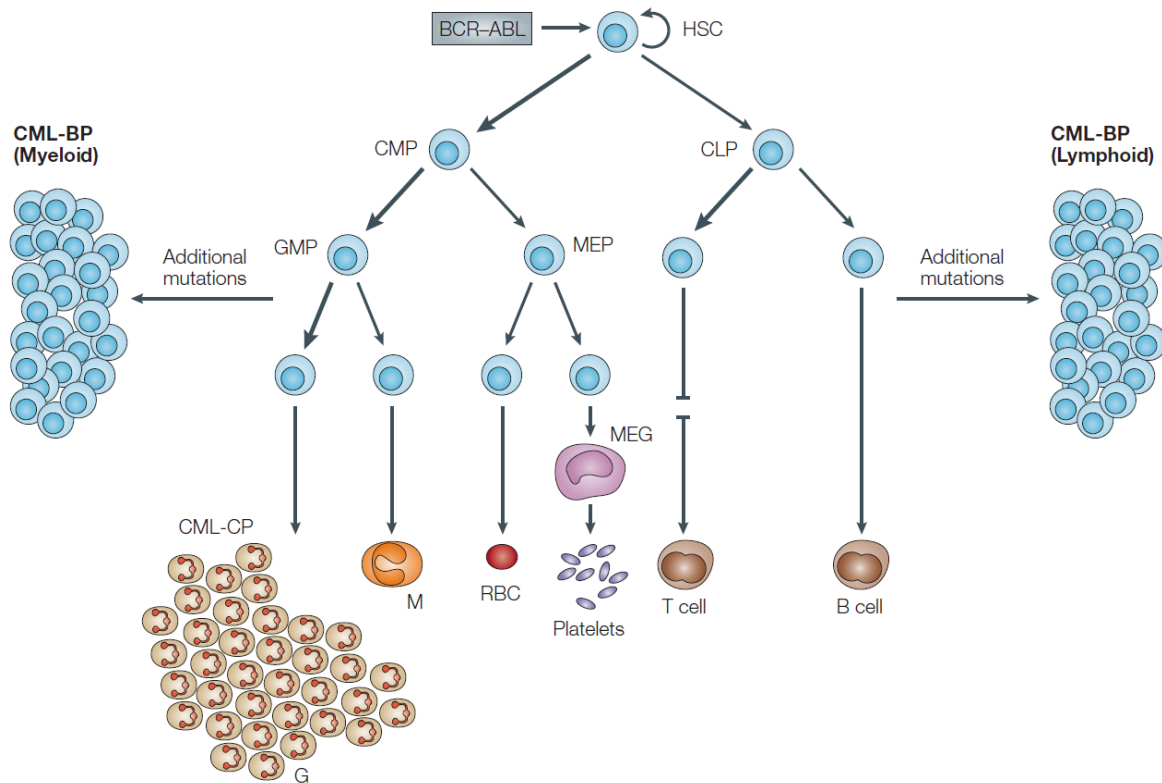


Figure 1.1 **Development of CML.** Hematopoietic stem cell (HSC), myeloid progenitor (MP), lymphoid progenitor (LP), granulocytic/macrophage progenitor (GMP), megakaryocyte/erythrocyte progenitor (MEG), macrophage (M). (Taken from Ref.4)

The exact mechanism responsible for the transition from CML chronic phase to blast phase is only incompletely understood. It seems however reasonable to assume that the unrestrained BCR-ABL activity is essential for the acquisition of additional cytogenetic and molecular defects that result in the manifestation of symptoms with increasingly malignant characteristics. For example it has been shown that BCR-ABL expression causes genomic instability by interfering with DNA repair pathways and thereby triggers chromosomal aberrations¹³⁻¹⁶. The most frequent oncogenic events associated with blast crisis include loss of p53 function^{17, 18}, trisomy 8, i(17q)^{19, 20}, *MYC* amplification¹⁹, *RB* (*RB1*) deletion/rearrangement²¹, *p16INK4A* (*CDKN2*) rearrangement/deletion²², and reduplication of the Ph chromosome²⁰. In addition, the increased levels of BCR-ABL in the advanced stages of the disease are likely to contribute to reduced apoptosis susceptibility, enhanced proliferation potential and differentiation arrest of the leukemic clone⁵.

1.2. BCR-ABL: regulation & signalling

C-Abl, the endogenous counterpart to BCR-ABL, is a ubiquitously expressed non-receptor tyrosine kinase which has been implicated in various cellular processes including proliferation, differentiation, apoptosis, stress response, cell migration and cell adhesion^{23, 24}.

Alternative splicing of the primary *ABL* transcript generates two messenger RNAs encoding different isoforms of the c-ABL protein. Isoform 1b is 19 amino acids longer than the 1a splice variant and is myristoylated on its second glycine residue, a site absent in isoform 1a and BCR-ABL²⁵ (Fig. 1.2).

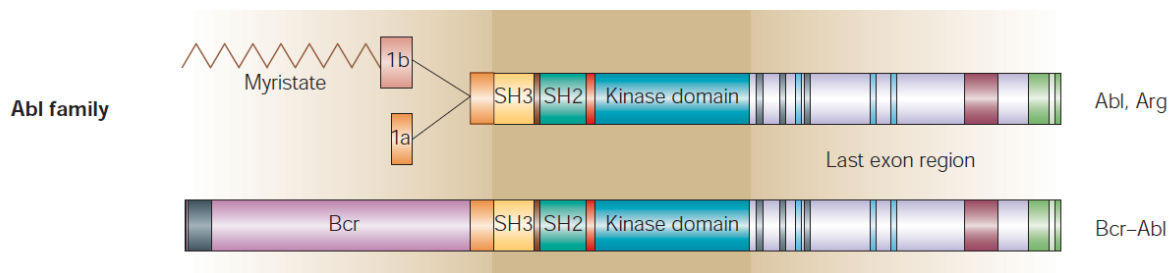


Figure 1.2 **Domain structures of the ABL family.** (Taken from Ref.25)

The kinase activity of c-ABL is tightly regulated and in its basal state is kept inactive by intramolecular interactions. The autoinhibited state of c-ABL is initiated by the intramolecular engagement of the N-terminal myristoyl modification of the 5' Cap region with a hydrophobic pocket of the kinase domain²⁶. This event induces a conformational change that allows the formation of the so called 'SH3-SH2 clamp'. Within this 'clamp' structure the Src homology domain 3 (SH3) and the Src homology domain 2 (SH2) of c-ABL bind to the distal side of the tyrosine kinase domain thereby causing a conformational change at the base of the activation loop and hence rendering the enzyme inactive^{25, 27}. The activation loop is a highly flexible stretch of amino acids that is projecting into the active site of the kinase domain and participating in the coordinated binding of ATP and peptide substrate²⁵.

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Although c-ABL isoform 1a is not myristoylated it appears to be regulated through an indistinguishable mechanism. It was suggested that hydrophobic residues in the Cap domain of c-ABL 1a may substitute for the myristoyl latching function^{26, 27}.

The full activation of c-ABL is thought to require a three step mechanism (Fig. 1.3). First of all, “unlatching” of the myristoyl group or hydrophobic residues in the kinase domain has to occur. Secondly, engagement of the SH2 and SH3 domains with tyrosyl phosphorylated peptides and proline rich proteins, respectively, is likely to open up the clamp structure. Phosphorylation of tyrosine 245 in the linker between SH2 and kinase domain is contributing to the disassembly due to the now impaired SH3-linker interaction of the autoinhibited structure. As a result the activation loop is reoriented and exposed outwards, leading in a third step to the phosphorylation of tyrosine 412 and to full kinase activation²⁶⁻³¹.

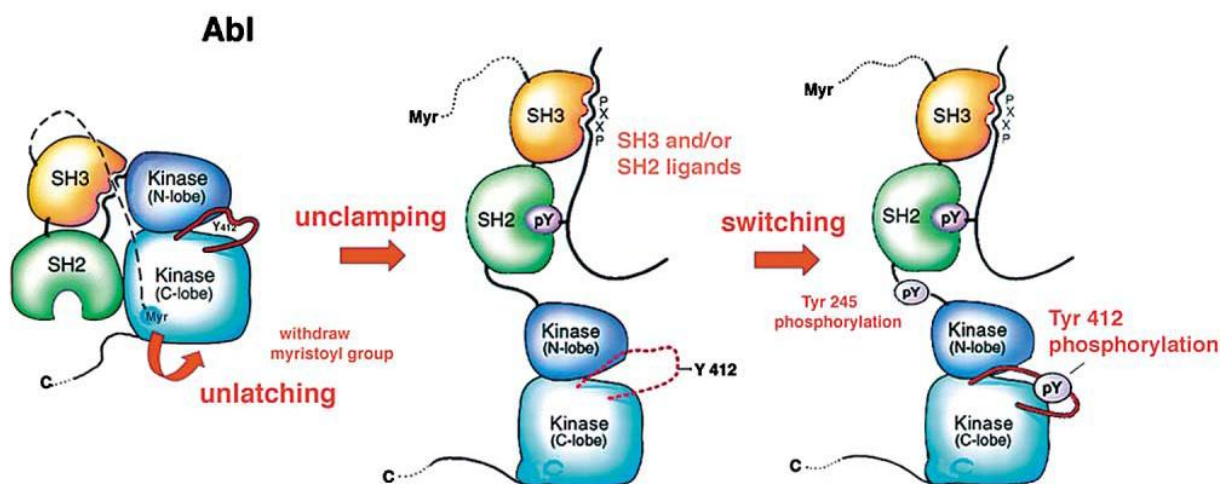


Figure 1.3 Mechanism of c-ABL activation. (Taken from Ref.28)

1.3. Mechanisms of BCR-ABL mediated malignant transformation

In order for BCR-ABL to exert its oncogenic function it relies on several features that were shown to be critical for cellular transformation and disease progression^{4, 32-35}.

1.3.1. Deregulated kinase activity

Obviously, deregulation of the otherwise tightly regulated kinase activity is one of them. Fusion of *BCR* sequences to *ABL* results in the partial deletion of the c-ABL Cap region that contains the myristoylation site and is absolutely required for c-ABL regulation³⁶. BCR-ABL as a consequence is able to evade the autoinhibitory mechanism observed in c-ABL, explaining its constitutive kinase activity and the increase in autophosphorylation and cellular total phosphotyrosine levels. Because of its catalytic activity, many substrates can be tyrosine phosphorylated by BCR-ABL. Even more important, autophosphorylation leads to a marked increase of phosphotyrosine on BCR-ABL itself, which creates docking sites for SH2 or PTB domain harboring proteins.

1.3.2. Oligomerization

Oligomerization is a further feature that is contributing to BCR-ABL induced cellular transformation. The amino-terminal coiled-coil domain (CC) mediates dimerization and tetramerization of the BCR-ABL protein³⁷ and enhances its kinase activity³⁸. It was shown in mice that lack of the BCR-ABL CC domain results in the inability of BCR-ABL to induce a myeloproliferative disorder (MPD), emphasizing its importance in the process of neoplastic transformation³⁹⁻⁴¹.

1.3.3. Aberrant signalling

Finally, BCR-ABL regulates a diverse range of signalling molecules and pathways whose contribution in leukemogenesis is based on the following features^{32, 34, 35}:

Mitogenic signalling Of all the signalling pathways with mitogenic potential in BCR-ABL-transformed cells, activation of the RAS MAP kinase system appears to play a central role in myeloid proliferation and survival. It was shown that impairment of

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RAS signalling severely suppresses BCR-ABL induced cell growth⁴²⁻⁴⁴ and that expression of an oncogenic form of RAS in mice induces an MPD^{45, 46}.

Absolutely required for the induction of an MPD is the GRB2-binding site in the Bcr-region of BCR-ABL^{39, 41, 47}. If phosphorylated, tyrosine 177 binds the SH2 domain of GRB2, which in turn recruits SOS and GAB2^{48, 49}. SOS is a guanine-nucleotide exchange factor of RAS and is therefore turning on the RAS MAP kinase pathway. GAB2 on the other hand is a scaffolding protein that recruits PI3K and SHP2 – the latter acting upstream of RAS and being required for full activation of the Erk MAP kinase pathway^{50, 51}.

Beside Y177 there exist two additional sites that are contributing to the activation of RAS⁵²: The SH2 domain of BCR-ABL was shown to recruit SHC, which, following tyrosine-phosphorylation, can, recruit GRB2⁵². For the second site – tyrosine 1294 – again a tyrosine-phosphorylation site, located in the activation loop of the kinase domain, the mechanism of RAS activation is still unsolved. It was however shown that mutation of the SH2 domain as well as of Y1294 attenuates leukemogenesis by BCR-ABL⁵³⁻⁵⁵.

Cell survival BCR-ABL promotes cell survival partly by activating the PI3K pathway⁵⁶ whose dysregulation has been implicated in contributing to the onset of a wide range of human cancers⁵⁷. The next relevant downstream substrate of PI3K in this cascade is the serine-threonine kinase AKT. AKT negatively regulates apoptosis by a multifactorial mechanism that is involving the phosphorylation of several components of the cell-death machinery (BAD, Caspase-9, FKHR, MDM2 and IKK)⁵⁷

Inhibition of the PI3K pathway by expression of dominant negative AKT or application of a PI3K inhibitor compromises BCR-ABL–induced cytokine-independent growth⁵⁸ and colony formation⁵⁹. There exist however also contradictory reports that show that expression of dominant negative AKT is not sufficient to reverse BCR-ABL induced cellular transformation^{60, 61}.

It is therefore likely that BCR-ABL relies on additional pathways to ensure undisturbed cell survival, like activation of STAT5^{54, 62}, inhibition of the p38 MAPK, or downregulation of JunB^{63, 64} and ICSBP⁶⁵ transcription.

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Loss of stromal cell adhesion In the bone marrow microenvironment, regulation of hematopoiesis normally involves adhesion of hematopoietic progenitor cells to marrow stroma cells and extracellular matrix components. This interaction negatively regulates cell proliferation and prevents premature circulation of primitive progenitors in the blood. In CML, however, malignant progenitor cells appear to escape this regulation by virtue of their altered adhesion properties^{66, 67}. Aberrant $\beta 1$ integrin signalling has been implicated in this process. Treatments with IFN α or an activating anti- $\beta 1$ -integrin antibody have been shown to reverse the adhesion and proliferation-inhibitory signalling defects observed in CML⁶⁷⁻⁶⁹.

Proteasomal degradation of c-ABL inhibitors Yet another, quite compelling, mechanism by which BCR-ABL is guaranteeing the progress of leukemogenesis is proteasome-mediated degradation of proteins with otherwise inhibitory function³². Mutational analysis of the c-ABL SH3 domain led to the discovery of point mutants with enhanced catalytic and transforming activities. These mutations were at residues predicted to obstruct binding of PxxP ligands⁷⁰. The Abl interacting proteins ABI-1 and ABI-2 contain such motifs. Binding of ABI-1 and ABI-2 to the SH3 domain of c-ABL negatively regulates its tyrosine kinase activity and antagonizes its oncogenic function⁷¹⁻⁷³. BCR-ABL seems to overcome this obstacle by inducing ubiquitination and proteasome-mediated degradation of ABI-1 and ABI-2⁷³. Significantly, degradation of the ABI proteins is specific for Ph-positive leukemias and is not seen in BCR-ABL-negative samples of comparable phenotype.

Inhibition of tumour suppressors Although still speculative, recent work suggests that loss of tumour suppression function is a further mechanism by which BCR-ABL is contributing to malignant propagation and progression. There is mounting data corroborating the theory that BCR-ABL is fostering oncogenesis through interaction and subsequent downregulation of tumour suppressors.

The cyclin-dependent kinase (CDK) inhibitors (CDKI) p16_{ink4a} and p19_{Arf}, for example, bind to CDK and are consequently promoting cell senescence by stalling the cycle³⁵. BMI1 is polycomb gene which represses p16_{ink4a} and p19_{Arf} mediated senescence to promote cell survival and self-renewal by epigenetic gene silencing^{74, 75}. Phosphorylated p38 MAPK has been implicated in causing the dissociation of BMI1 from chromatin via its downstream effector 3pK^{35, 76}.

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In normal cells c-ABL has stimulatory effects on p38 and the CDKIs and is hence directing the cell towards senescence. In CML cells, BCR-ABL is inhibiting the normal tumour suppressor activity of p38, which, following de-phosphorylation loses its inhibitory effect on BMI1. As a result p16_{ink4a} and p19_{Arf} expression is repressed and a shift away from senescence toward unrestrained cell proliferation and survival occurs^{35, 77, 78}.

The protein phosphatase 2A (PP2A) is another tumour suppressor that is inhibited by BCR-ABL^{34, 79}. In the chronic phase of the disease, PP2A antagonizes BCR-ABL through recruitment and activation of the tyrosine phosphatase SHP1. SHP1 catalyses the dephosphorylation of BCR-ABL, which in turn is downregulated through proteasomal degradation⁷⁹. While the activity of PP2A is only moderately impaired in the chronic phase of CML, its activity in blast phase cells is negligible. It was found that in CML blast crisis (CML-BC) progenitors, the phosphatase activity of PP2A is inhibited by the BCR-ABL-induced post-transcriptional upregulation of the PP2A inhibitor SET – a phosphoprotein that is frequently overexpressed in leukemias and solid tumours⁷⁹.

Differentiation arrest In CML progression a transition from mature, terminally differentiated cells to immature, undifferentiated cells can be observed. The differentiation arrest can be explained by the pathological interference of BCR-ABL with the corresponding differentiation programmes. It has been demonstrated that BCR-ABL suppresses the translation of the transcription factor CEBP and is thereby preventing the expression of the granulocyte colony-stimulating factor receptor (*GCSFR*) and *ID1* genes⁸⁰⁻⁸². The effect of BCR-ABL on CEBP is mediated by the translational regulator HNRNPE2. BCR-ABL increases the stability of HNRNPE2, which, following binding to the CEBP mRNA, inhibits the translation of the transcription factor⁸².

Genomic instability The transition from CML chronic phase to blast crisis is associated with increased genomic instability. The reasons for this are reduced capacity of CML cells to survey the genome for DNA damage, interference with DNA-repair proteins and progressive telomere shortening (reviewed in³⁴).

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Many more signalling molecules are regulated by BCR-ABL, but for the majority of them it is still rather unclear if and to what extent they are contributing to the onset of BCR-ABL induced leukemia.

In addition to the aforementioned proteins, the list of BCR-ABL interactors includes for example CRKL, CRK-II, STS-1, SHIP-2, CBL, p85, 3BP2, CAS, STAT5, PLC γ , FAK, RASA, paxilin, talin, synaptophysin, etc. ⁴. Also, signalling downstream of BCR-ABL is known to activate various transcription factors (e.g. MYC, STAT5, NF- κ b) and to induce the expression of different cytokines (e.g. IL-3, GM-CSF) ⁴.

1.4. BCR-ABL as a therapeutic target

Historically, first chemotherapy using hydroxyurea, cytosine arabinoside, arsenic trioxide and later INF- α was used to treat CML patients ⁸³. Chemotherapy was insufficient in delaying disease progression and was hence associated with a rather poor prognosis ^{84, 85}. Treatment with IFN α , often in combination with the DNA synthesis inhibitor cytarabine (Ara-C), represented a significant advance, producing hematologic and cytogenetic responses in patients with CP CML, and improving the survival rate compared with previous treatments ^{86, 87}.

A major breakthrough in CML treatment has arrived with the development of small molecule chemical inhibitors that allow molecular targeted therapies against specific oncogenic events. The discovery that BCR-ABL is required for the pathogenesis of CML, and that the tyrosine kinase activity of c-ABL is imperative for the transforming properties of BCR-ABL, made the BCR-ABL kinase domain an attractive target for therapeutic intervention ⁸⁸.

1.4.1. Imatinib

The first tyrosine kinase inhibitor (TKI) directed against BCR-ABL to be developed was Imatinib (also known as Gleevec, STI571, or CP57148B). Imatinib is a potent inhibitor of ABL, ARG, KIT, and PDGFR tyrosine kinases and has been shown to be remarkably successful in treating patients with CML as well as other blood

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neoplasias and solid tumors based on the deregulation of these tyrosine kinases' activity^{33, 89}. Imatinib binds to the cleft between the N- and C-terminal lobes of the kinase domain outside of the highly conserved ATP binding site thereby obstructing ATP binding. In doing so, Imatinib effectively inhibits the catalytic activity of ABL, trapping the activation loop of the kinase domain in an inactive conformation⁹⁰⁻⁹².

Imatinib selectively induces apoptosis of BCR-ABL positive cells and induces hematologic and cytogenetic remissions in all phases of CML, although treatment of patients with more advanced phases of CML is less effective and less durable than it is in the early phase of the disease⁹³⁻⁹⁷.

However, there are two major obstacles to Imatinib based therapies for patients with CML. One is the persistence of quiescent leukemic stem cells, which Imatinib fails to deplete despite the presence of higher levels of BCR-ABL transcripts and protein in these cells compared with more differentiated CML cells⁹⁸⁻¹⁰⁰. The insensitivity of these cells to Imatinib is believed to contribute to the relapse observed in some patients following the termination of Imatinib treatment, which is why the only way to suppress the disease is continuous Imatinib therapy.

The other major problem is Imatinib resistance. A significant proportion of patients is intrinsically resistant to Imatinib or develops resistance during treatment¹⁰¹. Several studies also suggest that a very small subpopulation of leukemic cells exists that harbors mutant subclones prior to Imatinib treatment and which grows in prevalence during therapy owing to the selective pressure of the drug^{102, 103}.

Imatinib resistance is often a result of single point mutations that impair drug binding in the tyrosine kinase domain and allow BCR-ABL to circumvent an otherwise potent anticancer drug^{101, 104, 105}. Usually this involves the re-emergence of BCR-ABL tyrosine kinase activity, indicating that the mutant BCR-ABL protein is still a putative target for inhibition in Imatinib-resistant patients¹⁰⁶⁻¹⁰⁸. Other mechanisms that contribute to Imatinib resistance include increased expression of BCR-ABL kinase through gene amplification, decreased intracellular Imatinib concentrations caused by increased expression of drug efflux proteins or decreased expression of drug influx proteins and Imatinib binding by plasma proteins⁸⁵.

The discovery of resistance mechanisms spurred the development of alternative therapies designed to overcome Imatinib resistance, including high-dose Imatinib, novel targeted agents, and combination treatments^{83, 109, 110}.

1.4.2. Dasatinib

Dasatinib (Sprycel, Bristol-Myers Squibb) has recently been approved for treatment of patients with CML following failure or with intolerance to Imatinib therapy. It is a highly potent multitargeted TKI that inhibits several critical oncogenic proteins, including BCR-ABL, Src family of kinases (SFKs), Kit, PDGFR, and ephrin A (EPHA2) receptor kinase ¹¹¹⁻¹¹³.

Dasatinib binds BCR-ABL with greater affinity than Imatinib and unlike Imatinib it binds primarily the active conformation of the enzyme ^{111, 114, 115}. As a result, Dasatinib has been shown to effectively inhibit all Imatinib-resistant kinase domain mutations tested, with the exception of the T315I mutation ^{114, 116, 117}.

A potential limitation of Dasatinib is the occurrence of untoward off-target toxicities, which probably relate to its inhibitory activity against a broader range of protein kinases than Imatinib ¹⁰⁹.

1.4.3. Nilotinib

Nilotinib (Tasigna, Novartis) is a derivative of Imatinib, and similar to Imatinib it binds BCR-ABL in its inactive conformation only ⁸³. Nilotinib is however even more selective, having only similar activity to Imatinib against KIT and PDGFR, and being devoid of activity against the related SFKs ¹¹⁰. Moreover, Nilotinib is about 30-fold more potent in inhibiting BCR-ABL than Imatinib and has demonstrated activity against 32 of 33 mutant BCR-ABL forms resistant to Imatinib, again with the exception of T315I ^{118, 119}.

1.4.4. Other inhibitors

Several other BCR-ABL inhibitors are currently in clinical trials including dual Src family and ABL kinase inhibitors (Bosutinib, INNO-404 and AZD0530), non-ATP competitive inhibitors of BCR-ABL (ON012380) and Aurora kinase inhibitors (MK-0457 and PHA-739358) ¹¹⁰. MK-0457 and ON012380 may even be capable of inhibiting the T315I mutant of BCR-ABL ¹²⁰⁻¹²².

1.5. SH2 & SH3 domains

The Src homology domains SH2 and SH3 are conserved protein domains that act as key regulatory participants in many different signalling pathways. Independent of surrounding sequences, both domains fold into functional and compact modules that recognize short peptide motifs containing either phosphotyrosine (pTyr) in the case of SH2, or proline-rich regions in the case of SH3.

Both domains belong to a group of conserved building blocks that are mediating protein-protein interactions and that are common to a variety of signalling proteins. Thus, they are assuming an important role in regulating many distinct cellular processes that are based on the coordinated interaction of proteins within complex signalling networks.

1.5.1. The SH3 domain

The SH3 domain has a characteristic fold which consists of five or six beta-strands arranged as two tightly packed anti-parallel beta sheets, resulting in a β -barrel-like structure ¹²³. The surface of the SH3 domain bears a relatively flat, hydrophobic ligand-binding surface, which is composed of two ligand-binding pockets (sites 1 and 2) defined by highly conserved aromatic residues (Trp, Tyr or Phe), and a third, more variable specificity pocket (site 3) ¹²⁴⁻¹²⁷.

The core of the proline-rich peptide ligand consists of approximately 10 amino acids and contains the consensus X-P-x-X-P, where X tends to be an aliphatic residue and the two prolines (P) are crucial for high affinity binding ¹²⁷⁻¹²⁹. Peptides associating with the SH3 domain adopt an extended, left-handed polyproline type II (PPII) helix conformation, with three residues per turn. This results in a roughly triangular shape in cross-section, where each of the X-P dipeptides at the base of the triangle is occupying one of the two hydrophobic ligand-binding pockets of the SH3 domain. The third groove of the SH3 domain makes specific contacts with a residue in the ligand distal to the X-P-x-X-P core. Although interactions at this position are more variable, they tend to involve an acidic residue in the SH3 domain and an Arginine, or less often Lysine, in the ligand ^{123, 126, 130-133}.

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Due to the symmetry of the PPII helix conformation, the presence of the basic “specificity” residue at an amino-terminal (R/K-x-X-P-x-X-P) or carboxy-terminal (X-P-x-X-P-x-R/K) position in the peptide gives rise to two possible interaction modes. Depending on the peptide orientation one distinguishes between class I (amino- to carboxy-terminal orientation, i.e. the Arginine fitting into site-3 pocket lies at the carboxy-terminal end of the peptide) and class II (carboxy- to amino-terminal orientation, i.e. the Arginine lies at the amino-terminal end) ligands^{130, 131, 133, 134}.

The Abl SH3 domain is atypical in that it prefers class I ligands in which hydrophobic residues such as methionine or tryptophan contact the specificity pocket. This is because it lacks the conserved acidic residues of other SH3 domains that usually dictate the selection of arginine at this site in the target sequence^{123, 128, 132, 135}.

Generally, the target specificity of particular SH3 domains has been addressed in a host of studies using phage display libraries and combinatorial peptide libraries. Apparently each SH3 domain has a distinct binding preference, the specificity being conferred by the interactions between the non-proline residues in the ligand and the site-3 specificity pocket formed by residues from the RT and n-Src loops, two variable SH3 loops flanking the main hydrophobic binding surface^{124, 127, 132, 136-141}.

Nevertheless, compared to hydrogen-bonding interactions, hydrophobic interactions are generally less specific. As a consequence SH3-peptide binding affinities are quite weak, with K_D values typically ranging from 1 μ M to approximately 10 μ M¹⁴². Often, SH3 domains achieve biological specificity only by exploiting multiple binding sites and tertiary interactions between the parent protein and its target¹⁴².

1.5.2. The SH2 domain

The SH2 domain is a compact structure containing approximately 100 amino acids and recognizing phosphorylated tyrosine residues in specific sequence contexts^{127, 143-148}. Its structure is composed of an amino-terminal α -helix (α A) and a central anti-parallel β -sheet (strands β A- β D), followed by a smaller β -sheet (β D', β E, β F), a second α -helix (α B) and a carboxy-terminal β -strand (β G)^{142, 149-154} (Fig. 1.4). The central β -sheet divides the SH2 domain into two functionally distinct sides, yielding a bipartite structure. One side, flanked by helix α A, is a conserved pTyr binding pocket which is separated from a second, more variable ligand binding surface, that typically engages residues C-terminal to the pTyr. This second side is flanked by helix α B, the

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smaller β -sheet and the loops between helix α B and strand β G, and between the β -strands E and F (called BG and EF loops, respectively).

The bound phospho-peptide usually lies perpendicular to the central β -sheet of the bound SH2 domain in an extended conformation¹⁵⁴. Therefore, the interaction between the SH2 domain and the peptide is largely independent of the context of the native protein from which the peptide is taken.

The interaction of the phosphotyrosine group with the conserved pocket of the SH2 domain is the major driving force of the SH2-ligand binding process¹⁴². Two Arginine residues, one from helix α A and one from strand β B (conserved in all SH2 domains) make key contacts with the pTyr by forming hydrogen bonds with the two phosphate oxygens^{127, 154}. Phosphotyrosine binding alone, however, provides only about half the free energy of binding¹⁵³. Efficient phospho-peptide binding therefore relies additionally on a series of weak interactions between the amino acids immediately C-terminal to the pTyr (P+1 – P+5) and the corresponding residues in the specificity pocket of the SH2 domain. These residues are determining the sequence specificity of the SH2 domain and are located in the EF and BG loops as well as at position β D5 (nomenclature according to the structure of the Src SH2 domain)^{142, 154}. β D5 is a residue that shows contacts with both the P+1 and P+3 residues of the phospho-peptide in many SH2-ligand complexes¹⁵⁵.

Huang et al. recently described the use of an oriented peptide array library (OPAL) to determine the phosphotyrosyl peptide-binding properties of the human SH2 domains¹⁵⁶. In the same publication it was proposed to categorize the human SH2 domains into three major groups, according to their β D5 identity. Group I SH2 domains contain an aromatic residue such as Tyr or Phe at this position and prefer a general consensus $p\phi Y\xi\xi\Phi$, where ξ and Φ denote a hydrophilic and a hydrophobic residue respectively. Group II SH2 domains contain a hydrophobic, but non-aromatic residue such as Ile, Leu, Val, Cys, or Met at β D5. The last major group of SH2 domain, group III, is composed solely of the STAT family of transcription regulators and has a hydrophilic β D5 such as Glu, Gln or Lys.

In summary, the SH2-peptide interaction is of moderate strength with a dissociation constant ranging between 0.1 μ M and 1 μ M, the affinity to phospho-peptides of random sequence being $\sim 1,000$ lower^{127, 142, 157-159}.

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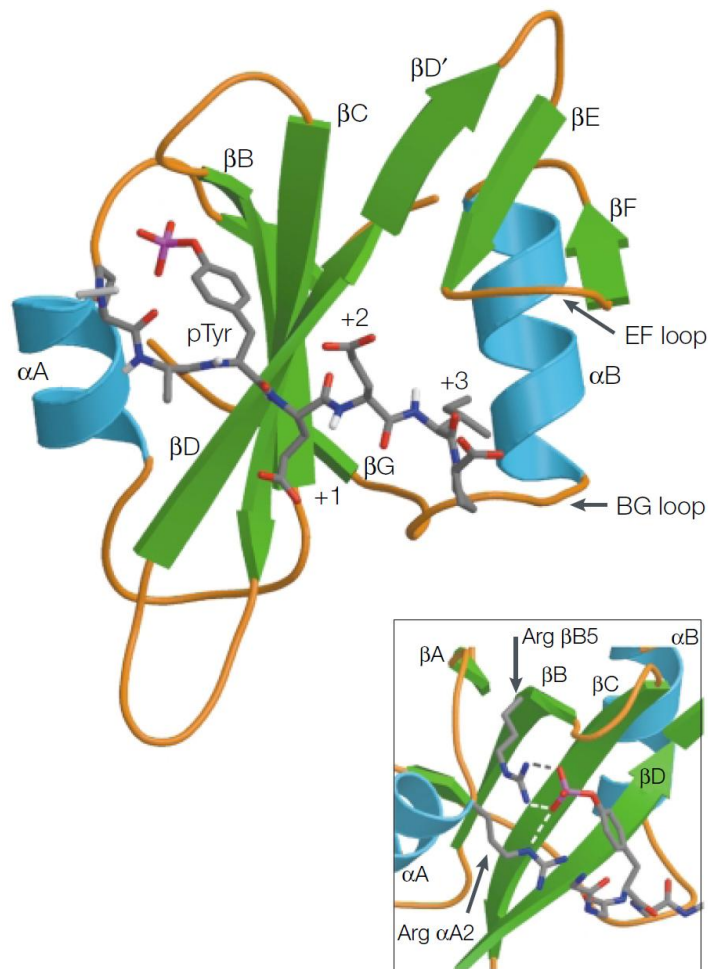


Figure 1.4 **The structure of SH2 domains.** (Taken from Ref.154)

1.6. MicroRNAs

MicroRNAs (miRNAs) are a family of single-stranded non-coding RNAs of 21–25-nucleotides that negatively regulate gene expression at the post-transcriptional level in a sequence-specific manner¹⁶⁰⁻¹⁶⁸. Bioinformatic predictions indicate that the human genome encodes approximately 800-1000 miRNAs which are estimate to regulate roughly ~30% of all protein-coding genes^{162, 169}.

Functional studies have implicated miRNAs as key players in the regulation of a host of cellular pathways and there is increasing evidence, suggesting that miRNA deregulation underlies several human pathologies, including cancer^{165, 166, 170-173}.

1.6.1. Biogenesis and mechanism

With some exceptions, miRNA transcription is usually mediated by RNA polymerase II (Pol II) ¹⁷⁴⁻¹⁷⁶. MiRNA genes are either located in intergenic regions and are transcribed as autonomous transcription units, or they are portions of introns of protein-coding open reading frames (ORFs) ¹⁷⁷⁻¹⁷⁹. Furthermore, many miRNAs are encoded in close proximity to other miRNAs and are transcribed from a single polycistronic transcription unit, ensuring their coordinated expression (e.g. in development) ^{177, 178, 180, 181}.

The primary miRNA transcripts (pri-miRNAs) have stemloop like shapes, contain 5' cap structures, are polyadenylated, and can range in size from a few hundred nucleotides to several hundred kilo bases ^{175, 176, 182}.

Within the nucleus pri-miRNA processing is initiated by a complex consisting of the RNase III type endonuclease Drosha, and its partner DGCR8 ¹⁸³⁻¹⁸⁶. The Drosha-DGCR8 complex catalyses pri-miRNA cleavage, leading to the excision of a ~70nt hairpin precursor RNA (pre-miRNA) with a 2-nucleotide overhang at the 3' end. This overhang is characteristic of RNase III mediated cleavage and serves as structural requirement for recognition by the nuclear export factor exportin5, which transports the pre-miRNA into the cytoplasm ¹⁸⁷⁻¹⁹⁰. There, the pre-miRNAs are cleaved by another RNase III enzyme, Dicer, which interacts with the dsRBD protein TRBP, to yield ~21-bp miRNA:miRNA*(star) duplexes with protruding 2-nucleotide 3' overhangs ¹⁹¹⁻¹⁹⁵. Only one strand is then selected to function as a mature RNA (referred to as the guide strand), while the other strand, known as the passenger strand, or miRNA*, is typically degraded. The strand with thermodynamically less stable base pairing at its 5' end (i.e., more A:U base pairs or mismatches) is usually destined to become the mature miRNA and is chosen for incorporation into a ribonucleoprotein complex (RNP) known as RNA-induced silencing complex (RISC) ^{192, 196, 197}. The so formed complexes are henceforth called miRNPs or miRISCs and their main component beside the miRNA guide strand is the Argonaute family protein AGO2 ^{167, 198-201}.

The assembled miRISC complex accomplishes gene silencing through two major mechanisms, depending on the degree of complementarity between a miRNA and its target mRNA ²⁰²⁻²⁰⁴. In plants most miRNAs exhibit nearly perfect complementarity to their target mRNAs and are therefore triggering RISC mediated endonucleolytic mRNA cleavage (slicing) by an RNAi like mechanism ²⁰⁵⁻²⁰⁷. Silencing is achieved

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through an Argonaute protein, possessing endonucleolytic activity. Mammals contain four Argonaute proteins (AGO1-AGO4), but only AGO2 has an RNaseH-like PIWI domain, which cleaves mRNA at the centre of the siRNA-mRNA duplex²⁰⁸⁻²¹¹. Most animal miRNAs however, usually base pair imperfectly with corresponding binding sites in the 3'UTR of target mRNAs, thereby promoting translational repression rather than active degradation of the mRNA^{165-168, 212} (Fig. 1.5).

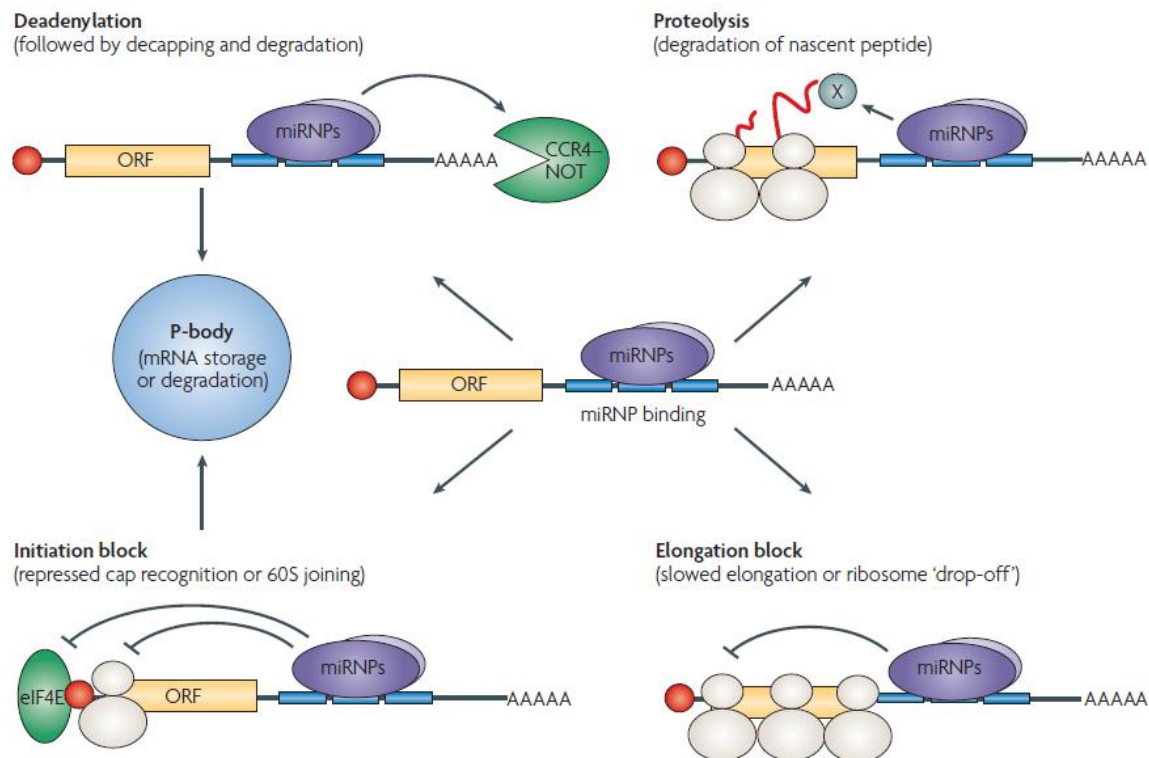


Figure 1.5 **Possible mechanism of microRNA mediated post-transcriptional gene silencing.** (Taken from Ref.167)

The precise mechanism that underlies post-transcriptional gene repression by miRNAs is still a subject of discussion. There is evidence suggesting that translational repression occurs at the initiation step, whereas other studies suggest that miRNAs interfere with translational elongation or termination. Possible mechanisms of miRNA-mediated gene repression include the induction of mRNA deadenylation and decay, the blocked initiation of translation at either the cap-recognition stage or the 60S subunit joining stage, the repression at post-initiations steps owing to either slowed elongation or ribosome “drop-off”, and the proteolytic

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cleavage of nascent polypeptides (extensively reviewed in ¹⁶⁷). Following translational repression, the affected mRNAs are probably moved to so-called P-bodies for either degradation or temporary storage ¹⁶⁷. P-bodies are discrete granules that are localized in the cytoplasm of eukaryotic cells and that were demonstrated to be enriched in mRNA-catabolizing enzymes, AGO proteins, miRNAs, miRNA repressed mRNAs and translational repressors ²¹³⁻²²².

1.6.2. MiRNA target prediction

In order to elucidate the function of a certain miRNA it is inevitable to identify their target genes whose expression it regulates. However, the identification of potential miRNA targets in metazoans is quite challenging, because, as mentioned before, miRNAs are usually imperfectly complementary to their targets.

The interaction between a given miRNA and its target mRNA follows a set of rules determined by experimental and bioinformatic analyses ²²³⁻²²⁷. In animals, the most consistent feature of miRNA-target interaction is base pairing at the 5' end of the miRNA. In particular, perfect and contiguous base pairing of the miRNA 'seed' region, which comprises nucleotides 2-8, is considered to be most important, although not always necessary. Another criterion is the presence of bulges or mismatches in the central region of the miRNA-mRNA duplex, precluding the AGO-mediated endonucleolytic cleavage of mRNA. A third rule is that there must be reasonable complementarity between mRNA and the miRNA 3' half to stabilize the interaction or to compensate for suboptimal matching in the seed region. Finally, effective post-transcriptional repression requires the presence of multiple binding sites for the same or different miRNAs in the mRNA 3'UTR.

Based on these principles, a diverse array of bioinformatic approaches have been applied to develop web-based tools that predict miRNA-target sites. The different algorithms utilize at least some of the following criteria to identify and prioritize putative targets: (a) complementarity between the miRNA seed sequence and the 3'UTR of the target mRNA; (b) the overall stability of putative miRNA-target duplexes; (c) miRNA target site conservation between closely related species; (d) multiple binding sites for a single miRNA within a given target 3'UTR; and (e) weak

or no secondary structure in the target at the miRNA-binding site¹⁶⁶. The advantages and caveats of the different prediction tools are discussed in²²⁸.

1.7. MiRNAs in cancer (leukemia)

MiRNAs are involved in the regulation of cancer-relevant processes such as proliferation, apoptosis and tissue differentiation^{162, 229}, establishing an important role for miRNAs in the pathogenesis of cancer. The examination of miRNA expression profiles in tumor-specific tissues has revealed a widespread dysregulation of miRNA molecules in a broad range of cancers^{230, 231}. Intriguingly, significantly differing miRNA profiles can be assigned to various types of tumors, illustrating the potential for miRNAs to act as novel diagnostic and perhaps prognostic markers^{230, 232}.

The differential expression of miRNA genes in malignant compared to normal cells can at least to some extent be attributed to the location of these genes in cancer-associated genomic regions, to epigenetic mechanisms, and to alterations in the miRNA processing machinery²³².

It has recently been shown that more than 50% of miRNA genes reside in particular genomic regions that are prone to alterations in cancer cells and are often harboring tumour-suppressor genes or proto-oncogenes²³³. Typical locations include minimal regions of amplification, minimal regions of loss of heterozygosity (LOH), common breakpoint regions as well as fragile sites (preferential sites of sisterchromatid exchange, translocation, deletion, amplification or integration)²³³.

Furthermore, the protein machinery that is involved in the biogenesis of miRNAs is quite complex and theoretically, alterations of its components should have dramatic effects on miRNA expression. In fact, altered expression levels of Dicer, Drosha and Argonaute proteins have already been associated with various types of cancer¹⁷⁰.

The third mechanism implicated in the regulation of miRNA expression involves modifications on the DNA and histone level, interfering with miRNA transcription^{232, 234}. Epigenetic markers like DNA hypomethylation, CpG island hypermethylation or aberrant histone-modification are a very common feature of malignant transformation and it is conceivable that such events also affect miRNA expression. Recent publications seem to support this notion^{233, 235, 236}.

Although, for most miRNAs it is still unknown whether they actually play an active role in tumorigenesis, there exist a few miRNAs that have been causatively linked to cancer formation.

1.7.1. MiRNAs as tumor suppressors

MiR-15 & 16 The initial evidence for the involvement of miRNAs in cancer came from a report by Calin *et al.* that showed that the chromosome region 13q14 is deleted in most cases of B-cell chronic lymphocytic leukemia (CLL) ²³⁷. This locus contains two clustered miRNA genes, encoding miR-15a and miR-16-1, and no other genes could be identified in this region. Accordingly, the expression levels of these miRNAs were shown to be reduced in over two thirds of CLL patients ^{237, 238}.

In a recent publication Cimmino *et al.* demonstrated that miR-15a and miR-16-1 negatively regulate BCL2 expression at a posttranscriptional level ²³⁹. BCL2 is an anti-apoptotic protein which is frequently overexpressed in CLL and whose expression levels in CLL are inversely correlated to those of miR-15a and miR-16-1 ²³⁹. This suggests that the deletion of the tumor-suppressor genes *mir-15a* and *mir-16-1* results in increased expression of BCL2, promoting abnormal survival of CLL cells.

Let-7 Humans possess twelve homologs of the *C. elegans let-7* miRNA in eight distinct clusters, four of which are located in genomic regions known to be deleted in cancer ²³³. Emerging evidence suggests that let-7 is a tumor suppressor that plays a critical role in the pathogenesis of lung cancer, reduced let-7 expression being associated with a rather poor prognosis ^{240, 241}. Let-7 is negatively regulating the expression of *MYC* and *RAS*, two key oncogenes in lung cancer that contain multiple let-7 binding sites in their 3' UTR ²⁴².

1.7.2. MiRNAs as proto-oncogenes

MiR-203 A miRNA with potential tumor suppressor activity, implicated in specific hematopoietic malignancies, is miR-203. The *miR-203* gene is located in the 14q32 region of human chromosome 14, a fragile chromosomal region that is especially rich in microRNAs, expressing 52 mature microRNAs²⁴³.

Expression profiling of T-cell lymphoma cells revealed significant silencing of miR-203 due to the loss of one allele and promoter CpG hypermethylation in the remaining allele²⁴³. Also, this miRNA gene was shown to be additionally hypermethylated in CML and B cell acute lymphoblastic leukemia (B-cell ALL)²⁴³, plus, the 14q32 region is frequently lost in CML BC patients^{244, 245}.

In the same study miR-203 was shown to target ABL and BCR-ABL, whose expression levels inversely correlated with the expression level of miR-203²⁴³. Reexpression of miR-203 reduced ABL and BCR-ABL protein levels and inhibited tumor cell proliferation. This antiproliferative effect was partially rescued by BCR-ABL overexpression and fully rescued by a BCR-ABL cDNA without the endogenous 3'UTR²⁴³.

It is therefore conceivable that there exists a specific pressure to inactivate miR-203 in Ph-positive tumors (CML, B-cell ALL) or tumors that overexpress ABL (T-cell lymphomas).

MiR-17-92 MiRNAs with oncogenic potential are expressed from the polycistronic *miR17-92* locus 13q31, which is amplified in lung cancer and several kinds of lymphoma, including diffuse large B-cell lymphoma²⁴⁶⁻²⁴⁸. Also, widespread overexpression of these miRNAs has been observed in diverse types of cancer, including CML^{231, 249}.

In the human genome, the *miR-17-92* cluster encodes six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1) and both, the sequences of these mature miRNAs and their genomic organization, are highly conserved in all vertebrates²⁵⁰.

Transcription of the *miR-17-92* cluster is activated by the oncogenic transcription factor MYC, which regulates cell growth, apoptosis and metabolism, and which is pathologically activated in a large fraction of human malignancies^{251, 252}. MYC is also directing the transcription of *E2F1*, a member of the E2F family of transcription

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factors that control the transition from G1 to S phase of the cell cycle by regulating genes that are involved in DNA replication, cell division and apoptosis²⁵³.

Interestingly, the E2F family transcription factors were among the first verified targets of the miR-17-92 miRNAs^{251, 254, 255}. Also, E2F1 can in turn directly activate the transcription of these miRNAs, establishing a negative feedback loop²⁵⁰. Considering that high levels of E2F1 can induce apoptosis, this circuitry allows MYC to simultaneously activate E2F1 transcription and limit its translation, thereby promoting cell division rather than cell death^{170, 246, 250}.

MiR-155 Another miRNA with oncogenic potential is miR-155, located in the only phylogenetically conserved region of the B cell integration cluster (BIC)²⁵⁶. Overexpression of BIC/miR-155 is linked to MYC overexpression and occurs frequently in B cell lymphomas including Burkitt lymphoma, Hodgkin lymphoma and diffuse large B cell lymphoma (DLBCL)²⁵⁶⁻²⁶¹. It therefore appears that miR-155 is promoting or accelerating lymphomagenesis in cooperation with MYC¹⁷⁰.

MiR-155 has also been reported to be upregulated in tumors of the breast, lung, colon and thyroid, indicating that there are further roles for this miRNA outside of the hematopoietic system^{231, 262, 263}.

MiR-21 Mir-21 is an oncogenic miRNA that has been linked to an array of human cancers. Volinia *et al.* demonstrated that miR-21 is overexpressed in tumors derived from breast, colon, lung, pancreas, stomach, and prostate²³¹. Further support for the function of miR-21 in tumorigenesis came from a series of studies implicating miR-21 overexpression in breast cancer²⁶⁴⁻²⁶⁷, colorectal cancer²⁶⁸, hepatocellular carcinomas (HCC)^{269, 270}, myelomas²⁷¹, cholangiocarcinomas²⁷² and glioblastomas^{273, 274}, as well as myeloproliferative disorders like CLL²⁷⁵, DLBCL²⁷⁶ and CML (data not published). The most prominent miR-21 targets that were identified in the course of these studies are known tumor suppressors like PTEN, TPM1, PDCD4 and maspin (SERPINB5), underlying the oncogenic potential of miR-21. Accordingly, miR-21 was shown to exert an anti-apoptotic function and to promote tumor growth, increased metastasis and increased invasion.

The *miR-21* gene is located on chromosome 17 immediately downstream of the *TMEM49* coding region. The promoter region of *miR-21* contains two STAT3 binding sites and it was shown by Loffler *et al.* that miR-21 expression is induced by IL-6 in a

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STAT3 dependent mechanism²⁷¹. STAT3 is a transcription factor whose activation is essential for cellular transformation and oncogenesis^{277, 278}.

Obviously, many more miRNAs were shown to be either upregulated or downregulated in cancer, but, as pointed out earlier, for the majority of them a causative link to tumorigenesis has yet to be established.

2. Aim of studies

2.1. Tyrosine-phosphorylation of the BCR-ABL SH3 domain

BCR-ABL is a very potent oncogene, which, due to its kinase activity and its multiple functional sites and domains, acts as a central signalling hub in CML cells. Being placed upstream of various signalling pathways it determines the cellular fate, guiding it towards neoplastic transformation. In order for this to happen BCR-ABL relies on its protein modules that undergo extensive interactions with a broad range of molecules, representing the first shell of the oncogenic signalling cascade.

In this study we are focusing on the BCR-ABL SH3 domain and are seeking to elucidate its role in leukemogenesis.

In 2001, in a publication by Li et al., it was shown that GRB2 is tyrosine-phosphorylated in BCR-ABL expressing cells on Tyr209, a site that is located within the GRB2 C-terminal SH3 domain²⁷⁹. Binding of the GRB2 SH2 domain to BCR-ABL phosphotyrosine Y177 was shown to be decisive for GRB2 phosphorylation. The same study revealed that binding of the guanine nucleotide exchange factor SOS to GRB2 was markedly decreased *in vivo* and *in vitro* upon Tyr209 phosphorylation.

Tyr209 of GRB2 is a highly conserved residue among different SH3 domains, and is directly involved in binding PxxP ligands¹³⁰. Bioinformatic analyses revealed that the BCR-ABL SH3 domain contains a tyrosine residue, Tyr134, which is located in the exactly same structural position as Tyr209 of the GRB2 C-terminal SH3 domain (Fig. 2.1).

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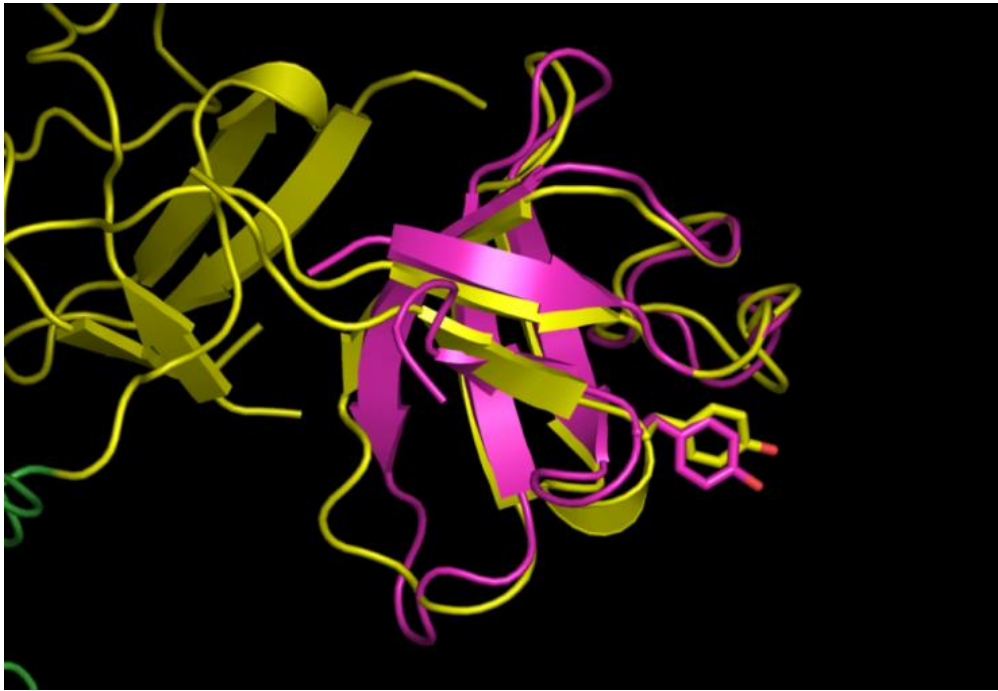


Figure 2.1 **Structural similarity between BCR-ABL SH3 domain and GRB2 C-terminal SH3 domain.** BCR-ABL SH3 domain (pink), GRB2 SH2 domain and C-terminal SH3 domain (yellow).

The resemblance between the two SH3 domains led to the question as to whether Tyr134 is also getting phosphorylated. This question was answered by Steen et al. who mapped BCR-ABL phospho-tyrosine residues, applying phospho-tyrosine specific immunonium ion scanning ²⁸⁰. They identified nine different phosphorylated tyrosine sites, one of which corresponded to Tyr134 of the SH3 domain. Supporting evidence for Tyr134 phosphorylation came from a recent report describing the SFK dependent phosphorylation of the BCR-ABL SH3 domain ²⁸¹. According to their results, Tyr134 is predominantly phosphorylated by the SRC family kinase HCK. Considering that phosphorylation of the GRB2 C-terminal SH3 domain interferes with the GRB2 binding pattern it is tempting to speculate that a similar effect might be observed for BCR-ABL. According to our model there exist two plausible scenarios of how Tyr134 phosphorylation could affect interactions mediated by the SH3 domain. One possibility is that phosphorylation of Tyr134, either through BCR-ABL (trans-)autophosphorylation or by SFKs, could lead to a 'loss-of-function' scenario, where, similar to the decreased SOS binding observed for the GRB2 C-terminal SH3 domain, SH3 dependent interactions of BCR-ABL with other proteins may no longer

2. AIM OF STUDIES

be possible or at least be markedly decreased. On the other hand, it is also conceivable that phosphorylation of the BCR-ABL SH3 domain results in a 'gain-of-function' situation, where phosphorylated Y134 serves as a docking site for a different set of interactors. In the latter case, new binding partners would be expected to contain an SH2 domain or a different phosphotyrosine recognizing module.

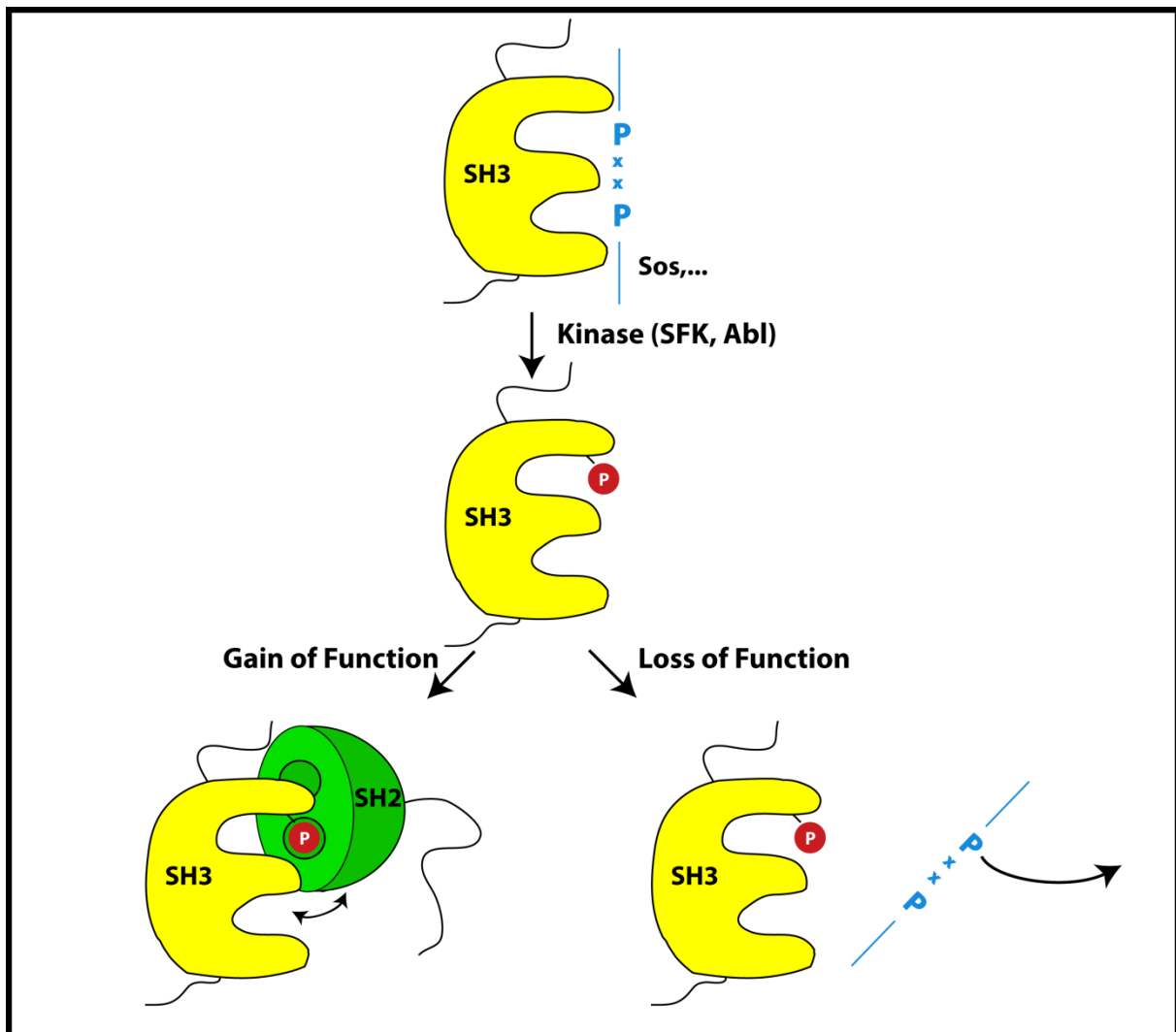


Figure 2.2 Project outline.

In a recent publication by Donaldson et al. an interaction between the ABL SH3 domain and the SH2 domain of the adaptor molecule CRK-II was shown to occur²⁸². Upon tyrosine-phosphorylation of CRK-II by ABL, autorecognition of the newly phosphorylated site by the CRK-II SH2 domain induced an intramolecular reorganization²⁸²⁻²⁸⁴. A conformational change in the CRK-II SH2 domain led to the

exposure of a proline-rich loop located between the β D and β E strands of the SH2 domain (DE loop)^{282, 285}. This extended 20-residue DE loop is unique to the mammalian CRK-II SH2 domain and was shown to be recognized by the regulatory SH3 domain of ABL, yielding a ternary complex consisting of the ABL SH3 domain, the CRK-II SH2 domain and a CRK-II phosphopeptide^{282, 285}.

Although the formation of this complex differs in its mechanism from the proposed interaction occurring between the phosphorylated BCR-ABL SH3 domain and SH2 domains of potential interactors, it nevertheless proves that such modular binding domains may have modes of recognizing biological partners that are beyond their canonical ligand binding.

Based on the observations made by Li et al. and Steen et al. we are predicting that phosphorylation of Y134 of the BCR-ABL SH3 domain affects the SH3 binding pattern. In particular we were interested in the 'gain-of-function' scenario, our focus being directed to the identification and characterization of novel, SH2 domain harboring, interactors of BCR-ABL, which bind in the presence of phosphorylated Y134 only.

2.2. MiR-21

MicroRNAs (miRNAs) are an abundant class of short nonprotein-coding RNAs (ncRNAs) mediating posttranscriptional silencing of target genes. They have been shown to play key regulatory roles in a diverse range of pathways, including proliferation, apoptosis, differentiation, and cell fate determination^{162, 229}. As a consequence, miRNA deregulation has immediate implications for a many physiologic processes. There is emerging evidence that aberrant miRNA expression is causatively linked to the pathogenesis of a variety of disorders, including human malignancies^{230, 231}. In this context miRNAs can function as tumor suppressors or oncogenes, depending on whether they specifically modulate the expression of tumor suppressor genes or oncogenes^{170, 173}.

Considering that characteristic miRNA expression profiles can be obtained for particular tumor types, miRNAs may serve as biomarkers for diagnostics and to

2. AIM OF STUDIES

predict clinical outcomes, and may eventually be exploited as therapeutic targets^{230, 232}.

Besides the diagnostic and prognostic significance of miRNAs, defining disease specific miRNA signatures will hopefully provide new insights in the pathogenesis of diseases in which the etiology is only incompletely understood.

In the present study we are focusing on the identification and eventual characterization of miRNAs that are deregulated in the presence of BCR-ABL in chronic myeloid leukemia.

It is known that miRNAs play an important role in the regulation of hematopoiesis, and there is rapidly accumulating evidence to suggest that dysfunctional expression of miRNAs is a common feature in hematological malignancy^{286, 287}.

In a recent publication by Venturini et al. aberrant expression of the miR-17-92 polycistron in CML has been reported²⁴⁹. BCR-ABL positive cell lines were treated with either Imatinib or anti-BCR-ABL shRNA, followed by subsequent microarray analysis and miRNA-specific quantitative real-time reverse transcriptase PCR (miR-qRT-PCR)²⁴⁹. According to this study, inhibition of BCR-ABL tyrosine kinase activity results in decreased expression of the miR-17-92 miRNAs, indicating that miRNAs from this cluster are upregulated in CML.

In an earlier study we investigated how Dasatinib treatment affects the gene expression pattern of K562 cells, a BCR-ABL positive CML cell line. Microarray-based gene expression profiling showed that many genes are subject of significant deregulation in these cells. According to the microarray data, expression of the transmembrane protein *TMEM49* is supposedly downregulated by a factor of 3.5 in Dasatinib treated cells compared to normal K562 cells. However, sequence analysis of the corresponding probe revealed that it actually measured the expression of miR-21, a microRNA, whose coding sequence is located immediately downstream of *TMEM49*. Therefore, we speculated that miR-21 is upregulated in CML, the increased expression being caused by the catalytic activity of BCR-ABL.

3. Materials and Methods

3.1. Cell biology

3.1.1. Cell lines and cell culture

K562 human CML cell line expressing endogenous BCR-ABL, established from a CML patient in blast crisis, was obtained from DSMZ (Braunschweig, Germany) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Invitrogen) at 37°C in a 5% CO₂ incubator. Cells were kept at subconfluency by splitting them every 2-3 days, three times a week.

3.1.2. Kinase inhibitors

Dasatinib (Sprycel, BMS-354825) and Nilotinib (Tasigna, AMN107) were synthesized by WuXi PharmaTech (Shanghai, China). Kinase inhibitors were prepared as 20 mM stock solution in DMSO and diluted in the respective cell culture medium to final concentrations of 100 nM for dasatinib and 1 µM for nilotinib for all experiments. Subconfluent cells were treated with the drugs for 4 hours for RNA isolation.

3.2. Protein biochemistry

3.2.1. Cell lysates for protein gels

Lysis buffer was prepared by mixing the following protease inhibitors to immunoprecipitation buffer (IPB; 50 mM Tris/HCl, pH 7.5; 150 mM NaCl; 1% (v/v) Nonidet-P40; 5 mM EGTA, pH 8.0; 5 mM EDTA, pH 8.0, 50 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF (Sigma), 5 µg/ml TLCK (Roche), 10 µg/ml TPCK (Biomol), 1 µg/ml leupeptin, 1 µg/ml aprotinin and 10 µg/ml soybean trypsin inhibitor (all Roche)). Cells were harvested and washed in 10 ml PBS (Invitrogen). Then the pellet was resuspended in 50 µl to 200 µl ice cold lysis buffer, to reach a concentration of approximately 1×10^5 cells/µl. The cell pellet was resuspended well to achieve good cell lysis and the mixture was immediately centrifuged for 10 min at 13 000 rpm at 4°C. The supernatant containing the proteins was transferred into a new vial and the protein concentration was measured using Bradford assay (Bio-Rad) according to the manufacturer's instructions using a solution of 1 µg/ml BSA as standard. For normalization, the samples were diluted in IP-buffer and the appropriate amount of 4x SDS sample buffer (200 mM Tris/HCl, pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, brom phenol blue, 1.4 M β-mercaptoethanol) was added to reach a protein concentration of 100 µg/ 20 µl. The samples were heated at 95°C for 4 minutes and directly loaded on the acrylamide gel or stored at -20°C. Cell lysates containing 100µg total protein were loaded on a discontinuous polyacrylamide gel consisting of 7% - 15% separating gel (depending on size of protein of interest) and 5% stacking gel. As weight marker 5 µl PageRuler Prestained Protein Ladder (Fermentas) was used.

3.2.2. Recombinant proteins

To construct GST-SH3 fusion proteins, cDNAs encoding the full-length BCR-ABL SH3 domain and the GRB2 C-terminal SH3 domain were inserted into pETM30 in-frame with GST. GST and GST-SH3 constructs were expressed in *E. coli* BL21(DE3), and extracted using a French Press in Ni-wash buffer (50 mM Tris pH

3. MATERIALS AND METHODS

7.5; 500 mM NaCl; 20 mM imidazole; 2 mM β -mercaptoethanol; 10% (v/v) glycerol). Each extract was purified via FPLC using HisTrap HP Columns (1ml, GE Healthcare). After washing the column, adsorbed proteins were eluted with a linear gradient from 20 mM imidazole to 500mM imidazole (elution buffer: 50 mM Tris pH7.5; 500 mM NaCl; 5% (v/v) glycerol; 5 mM β -mercaptoethanol; 500 mM imidazole). The resultant proteins were exchanged into storage buffer (20mM Tris/HCl pH7.5; 150 mM NaCl; 1 mM DTT) by dialysis. Concentrations of proteins were determined using Bradford assay (Bio-Rad) according to the manufacturer's instructions using a solution of 1 μ g/ml BSA as standard.

3.2.3. Peptide pull-downs

All peptides used in the pull-down assays were synthesized and quantified by Eurogentec (Belgium). Peptide immobilization was performed using the SulfoLink® Immobilization Kit for Peptides (Pierce), according to manufacturer's instructions. Each peptide had a single carboxy-terminal cysteine residue that was used for coupling to the SulfoLink coupling resin with a free sulfhydryl group at a concentration of 1 mg of peptide per 1ml of gel. In the peptide binding assays, coupled peptides were incubated with K562 cell lysates for 4 h and after washing three times with 1x PBS (Invitrogen) the protein complexes were eluted by 1% SDS and analyzed by LC-ESI-MS/MS and SDS-PAGE.

3.2.4. *In vitro* protein-binding assay (GST pull-down)

Glutathione-Sepharose resin (Amersham Biosciences) was incubated with purified recombinant GST or GST-SH3 proteins (each 500 μ g) in the binding buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5% (vol/vol) glycerol, 0.2% (vol/vol) Nonidet-P40, 1.5 mM $MgCl_2$, 25 mM NaF, 1mM Na_3VO_4 and protease inhibitors) for 1 h. The coupled proteins were incubated with K562 cell lysates for 4h and after washing three times with the binding buffer the protein complexes were analyzed by LC-ESI-MS/MS and SDS-PAGE.

3.2.5. Coimmunoprecipitations

HEK-293-null cells (InvivoGen, San Diego, CA) were transfected with pCS2-6xmyc-BCR-ABL using Lipofectamine (Invitrogen). 48 h later, cells were lysed in immunoprecipitation buffer (IPB; 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 5mM EGTA, 5 mM EDTA, 50 mM NaF, 1mM Na₃VO₄, 1 mM PMSF, 5 µg/ml TLCK, 10 µg/ml TPCK, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 10 µg/ml soybean trypsin inhibitor). After centrifugation, soluble cell lysates (5mg or 10mg) were immunoprecipitated for 5-6 h with anti-MYC antibodies precoupled to agarose (Sigma). Beads were washed twice with IPB. Proteins were eluted with SDS sample buffer, subjected to SDS/PAGE, and immunoblotted with anti-MYC, anti-c-ABL, anti-PLCG1 and anti-SHP2 antibodies.

3.2.6. *In vitro* phosphorylation assay

Recombinant His-GST-SH3 fusion proteins were phosphorylated *in vitro* by incubating with the catalytic subunit from Abelson tyrosine kinase (ABL-CD). *In vitro* phosphorylation was performed for 30 min at room temperature in kinase assay buffer (20 mM Tris/HCl pH7.5, 10 mM MgCl₂, 1 mM DTT) in the presence of unlabeled ATP (50 µM) and radioactively labeled [γ -³²P]ATP (3000Ci/mmol, 10mCi/ml). After 30 min TEV protease mediated cleavage of the fusion proteins was performed for additional 90 min at room temperature. The reactions were terminated by adding 4× SDS sample buffer and boiling for 4 min. The samples were analyzed by Tricine-SDS-PAGE on 16% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue and dried, followed by autoradiography. To prevent gel cracking during the drying process the gel was kept in methanol solution (40% (vol/vol) MeOH, 10% (vol/vol) acetic acid, 3% (vol/vol) glycerol) for at least 1 h before using the gel-drier. Tricine-SDS-PAGE was performed as described ²⁸⁸.

3.2.7. Immunoblotting

After being resolved by SDS-PAGE samples were transferred to a nitrocellulose membrane (Whatman Schleicher & Schuell) using a semi-dry blotting apparatus. Membrane and gel were sandwiched by 2 layers of Whatman paper and two sponges. The transfer was carried out for 1 – 1.5 h at 1 mA/cm² (transfer buffer: 2.5 mM Tris, 15 mM Glycine, 10% (v/v) methanol). The quality of the transfer was assessed by Ponceau staining. The membrane was blocked in blocking solution for 20 min at least. Primary antibodies were diluted in blocking solution and incubated with the membrane overnight at 4°C. After washing the membrane three times in PBST (0,1% Tween-20 (Sigma) in PBS), the primary antibody was detected using an Alexa Fluor 680-labeled goat anti-mouse antibody (Invitrogen) diluted 1:7000 in PBST and incubated 1 h at room temperature in the dark. Finally, the blot was again washed in 3x in PBST and scanned by the Odyssey Infrared Imaging System (Li-Cor Biosciences). BCR-ABL was detected using the mouse monoclonal antibody (clone 21-63) directed against c-ABL (diluted 1:7000 in 3% (w/v) BSA in PBST). Mouse anti-PLCG1 (#610028, 1:250 in 5% (w/v) dry milk in PBST) and mouse anti-SHP2 (#610622, 1:2500 in 5% (w/v) dry milk in PBST) antibodies were obtained from BD Transduction Laboratories.

3.3. Genomic analysis

3.3.1. MicroRNA extraction

For isolating the miRNA, mirVana™ miRNA Isolation Kit from Ambion was used, extraction was performed by following the manufacturer's protocol. The kit allows the isolation of small RNA-containing total RNA through organic extraction followed by glass fiber filter mediated purification.

3.3.2. Quantitative Real-Time PCR (qRT-PCR) of miR-21

Reverse transcription of miRNAs was performed using the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's recommendations. Briefly, 10 ng of total RNA were combined with dNTPs, MultiScribe™ reverse transcriptase, and the miR-21 specific primers. The resulting cDNA was diluted 15-fold and used in PCR reactions. PCR was performed according to the manufacturer's recommendations (Applied Biosystems). Briefly, cDNA was combined with the TaqMan™ assay specific for the miR-21, and PCR reaction was done using the Rotor-Gene 6000 (Corbett). Expression of mature miR-21 was normalized using the $\Delta\Delta C^T$ method relative to RNU43 snoRNA, which was nearly equally expressed in untreated and Dasatinib and Nilotinib treated cells. TaqMan PCRs for miR-21 and RNU43 were performed in triplicate for each condition (i.e. untreated, Dasatinib, Nilotinib).

3.3.3. MiR-21 mimics and antagomirs

MiRIDIAN™ mimic, inhibitor, and negative control (Dharmacon) for miR-21, were transfected into K562 cells using Hiperfect (Qiagen). According to the manufacturer's instructions the molecules were dissolved in ultra pure water to obtain a 20 μ M stock solution and kept at -20°C. Briefly, 1×10^6 cells were suspended in 500 μ l of RPMI 10% FCS and combined with a mixture of 500 μ l RPMI w/o FCS, 18 μ l Hiperfect and 5 μ l of the 20 μ M miRIDIAN™ stock solutions (leading to 100 nM miRIDIAN™ solutions). After vortexing and 10 min incubation the cell suspensions were transferred into 6-well plates and incubated at 37°C. After 6 h 2 ml of pre-warmed RPMI 10% FCS were added to the cell suspensions. Transfection was repeated after 24 h. Cells were harvested 72 h after the first transfection.

3.3.4. MicroRNA profiling

Total RNA was isolated using Trizol reagent (Invitrogen) and further purified using by purification over a glass fiber filter column (mirVana; Ambion). Purified RNAs were

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sent to EXIQON (Denmark) for miRNA expression analysis. MiRNA microarray including labeling, hybridization, scanning, normalization and data analysis was carried out by EXIQON. Briefly, RNA Quality Control is performed using Bioanalyser 2100 and Nanodrop. The samples were labeled using the mercury[™] Hy3[™]/Hy5[™] labeling kit and hybridized on the miRCURY[™] LNA (locked nucleic acid) Array (v.10.0). Three independent hybridizations for each sample were performed on chips with each miRNA spotted in quadruplicate. Labeling efficiency was evaluated by analyzing the signals from control spike-in capture probes. LNA-modified capture probes corresponding to human, mouse, and rat mature sense miRNA sequences were spotted on slides. The resulting signal intensity values were normalized to per-chip median values and then used to obtain geometric means and standard deviations for each miRNA. Triplicate arrays were performed under each treatment condition.

4. Results

4.1. Role of the tyrosine-phosphorylated BCR-ABL SH3 domain

The aim of the present study was to assess the potential interference of SH3 domain phosphorylation with the binding profile of BCR-ABL. In this regard we initially aspired to address both, the gain-of-function scenario as well as the loss-of-function scenario, upon Tyr134 phosphorylation.

4.1.1. *In vitro* phosphorylation and GST pull-down

First, the SH3 domain of BCR-ABL and the carboxy-terminal SH3 domain of GRB2 were cloned into a bacterial expression vector, and following IPTG induction, the amino-terminal His-tag and Glutathion-S-Transferase (GST) tagged SH3 constructs were purified via Fast Protein Liquid Chromatography (FPLC) (Fig. 1A). In addition to the wild-type forms of the two SH3 domains, constructs were generated, where the corresponding tyrosine residues, Tyr134 of BCR-ABL and Tyr209 of GRB2, respectively, were replaced by a phenylalanine.

In a next step we intended to *in vitro* phosphorylate the purified SH3 domains stoichiometrically with a recombinant and constitutively active ABL kinase domain (ABL-CD). The phosphorylated SH3 constructs were then supposed to be immobilized on glutathione-coupled sepharose, exploiting the GST tag of the SH3 fusion proteins. Total cell lysates from K562 cells were then planned to be subjected to GST pull-down analyses, allowing the identification of specific interactors of the

4. RESULTS

phosphorylated and unphosphorylated BCR-ABL and GRB2 SH3 domains by mass-spectrometry (Fig. 4.1).

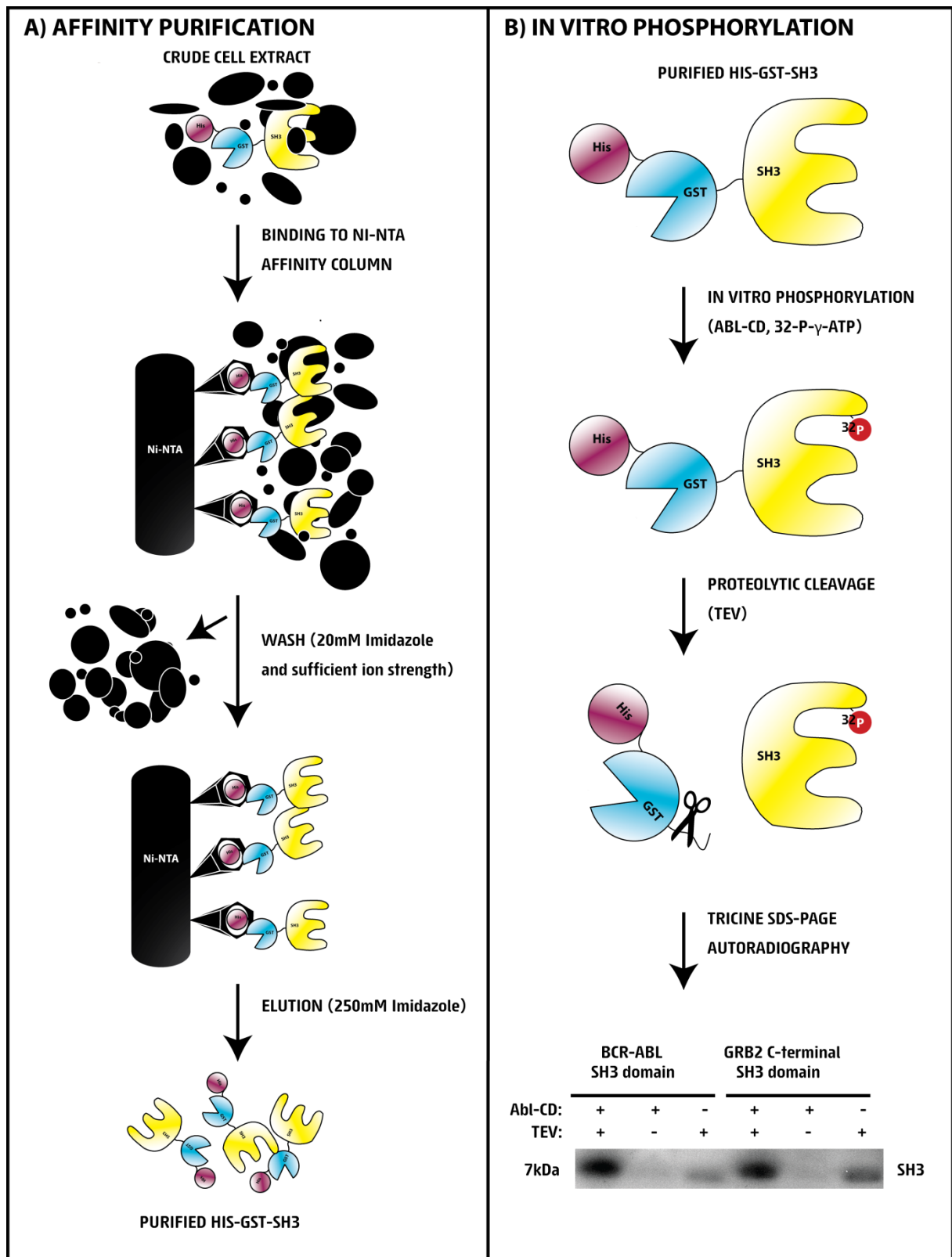


Figure 4.1 Recombinant protein purification and in vitro phosphorylation.

4. RESULTS

To test the efficiency of SH3 *in vitro* phosphorylation, the purified SH3 domains were incubated with ABL-CD and either cold or radioactively labeled ATP. The presumably phosphorylated SH3 domains were subsequently separated from their amino-terminal tags by the action of recombinant TEV protease. The TEV cleavage site usually consists of the amino acid sequence ENLYFQG, the cleavage occurring between the Q and the G residues. The tyrosine residue within this sequence was mutated beforehand to a phenylalanine, preventing its interference with the *in vitro* phosphorylation of the SH3 domain.

Finally, detection of phosphorylated SH3 was accomplished by immunoblotting or autoradiography. *In vitro* phosphorylation with unlabeled ATP did not yield any SH3 assignable signal on a Western Blot (data not shown). Radiolabeling on the other hand, proved to be more reliable, showing a clear difference in the signal intensity between phosphorylated and unphosphorylated SH3 constructs (Fig. 1B). Nevertheless, phosphorylation efficiency was significantly lower for the SH3 domain than for either the GST part of the protein or the positive control CRK-II, a known substrate of BCR-ABL (data not shown).

To summarize, the stoichiometric phosphorylation of the SH3 domain as well as the detection of phosphorylated SH3 revealed to be rather problematic. The relatively low molecular weight of the BCR-ABL and GRB2 SH3 domains (6.6 and 7.4 kDa, respectively) and the probable inefficiency of SH3 domain phosphorylation by ABL-CD were decisive in reasoning against this approach.

4.1.2. Peptide pull-downs

Because of the setbacks encountered in the GST pull-down approach, a different strategy had to be designed to analyze the relationship between SH3 domain phosphorylation and the BCR-ABL interaction profile. As a consequence we decided to merely concentrate on the gain-of-function situation where SH2 domain containing proteins are supposed to recognize the tyrosine-phosphorylated form of the BCR-ABL SH3 domain.

The interaction between SH2 domains and their respective phosphopeptide ligands can be described as an interaction occurring between short unstructured amino acids

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and modular protein domains that are specialized on peptide binding. In search for BCR-ABL interactors that contain such SH2 modules and are recognizing phosphorylated Y134, the use of synthetic peptides as baits in affinity pull-down experiments appeared to be a straightforward method.

Because of their bipartite binding surface, SH2 domains tend to bind phosphotyrosine residues depending on their sequence context. In a recent publication the sequence specificity of 76 human SH2 domains was assessed using an oriented peptide array library (OPAL). Based on the obtained data a web based prediction tool was established (<http://lilab.uwo.ca/SMALL.htm>), predicting which SH2 domains potentially interact with a query phosphopeptide. For the Y134 phosphopeptide of the BCR-ABL SH3 domain it predicted to be recognized by the carboxy-terminal SH2 domains of RASA1 and PLCG1, and the CRK SH2 domain.

In order to verify those predicted interactions, and to see in general which other SH2 domains might be recruited to the BCR-ABL SH3 domain, synthetic peptides, 12 amino acids in length, were ordered from Eurogentec. The sequence of the peptides corresponded to the region of interest of the BCR-ABL SH3 domain and the GRB2 carboxy-terminal SH3 domain, harboring Y134 and Y209, respectively (Fig. 4.2). The synthetic peptides were used in 'active' (i.e. phosphorylated) and 'control' (i.e. non-phosphorylated) forms as baits in affinity pull-down experiments to reveal direct binders of the phosphorylated SH3 domains. In the 'active' peptides a phosphate was covalently attached to the tyrosine's hydroxyl group, whereas in the 'control' peptides the respective tyrosine residue was substituted by a phenylalanine.

The peptides were subsequently coupled to agarose beads using the SulfoLink® Immobilization Kit for Peptides from Pierce Biotechnology. All peptides contained a cysteine residue at position 12, allowing the formation of a thioether bond with the iodoacetyl-group of the agarose beads.

Pull-down experiments were performed exposing the immobilized bait peptides to total cell extracts from K562 cells. Following LC-ESI-MS/MS analysis of the eluted protein complexes and subtraction of the proteins found in the respective control pull-downs, a list of potentially specific interactors was obtained for the BCR-ABL and the GRB2 SH3 phosphopeptides. According to our model, such specific phosphopeptide interactors are expected to contain at least one phospho-tyrosyl binding domain.

4. RESULTS

Therefore, in a final step, domain analyses of the potential interactors was performed, yielding two SH2 harboring proteins in the case of the BCR-ABL phosphopeptide and three SH2 harboring proteins for the GRB2 phosphopeptide.

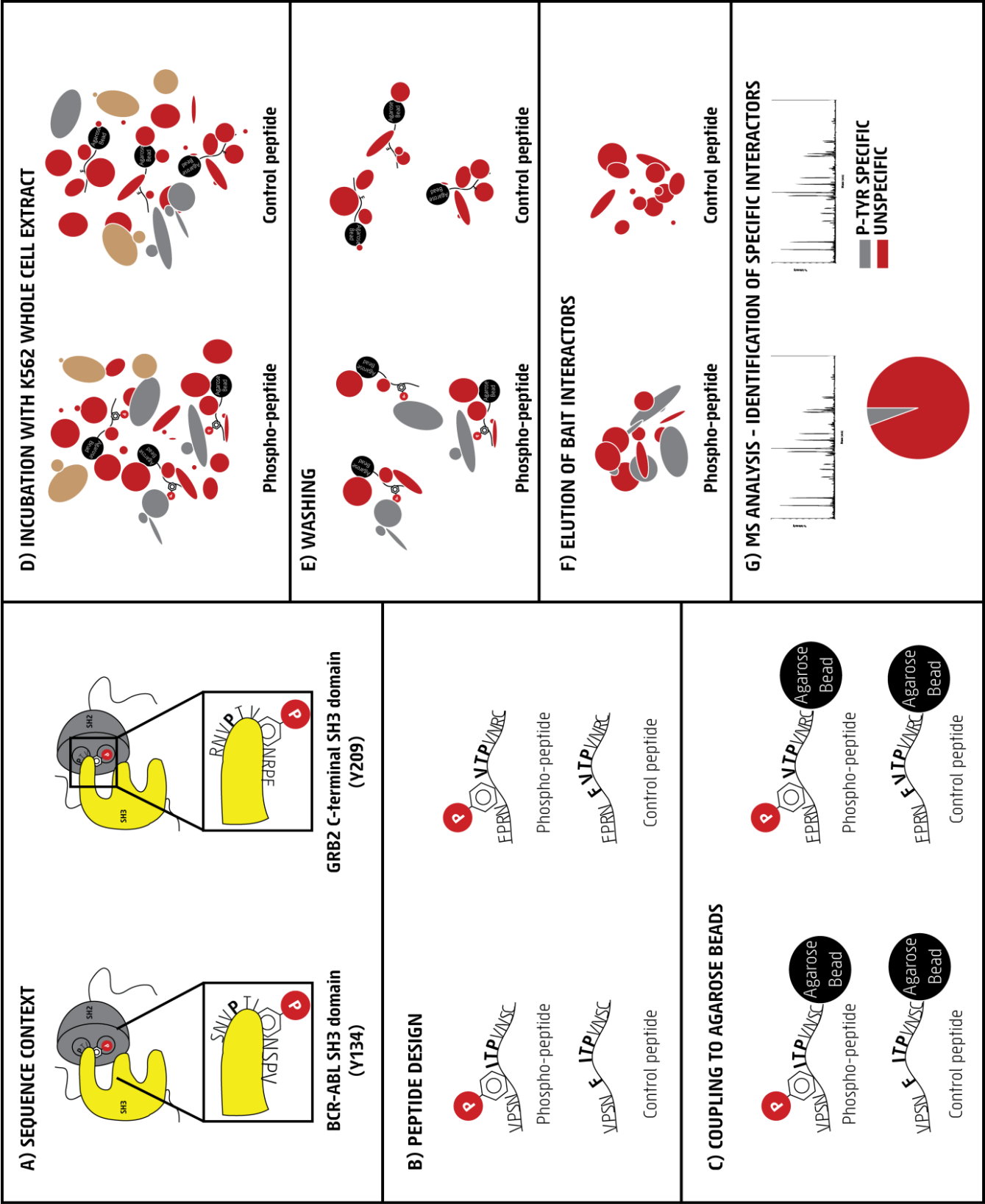


Figure 4.2 Peptide pull-down.

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For the BCR-ABL SH3 domain, 659 protein groups were identified in the phosphopeptide pull-down and 638 protein groups were found in the control pull-down. In the case of the GRB2 carboxy-terminal SH3 domain the number of identified protein groups amounted to 712 for the phosphopeptide pull-down, and 641 for the control pull-down.

After subtraction of the corresponding control pull-downs, 44 and 77 protein groups were listed in the dataset of the BCR-ABL and GRB2 phosphopeptide pull-downs, respectively. Following domain analyses all proteins that were not containing a phospho-tyrosyl binding domain were excluded from that list, leaving us with those proteins whose interaction with the SH3 domains of BCR-ABL and GRB2 is likely to require a phosphotyrosine docking site.

The phospholipase PLCG1 and the protein phosphatase PTPN11 (also called SHP2) were the only two proteins fulfilling those criteria for the BCR-ABL phosphopeptide. Both candidates are multidomain proteins that in addition to their catalytic domains contain two SH2 domains. Strikingly, both proteins were found to bind also to the Tyr209 phosphopeptide of GRB2, which was additionally recognized by the adaptor protein CRK-II.

PLCG1 and SHP2 were the most frequent proteins in the Tyr134 phosphopeptide pull-down sample, the unspecific interactors not taken into account (Table 4.1). A similar situation was observed for the GRB2 phosphopeptide, where PLCG1 and CRK-II were among the most frequent of the specific interactors (Table 4.2)

To confirm the interaction between the BCR-ABL and GRB2 phosphopeptides and PLCG1 and SHP2, 2% of the pull-down eluates were subsequently analyzed for the presence of PLCG1 and SHP2 on a Western Blot (Fig. 4.3). Both proteins were detected in the phosphopeptide pull-down samples only, with no signal being spotted in the control samples. The detection of PLCG1 and SHP2 in the Tyr134 and Tyr209 phosphopeptide eluates came not unexpectedly, given that, depending on the antibody, the immunoreactive detection of a certain protein is expected to be more sensitive than the mass spectrometry based approach. However, the renewed failure to detect PLCG1 and SHP2 in the control pull-downs, even by this, more sensitive, method, was corroborating the initial notion that both proteins bind to the SH3 domains of BCR-ABL and GRB2 in the tyrosine phosphorylated state only.

4. RESULTS

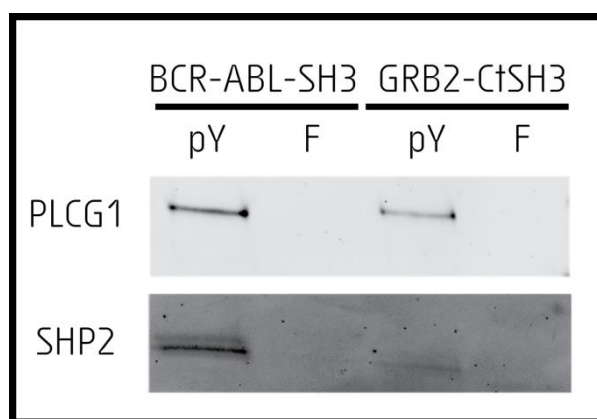


Figure 4.3 Immunoblot analysis of pull-down eluates.

Beyond the structural similarity between the two SH3 domains, Tyr134 of the BCR-ABL SH3 domain and Tyr209 of the GRB2 carboxy-terminal SH3 domain are in addition located in a comparable sequence context. As mentioned earlier, the interaction between an SH2 domain and a phospho-tyrosine containing site is depending on the identity of the amino acids immediately C-terminal to the tyrosine residue. Therefore, we expected that SH2 domains binding to phosphorylated Tyr134 of BCR-ABL are also recognizing, at least to some extent, the phosphorylated form of Tyr209 of GRB2. The fact that PLCG1 and SHP2 were indeed recruited by both phosphopeptides confirmed us in our intention to further evaluate their interaction with the BCR-ABL SH3 domain.

To confirm the specific interaction between the tyrosine-phosphorylated BCR-ABL SH3 domain and PLCG1 and SHP2, additional peptide pull-downs were conducted, the experimental design remaining unchanged, with the exception of the GRB2 peptides that were no longer included.

Although peptide count and sequence coverage did not measure up to the numbers of the initial run, PLCG1 and SHP2 were nevertheless unambiguously shown to specifically bind the phosphorylated form of Tyr134 of the BCR-ABL SH3 domain (data not shown). The apparently decreased affinity of PLCG1 and SHP2 to the BCR-ABL phosphopeptide might be on account of the instability of the phosphopeptides after an extended period in solution.

4. RESULTS

Table 4.1 Specific interactors of the BCR-ABL Y134 phosphopeptide.

Potein-Name	Peptide Count	Sequence-Coverage (%)	CDD-Domains	SMART-Domains
PLCG1	31	22,64	PLCc, SH2, SH3, PH_PLC, C2_2	PLCYc, PLCXc, C2, SH3, PH, SH2
PTPN11	10	18,89	SH2, PTPc, PTPc	PTPc, SH2
CAP1	6	16,63	CARP, CAP	CARP
PHGDH	5	10,32	2-Hacid_dh_C, 2-Hacid_dh, SerA	
GLUD1	5	8,78	GLFV_dehydrog_N, GdhA	
GLUD2	5	8,78	GLFV_dehydrog_N, GdhA	
TKT	5	8,51	Transketolase_C, Transket_pyr, TPP_TK, TktA	
SFPQ	5	7,92	RRM, RRM_1	RRM
G6PD	5	8,07	G6PD_C, G6PD_N	
EEA1	5	3,61	COG4372, Smc, FYVE	FYVE, ZnF_C2H2
LACTB	3	8,04	Beta-lactamase	
GTPBP6	3	9,18	HflX, HflX	
HRMT1L2	3	9,62	COG4076	
FAM98A	3	7,92		
FKBP5	3	7,22	TPR, FKBP_C	
DLD	3	6,29	Pyr_redox_dim, Lpd, THI4	
DDX28	3	6,30	DEADc, HELICc	HELICc, DEXDc
XPNPEP3	3	6,11	PepP, AMP_N, Prolidase	
MRE11A	3	5,29	Metallophos, Mre11_DNA_bind	
TRIP4	3	4,65	Zf-C2HC5	
SOD1	2	18,18	Cu-Zn_Superoxide_Dismutase	
TCEB2	2	13,66	ElonginB	UBQ
SNRPB	2	12,08	Sm_B	Sm
GSTO1	2	9,96	GST_N_Omega, GST_C_Omega, Gst	
UFD1L	2	8,79	UFD1	
SF3B4	2	5,90	RRM	RRM
KPNA6	2	5,22	IBB, ARM, SRP1	ARM
LOC728340	2	5,06		VWA
LOC730394	2	5,06		VWA
GTF2H2	2	5,06	vWA_transcription_factor_IIH_type, SSL1	VWA
LOC653866	2	5,06		VWA
DKFZP686 M0199	2	5,00		VWA
SGPL1	2	4,58	Pyridoxal_deC	
KPNA1	2	4,65	IBB, ARM, SRP1	ARM
PSMD5	2	4,37		
CBS	2	3,81	COG3620, PALP, COG4109, CBS, CysK	CBS
GMPS	2	3,46	GMP_synthase_C, GuaA, GATase1 GMP_Synthase	
ZNF689	2	3,20	COG5048, KRAB	KRAB, ZnF_C2H2
HYOU1	2	2,90	HSP70	
APLP2	2	2,62	KU, A4_EXTRA	KU, A4_EXTRA
SEC3L1	2	2,28		
LOC91664	2	1,77		KRAB, ZnF_C2H2
KTN1	2	1,69	Smc	
SMARCA4	2	1,34	DEXHc, SNF2_N, HELICc, BRK, BROMO, HSA	HSA, BROMO, HELICc, BRK, DEXDc

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Table 4.2 Specific interactors of the GRB2 Y209 phosphopeptide.

Protein-Name	Peptide Count	Sequence-Coverage (%)	CDD-Domains	SMART-Domains
PLCG1	36	29,38	PLCc, SH2, SH3, PH_PLC, C2_2	PLCYc, PLCXc, C2, SH3, PH, SH2
DSP	8	3,45	Smc, SPEC, PLEC	PLEC, SPEC
RENT1	7	5,81	DEXDc, UvrD-helicase, COG1112	
CRK	6	36,27	SH2, SH3	SH3, SH2
VSIG8	6	20,05		IG, IGc2
LRRC15	6	14,63	LRR_RI	LRR_TYP, LRRNT, LRRCT
JUP	6	11,54	VATPase_H, ARM	ARM
MCM5	6	9,67	MCM	MCM
MRE11A	5	7,94	Metallophos, Mre11_DNA_bind	
TBL2	5	13,65		WD40
TOE1	5	12,75	CAF1	
G6PD	5	9,54	G6PD_C, G6PD_N	
KRT39	4	14,66	Filament	
LOC728765	4	14,66		
COPS4	4	12,32	RPN5	PINT
GOT1	4	11,38	Aminotran_1_2, TyrB	
ACADVL	4	7,79	CaiA, VLCAD	
DSG4	4	5,58	Cadherin_C, CA	CA
S100A3	3	33,66	S-100/ICaBP-like	
KRTAP13-2	3	28,57	PMG	
PARK7	3	17,46	GATase1_DJ-1	
PSMB5	3	14,07	proteasome_beta_type_5	
MRPL38	3	8,09	RKIP	
AHCY	3	6,71	AdoHcyase, SAM1	
PTPN11	3	6,24	SH2, PTPc, PTPc	PTPc, SH2
HBS1L	3	6,14	EF1_alpha, eRF3_II_like, TEF1, HBS1_C	
SLC3A2	3	6,03	Aamy	
ZCCHC4	3	5,65		ZnF_C2HC
ATP6V1B2	3	5,48	ATP-synt_ab_C, ATP-synt_ab_N, NtpB, V_A-ATPase_B	
PLOD3	3	4,34	P4Hc	P4Hc
ACO2	3	4,36	AcnA_Mitochon_Swivel, AcnA, AcnA_Mitochondrial	
CAPN1	3	4,20	Calpain_III, EFh, CysPc	EFh, CysPc, calpain_III
NPEPPS	3	4,00	PepN, Peptidase_M1	
VDP	3	3,22	Uso1_p115_C, Smc, Uso1_p115_head	
OGDHL	3	2,97	Transket_pyr, SucA, TPP_E1_OGDC_like	
OGDH	3	2,93	Transket_pyr, SucA, TPP_E1_OGDC_like	
COPB	3	2,83	COG5096, Adaptin_N	
SEC31L1	3	2,62	WD40	WD40
KRTAP3-1	2	24,49		
KRTAP9-3	2	20,13		
KRTAP9-8	2	20,13		
CALML3	2	19,46	FRQ1, EFh	EFh
KRTAP9-5	2	18,93		
KRTAP9-2	2	18,39		

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SPRR1B	2	17,98		
SPRR1A	2	17,98		
KRTAP11-1	2	15,34	PMG	
KRTAP4-3	2	12,82		
LOC643287	2	12,71		
RAN	2	11,11	RAN, Ran	RAN
LGALS3	2	8,80	GLECT	GLECT
MAT2B	2	7,49	RfbD, FabG, RmlD_sub_bind	
PHB2	2	7,36	Band_7_prohibitin	PHB
LASP1	2	8,81	SH3, LIM, NEBU	SH3, LIM, NEBU
DNAJA1	2	6,55	DnaJ_C, DnaJ, DnaJ, DnaJ_CXXCXGXG	DnaJ
ERAL1	2	6,18	Era, Era	
SERPINB5	2	5,60	maspin_like	SERPIN
VASP	2	5,53	Ena-Vasp	WH1
FKBP5	2	5,03	TPR, FKBP_C	
SMARCE1	2	4,87	HMGB-UBF_HMG-box	HMG
CANX	2	4,73	Calreticulin	
LOC728340	2	4,56		VWA
LOC730394	2	4,56		VWA
GTF2H2	2	4,56	vWA_transcription_factor_IIH_type, SSL1	VWA
LOC653866	2	4,56		VWA
DKFZP686 M0199	2	4,50		VWA
FDFT1	2	4,08	Trans_IPPS_HH	
ZCCHC8	2	3,82	zf-CCHC, PSP	ZnF_C2HC, PSP
FLJ21908	2	3,61	TPR	TPR
LOC652798	2	3,48		
LRRC40	2	3,32	COG4886, LRR_RI	LRR_TYP
COPG	2	3,32	SEC21	
PKP1	2	2,81	ARM	ARM
HNRPUL2	2	2,41		SPRY, SAP
IARS2	2	2,17	IleRS_core, zf-FPG_IleRS, IleS	
SMARCC1	2	1,90	SWIRM, MDN1, RSC8	CHROMO, SANT
PFAS	2	1,87	AIRS_C, GATase1_FGAR_AT, PurL_repeat2, PurL_repeat1, PurL	

4.1.3 Validation of the peptide pull-down approach

The application of a phosphopeptide pull-down strategy for the identification of proteins docking to specific phospho-tyrosyl sites is a rather novel approach. It was hence our ambition to prove its validity.

In the already mentioned study by Huang *et al.* the peptide ligand consensus sequence of 76 human SH2 domains was either established or redefined. Each SH2 domain was analyzed for its sequence preference. More specifically, it was

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established which amino acids a given SH2 domain favours in the P-2 to P+4 region that is flanking the phospho-tyrosyl site of the peptide ligand.

We utilized these findings for the design of two phosphopeptides that based on the published consensus motifs are predicted to be bona fide targets for the SH2 domains of PLCG1 and SHP2, and SRC (Table 4.3). Because of the overlapping sequence preferences of the PLCG1 and SHP2 SH2 domains, we managed to design a single phosphopeptide ligand that is presumably recognized by both proteins (Table 4.3). The second phosphopeptide was designed to be bound primarily by SRC.

Table 4.3 **Peptide design.**

Established SH2 Binding Motifs

	P-2	P-1	P+1	P+2	P+3	P+4
PLCG1_N	?	?	?	?	?	?
PLCG1_C	x	x	L ≥ V ≥ I > T	L > M	P ≥ I ≥ L	x
PTPN11_N	L > H > V	x	V ≥ L > I	x	L ≥ M	L ≥ M
PTPN11_C	x	M	V > I > T > L ≥ S ≥ E	D > M	I > M ≥ V > A	M
SRC	P > H	I > P	E > D > V > I	L > I > E	I > L > V	D

Designed Phosphopeptides

	P-2	P-1	P+1	P+2	P+3	P+4
PLCG1/SHP2 ligand	L	M	V	M	L	M
SRC ligand	P	I	E	E	I	D

We performed two additional peptide pull-downs, both times using the Tyr134 phosphopeptide and control peptide, as well as the two newly designed ‘validation peptides’. The pull-down protocols were identical to the initial pull-down; the only exception was that for one of the two pull-downs we used 0.1 M formic acid instead of 1% SDS in the elution step.

We subsequently tested the eluates for the presence of PLCG1 and SHP2 by Immunoblotting (Fig. 4.4). We detected two bands that corresponded to PLCG1, only the upper band being specific to the phosphopeptide pull-down. SHP2 was neither detected in the phosphopeptide nor in the control pull-down. Again, the prolonged storage in solution is likely to be responsible for the decreased phosphopeptide affinity for PLCG1 and SHP2.

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As far as the 'validation peptides' are concerned, the PLCG1/SHP2 motif peptide was bound by the PLCG1 and SHP2, the SHP2 signal being considerably stronger. The SRC motif peptide was only bound by PLCG1, indicating that the PLCG1 SH2 domains are less sequence specific. It is however noteworthy that the amino-terminal SH2 domain of PLCG1 was shown to be required for PDGF-induced enzyme activation, while the carboxy-terminal SH2 domain was not ²⁸⁹. As the sequence specificity of the amino-terminal SH2 domain has so far not been described, we could only consider the sequence preferences of the carboxy-terminal SH2 domain when designing the PLCG1/SHP2 motif peptide.

Based on the SHP2 data it is however safe to assume that the peptide pull-down approach is valid for the identification of phospho-tyrosine specific SH2 domain harbouring interactors.

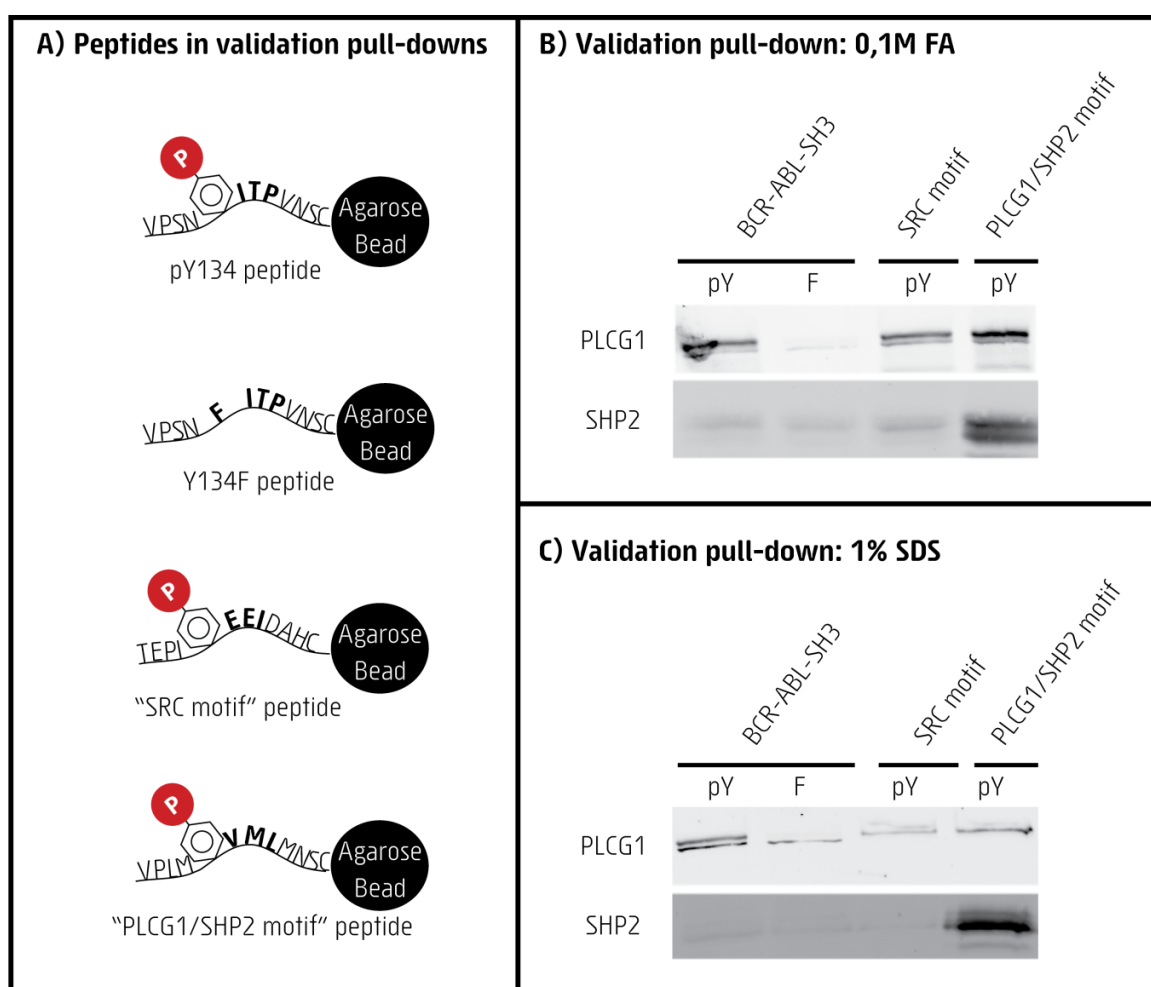


Figure 4.4 Validation pull-downs.

4.1.4 GST pull-down

Next, we sought to verify the interaction between PLCG1 and SHP2 and the SH3 domain of BCR-ABL in a less artificial system. We therefore decided to fall back on the initial GST pull-down approach, although in a slightly modified version (Fig. 4.5). Instead of being subjected to an *in vitro* phosphorylation step prior to the actual pull-down procedure, the FPLC purified His-tag and GST tagged BCR-ABL SH3 constructs were immediately immobilized on glutathione-coupled sepharose. As described earlier, the immobilized SH3 constructs were subsequently incubated with a freshly prepared K562 cell extract. In this context, the cell lysate is not only serving as a pool of potential protein interactors, but is also supplying the kinases that are presumably involved in SH3 domain phosphorylation (e.g. BCR-ABL or SFKs).

Compared to the *in vitro* phosphorylation approach, this approach is more likely to reflect the actual percentage of phosphorylated BCR-ABL SH3 domains normally found in K562 cells. However, a decrease in the efficiency of SH3 domain phosphorylation goes hand in hand with a reduced likelihood of identifying specific alterations in the SH3 domain binding pattern.

Considering the relative abundance of PLCG1 and SHP2 in the phosphopeptide pull-downs, we were nevertheless optimistic to detect different levels of PLCG1 and SHP2 in the respective GST pull-downs.

We included five different SH3 constructs in the pull-down experiment (Fig. 4.6A). In the wild-type construct (WT) the sequence of the BCR-ABL SH3 domain was unchanged. In the Y134F construct Tyr134 was replaced by a phenylalanine. Construct number three was a triple mutant where all three tyrosine residues that are located within the BCR-ABL SH3 domain were replaced by a phenylalanine (Y89/112/134F). In the remaining two constructs, the tryptophan residue involved in the polyproline type II helix recognition of the SH3 domain, W118, was mutated to an alanine, Tyr134 either being present or being replaced by a phenylalanine (W118A and W118A Y134F).

The substitution of the functionally important tryptophan residue was carried out to increase the probability of Tyr134 phosphorylation by the endogenous kinases present in the K562 lysate. It is conceivable that the interaction of the SH3 domain with conventional ligands is interfering with Tyr134 phosphorylation as the kinases

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might be prevented from gaining access to the phosphorylation site. Thus, we hoped to undermine such a steric effect by interfering with the classical binding properties of the SH3 domain.

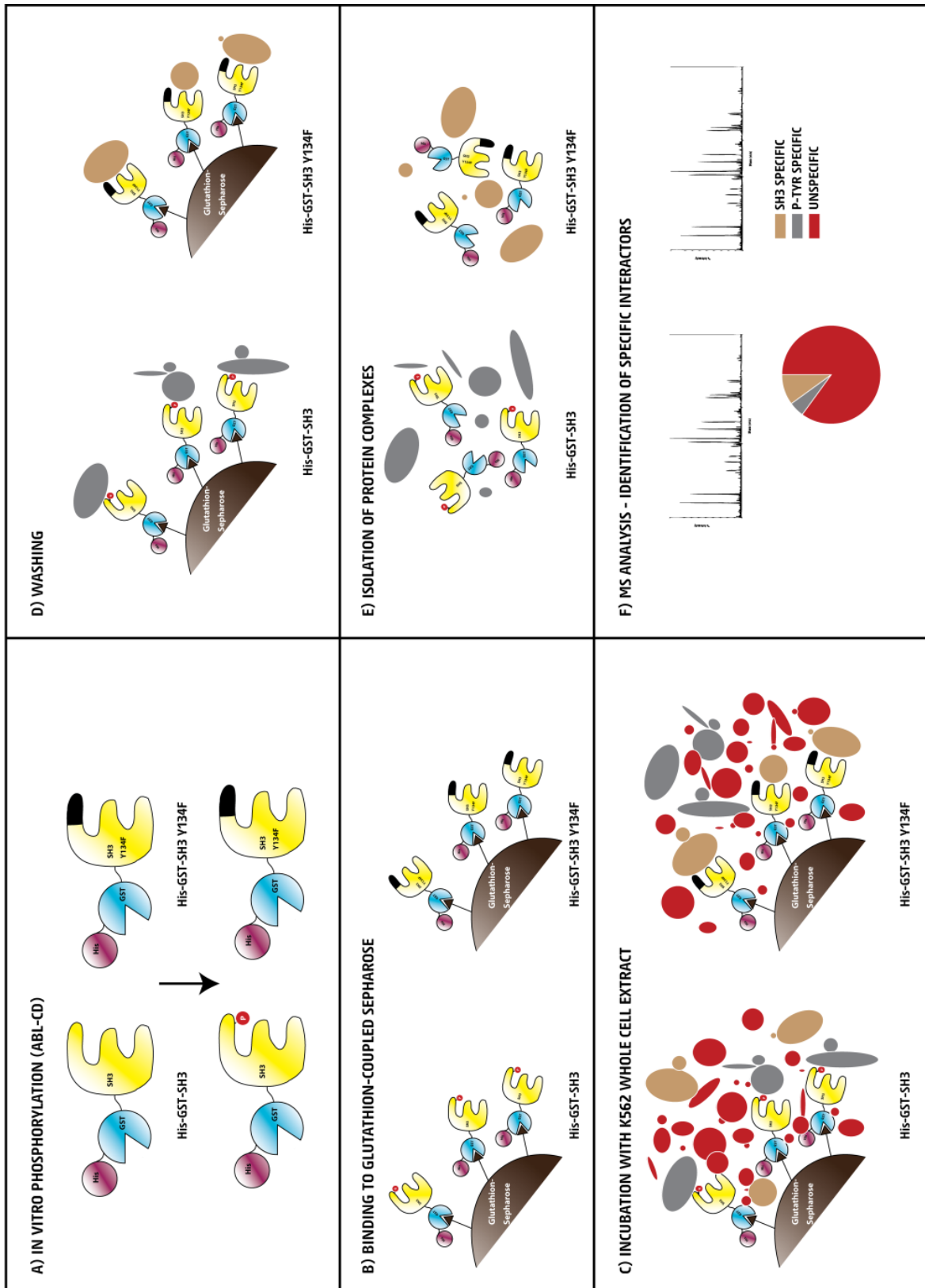


Figure 4.5 GST pull-down assay.

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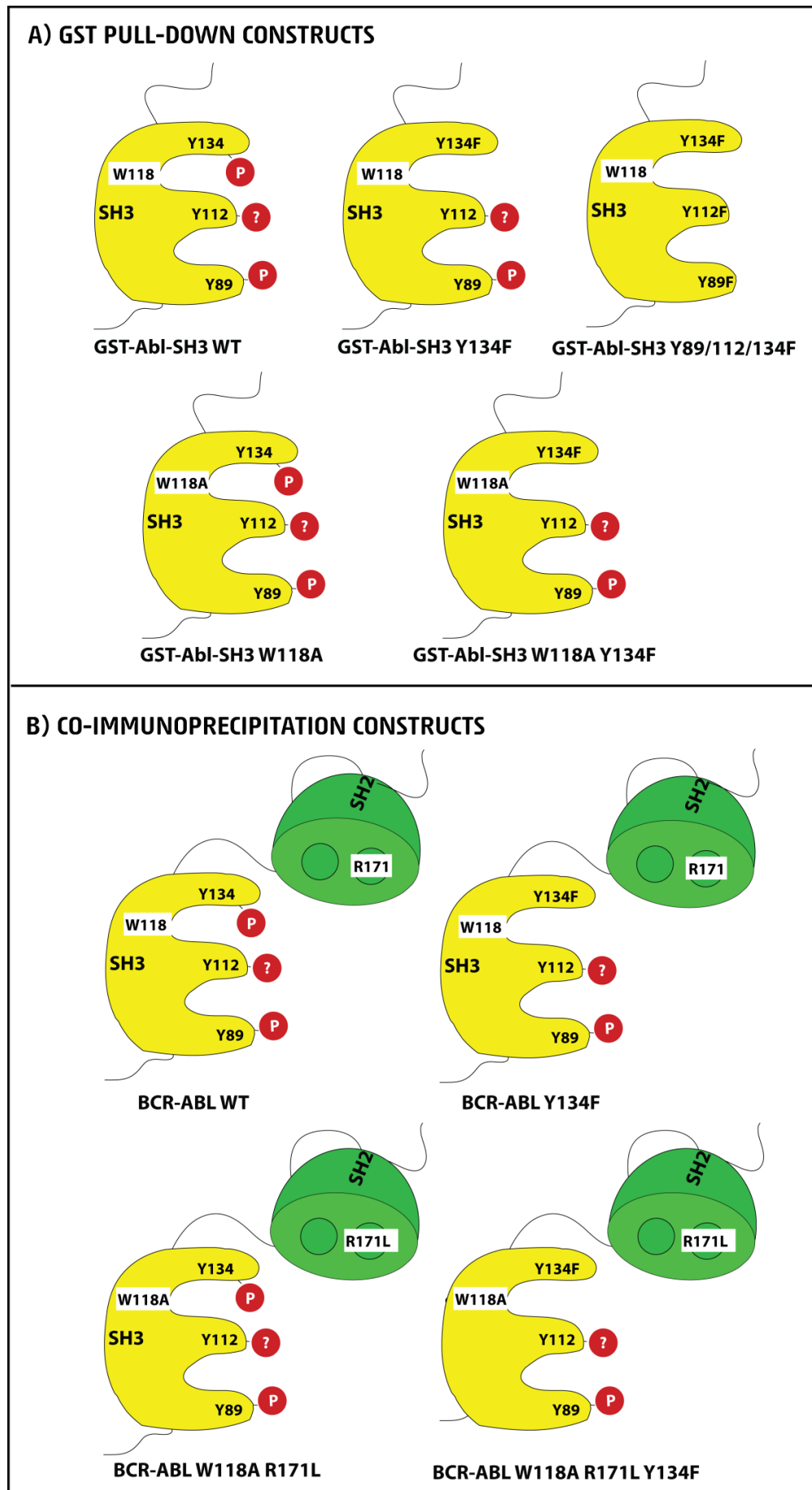


Figure 4.6 Constructs used in GST pull-down and Co-IP assays.

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Western Blot analysis of the pull-down eluates showed that PLCG1 interaction with the BCR-ABL SH3 domain is significantly reduced in the Y134F mutant compared to the wild-type form, and is even undetectable in the triple mutant (Fig. 4.7). PLCG1 seems therefore to be recruited to the phosphorylated form of the BCR-ABL SH3 domain via its SH2 domains, given that the interaction requires the presence of tyrosine residues. Moreover, the specificity pockets of the PLCG1 SH2 domains evidently prefer the Tyr134 phosphopeptide over the other two tyrosine containing sites of the BCR-ABL SH3 domain. It has to be noted that while Tyr89 has been reported to be phosphorylated, there exist no such reports involving Tyr112.

PLCG1 could not be detected in the pull-down samples of the W118A and W118A Y134F constructs. While this might be expected for the double mutant it came as a surprise for the W118A construct. At least according to our model, Tyr134 phosphorylation and hence PLCG1 recruitment should be facilitated in the W118A mutant. A possible explanation for the absence of such an effect could be improper domain folding upon introduction of the W118A point mutation.

Unfortunately, we could not confirm the interaction of SHP2 with the phosphorylated SH3 domain of BCR-ABL by this approach.

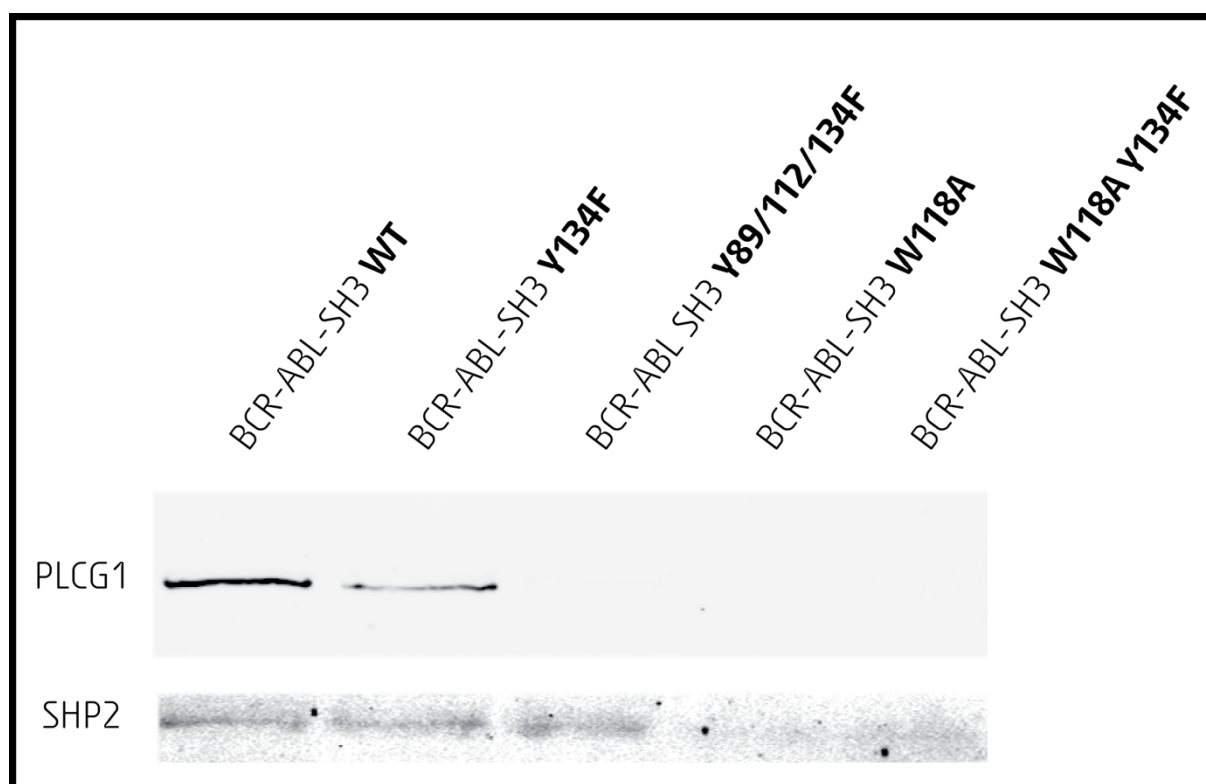


Figure 4.7 Immunoblot analysis of GST pull-down eluates.

4.1.5 Co-immunoprecipitation

Finally, we sought to prove the interaction occurring between our candidates and the BCR-ABL SH3 domain in the context of the full-length BCR-ABL protein. Co-immunoprecipitation (Co-IP) experiments were designed to address the role of Tyr134 phosphorylation in the recruitment of PLCG1 and SHP2.

Different full-length BCR-ABL constructs were generated and used in the Co-IP (Fig. 4.6B), similar to the SH3 constructs used in the GST pull-downs. The constructs, listed in Fig. 4.6B, were denoted as BCR-ABL WT, BCR-ABL Y134F, BCR-ABL W118A R171L and BCR-ABL W118A Y134F R171L. The W118A and R171L point mutations were introduced for the same reason for which W118A was included in the GST pull-down constructs. Both residues, W118A of the BCR-ABL SH3 domain, and R171 of the BCR-ABL SH2 domain, are thought to be involved in the cooperative binding of either SH3 or SH2 domain ligands. Replacement of these residues by non functional amino acids was therefore expected to abrogate conventional SH3 mediated interactions, thus making Y134 more accessible for phosphorylation.

The MYC-tagged BCR-ABL constructs were transiently transfected in HEKnull cells. Then, immunoprecipitation of BCR-ABL and immunoblot analysis were conducted to detect endogenous PLCG1 and SHP2 in the compound of the BCR-ABL protein complexes (Fig. 4.8).

The association of PLCG1 and SHP2 with BCR-ABL was significantly reduced in the BCR-ABL Y134F and BCR-ABL W118A R171L Y134F mutants compared to the BCR-ABL wild-type form. For BCR-ABL W118A R171L the results are rather inconclusive; in Fig. 4.8A PLCG1 interaction with BCR-ABL W118A R171L was ~1.5 fold increased compared to BCR-ABL WT, whereas in Fig. 4.8B the levels of detected PLCG1 were reduced to ~40% of the wild-type situation. Analogously, the levels of co-immunoprecipitated SHP2 were comparable for BCR-ABL WT and BCR-ABL W118A R171L in Fig. 4.8A, whereas in Fig. 4.8B SHP2 could only be detected in the BCR-ABL WT Co-IP.

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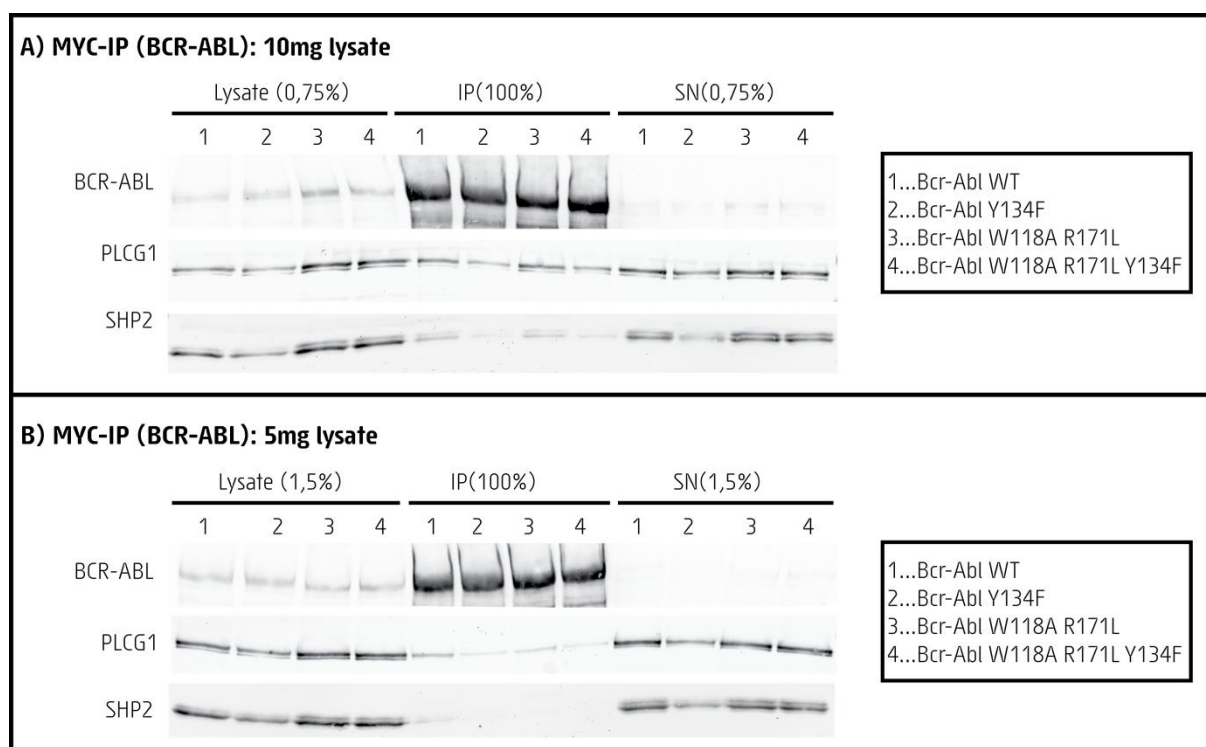


Figure 4.8 Co-immunoprecipitation assay.

We assume that only a small percentage of BCR-ABL molecules is actually phosphorylated on Tyr134. This being said, it is not unexpected that the band intensities of PLCG1 and SHP2 in the Co-IP experiments are generally quite low.

Furthermore, it is possible that the lower levels of PLCG1 and SHP2 in the Y134F Co-IPs are mere background signals that derive from unspecific binding. Although this issue might be solved by applying a stricter washing procedure, at the same time the immunoreactive detection of both candidates would probably be rendered impossible.

Overall, these data provide convincing evidence that the interaction of PLCG1 and SHP2 with BCR-ABL is largely dependent on the presence of Tyr134, indicating a binding interface involving the SH2 domains of PLCG1 and SHP2 and a phosphorylated Tyr134 residue. Even so, because of the numerous tyrosine residues of BCR-ABL it cannot be excluded that other sites are contributing to the interaction, albeit to only a minor extent.

4.2. BCR-ABL mediated microRNA deregulation

4.2.1 Reduced miR-21 levels

MiRNA-21 levels in K562 cells were observed to be ~3.5 fold reduced after Dasatinib treatment. To validate miR-21 expression as determined by the microarray-based gene expression profiling and to acquire more quantitative information, miR-qRT-PCR was performed on RNA isolated from K562 cells treated for four hours with either 100nM Dasatinib or 1 μ M Nilotinib. As shown in Fig. 4.9, miR-qRT-PCR confirmed the BCR-ABL dependent expression of miR-21 for both Dasatinib and Nilotinib treated cells. The reduced miR-21 levels result in higher Ct values compared to the untreated samples, thus decreasing the measured Δ Ct. As reference served RNU43 (SNORD43), a small nucleolar RNA (snoRNA) involved in pre-rRNA processing and modification. The Ct values of RNU43 were higher than for miR-21 and were supposed to stay unaffected by the drug treatment. As a consequence, the higher the Δ Ct (RNU43-miR-21) values are, the more miR-21 molecules are present in the sample.

To better visualize the altered miR-21 levels, the relative fold changes of miR-21 expression, untreated versus treated K562 cells, determined by miR-qRT-PCR, were assessed using the $\Delta\Delta$ Ct method. As shown in Fig. 4.9, both Dasatinib and Nilotinib treatment reduced miR-21 expression to similar extents. A 40% reduction of miR-21 levels was observed after Dasatinib treatment and a 50% reduction after Nilotinib treatment.

These results confirm the initial observations that miR-21 expression is indeed downregulated upon inhibition of the BCR-ABL kinase activity by small molecule chemical inhibitors like Dasatinib or Nilotinib. Formulated in a different way, miR-21 expression is upregulated in CML cells in a BCR-ABL dependent manner.

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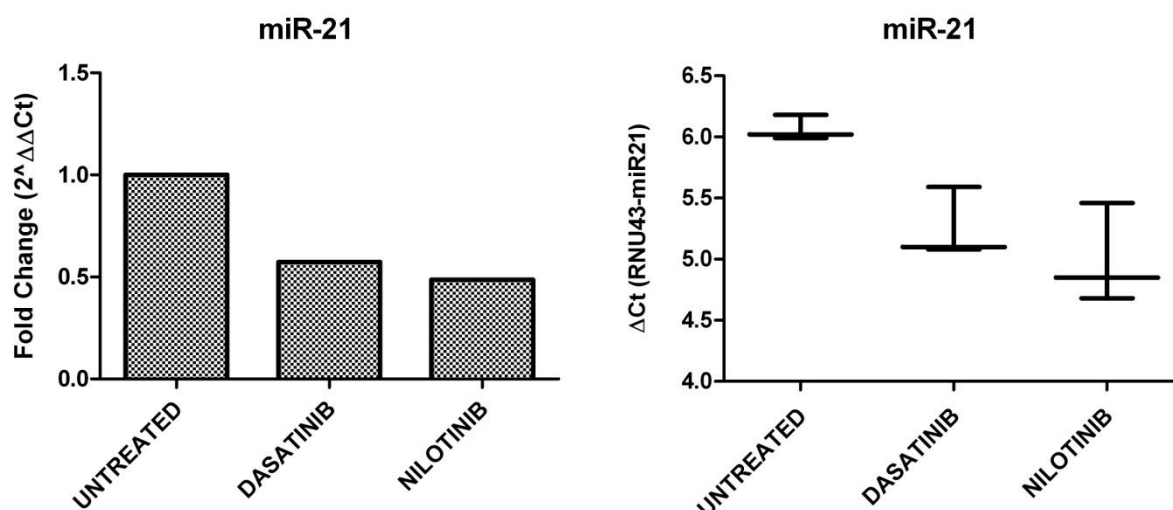


Figure 4.9 **MiR-21 downregulation in Dasatinib and Nilotinib treated K562 cells (miR-qRT-PCR).**

To study the biological significance of elevated miR-21 expression, we applied a gain-of-function, as well as a loss-of-function approach in K562 cells. We manipulated miR-21 levels by transfecting miR-21 mimics in K562 cells to supplement miR-21 activity. Analogously, K562 cells were transfected with anti-miR-21, which bind to endogenous miR-21 and thereby antagonize its activity. To allow a distinction between inhibitory activity and background effects, we included a non-targeting anti-miR as a negative control in the experiment. K562 cells were harvested 72 hours after treatment and mir-21 levels were quantified by miR-qRT-PCR. As expected, miR-21 mimic treatment caused a marked increase of miR-21 levels and while the negative control showed no effect, application of anti-miR-21 led to a 0.4 fold reduction of miR-21 levels (Fig. 4.10).

Yet, when we tested the differently treated K562 cells in a preliminary experiment for the differential expression of proposed miR-21 target genes (Bcl-2, STAT3, p21 and p-AKT downstream of PTEN) no effect could be observed (data not shown). Cell viability and apoptotic rate were also unaffected (data not shown).

Although this data has yet to be verified and microRNA target genes are likely to vary from cell type to cell type, it is possible that the redundant activation of different growth and survival pathways in CML cells is concealing potential miR-21 mediated alterations.

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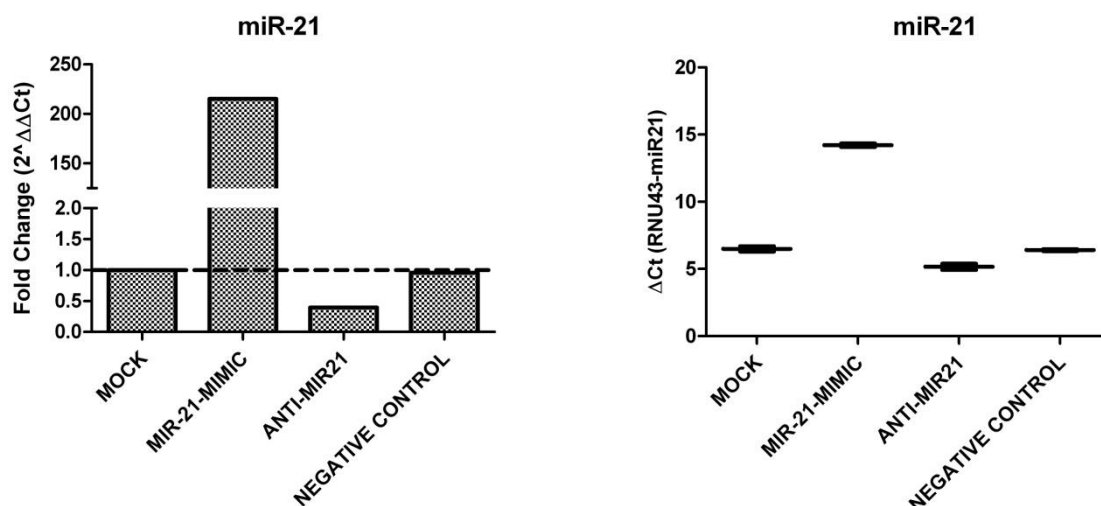


Figure 4.10 **MiR-21** antagomirs and mimics in K562 cells (miR-qRT-PCR).

4.2.2. MicroRNA microarray

With the aim of characterizing the miRNA expression profile of untreated K562 cells and Dasatinib or Nilotinib treated K562 cells, a miRNA-based microarray chip assay (miCHIP), conducted by Exiqon, was used to screen for differentially expressed miRNAs.

To search for BCR-ABL dependent miRNA expression, K562 cells were treated with either Dasatinib (100nm for 4h) or Nilotinib (1μM for 4h) to inhibit BCR-ABL tyrosine kinase activity. Each treatment was performed in triplicate. In a next step, total RNA was isolated using the mirVana miRNA Isolation Kit from Ambion. The quality of the RNA samples was assessed using the Agilent 2100 Bioanalyzer, allowing the standardization of RNA integrity interpretation. The RNA Integrity Number (RIN) measures RNA quality and grades it on a quantitative scale of 1 (poor) to 10 (high).

After submission of all nine RNA samples, three for each treatment, miRNA expression profiling was performed by Exiqon, applying the most recent version of the miRCURY LNA™ microRNA Array. According to Exiqon the array covers all human, mouse and rat microRNA sequences annotated in miRBase 10.0, including 94 miRNAs of the viruses related to these three species. Additionally the array

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contains 43 capture probes for the detection of miRPlus microRNAs, which are human miRNAs unique to Exiqon. In total, 758 human microRNAs can be detected using the array.

Among the miRNAs analyzed, 31 were found to be differentially expressed in the different samples comparing drug treated groups and untreated samples (Fig. 4.11, Fig. 4.12). The Nilotinib sample group showed to be significantly more different to the untreated group than Dasatinib treated samples.

Strikingly, for the majority of the downregulated miRNAs only the microRNA star form (miRNA*) was detected to be affected by BCR-ABL inhibition. In theory, cleavage of pre-miRNA hairpins produces a duplex composed of two small RNAs. One of the two strands initially produced in a 1:1 ratio by transcription usually accumulates to a higher level than its partner. Because of the preferred stability of miRNA species and the concomitant turnover of miRNA* species the mature miRNA/miRNA* ratio is asymmetric at steady state, sometimes at a discrepancy of >10 000:1^{290, 291}. The mature miRNA strand serves as guide strand in the miRISC complex whereas the poorly expressed partner strand, termed miRNA*, was thought to be functionally irrelevant. However, recently miRNA* species were implicated as players in the miRNA regulatory network²⁹²⁻²⁹⁴. It was shown that some miRNA* sequences are inhabiting AGO complexes and exert a regulatory function. Also, many miRNA* sequences are present at physiological relevant levels, and most miRNA* seed regions and their complementary 3'UTRs are substantially constrained during evolution.

We are assuming that drug treatment for four hours is not sufficient to reduce the levels of the mature miRNA strands in a significant and hence detectable way. MiRNA* species on the other hand are less stable and have a higher turnover rate, resulting in a marked drop of the corresponding miRNA* strand levels, even after drug treatment for only four hours. Thus, downregulation of miRNA expression is initially causing a reduction in the number of miRNA* strands, and is only later manifesting on the mature miRNA level. MiRNA hairpins whose physiological levels are high enough to be detected by miCHIP are likely to produce two distinct regulatory RNAs, thereby broadening their regulatory network and their spectra of potential target genes.

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20 human microRNAs were shown to be downregulated in K562 cells following drug treatment. Importantly, downregulation of miR-21 expression was confirmed by the miCHIP assay. MiR-21* levels were reduced ~0.5 and ~0.6 fold in the Dasatinib and Nilotinib treated cells, respectively. Based on the assumption that a reduction in miRNA* levels is a harbinger of the ensuing decrease of the mature miRNA levels, miR-21 expression was found to be BCR-ABL dependent. Strikingly, miR-590-5p, the only miRNA containing the same 7nt seed region and therefore the same target genes as miR-21, was also shown to be downregulated.

Consistent with the findings of Venturini et al., expression of miRNAs within the polycistronic miR-17-92 cluster (encoding miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1) and one of its paralog clusters, miR-106a-363 (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2, and miR-363), is specifically downregulated by both Dasatinib and Nilotinib treatment.

Among the 758 human miRNAs analyzed, 11 were found to be upregulated by both Dasatinib and Nilotinib treatment. Interestingly, the levels of miR-155, a presumably oncogenic miRNA, were also increased in the drug treated compared to the untreated cells.

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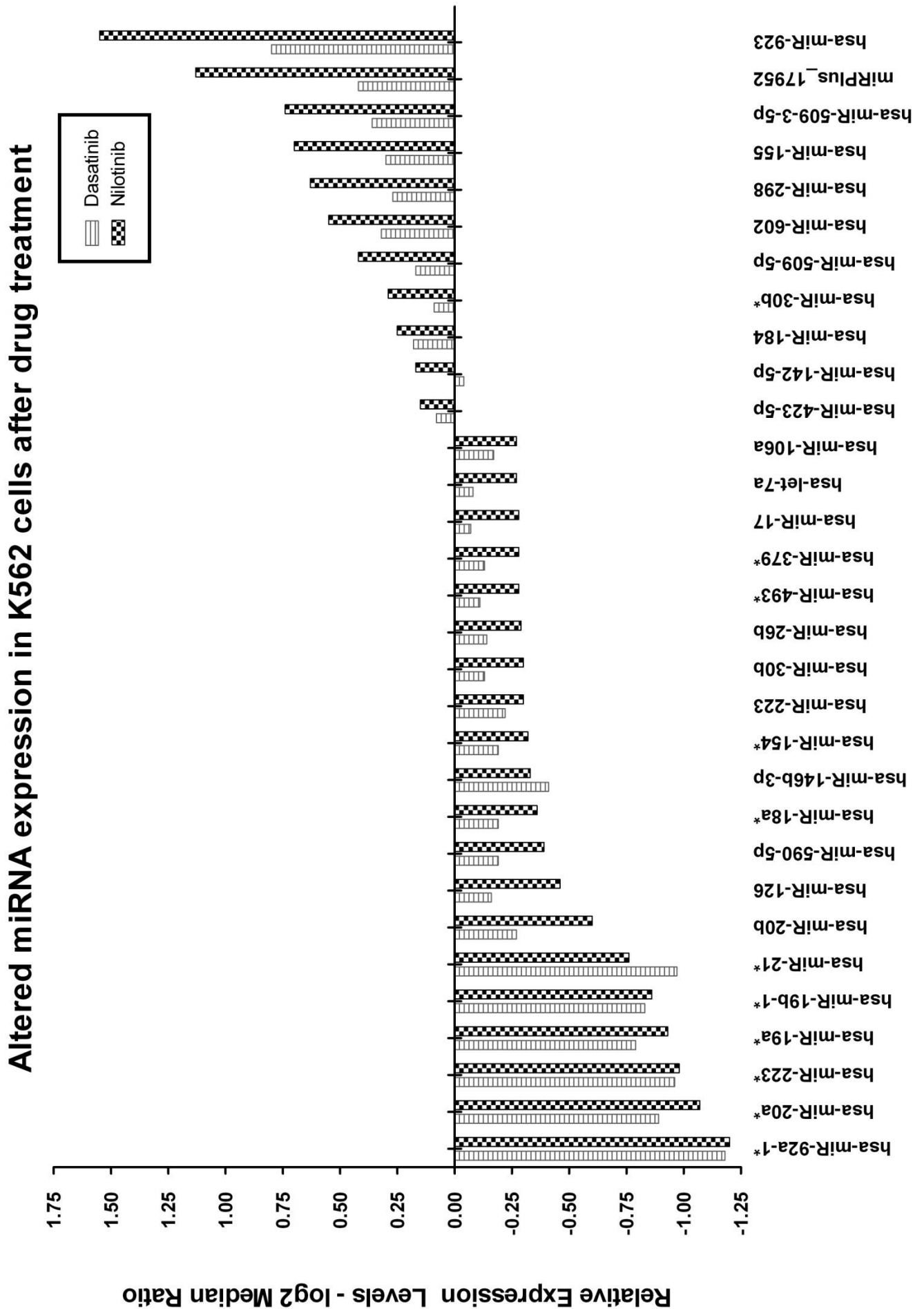
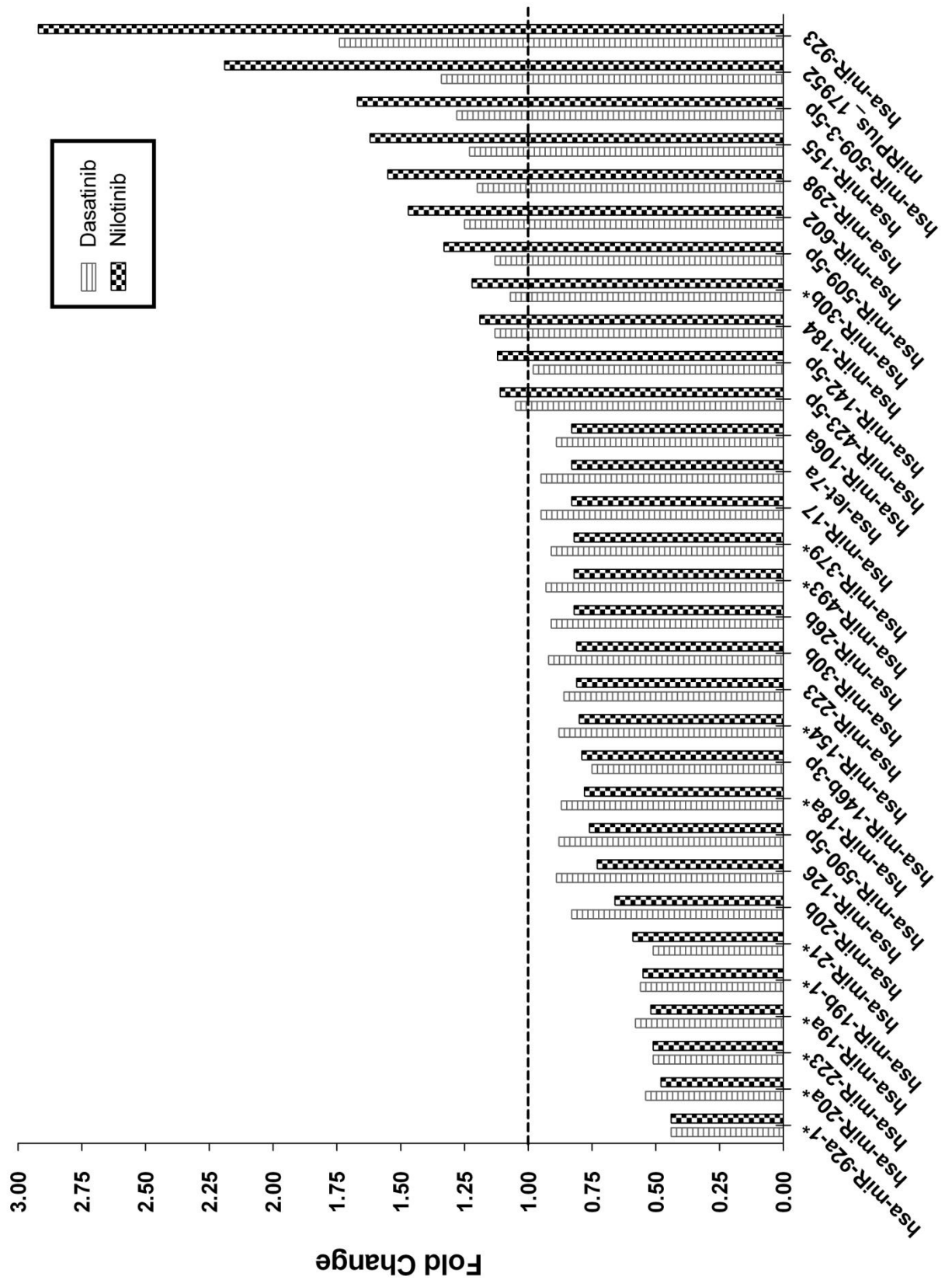


Figure 4.11 Differentially regulated miRNAs (log2 median ratios).



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We then sought to elucidate the role of miRNA deregulation in BCR-ABL signalling. We compared the list of the predicted target genes of the deregulated miRNAs we identified by miCHIP with the list of genes whose expression profile was shown to be altered following Dasatinib treatment in the initial microarray experiment (i.e. the one that led to the discovery of miR-21 downregulation in Dasatinib treated K562 cells) (Table 4.3).

Genes whose expression levels were increased in Dasatinib treated cells were matched against the predicted target genes of those miRNAs whose expression levels were reduced by the drug treatment. Contrariwise, genes whose expression was repressed following Dasatinib treatment were matched against the targets of the upregulated miRNAs.

Especially for the downregulated miRNAs we detected a redundancy concerning their target genes. This was not unexpected, given that almost half of them belonged to the miR-17-92 family and considering that polycistronic miRNAs often share identical seeds.

Table 4.4 and 4.5 list all the genes that are potentially targeted by more than one miRNA. Nonetheless, it remains to be seen which genes are really regulated by the corresponding miRNAs and to which extent their differential expression in Dasatinib treated cells can be attributed to the relief or onset of miRNA mediated post transcriptional gene silencing (PTGS).

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Table 4.3 MiRNAs and target genes inversely regulated by drug treatment

MicroRNA	Seed (5'-3')	Predicted Targets (TargetScan)
hsa-miR-17	AAAGUGC	AHNAK, C1orf63, C9orf5, CCNG2, CENPO, CNN1, CYBRD1, EPB41, ERBB3, FAM46C, FRMD4A, FZD4, HBP1, KIAA0831, MARCH8, MGEA5, MLL3, PGM2L1, PIK3R1, RRAGD, SH3BP5, SLC16A9, SLC40A1, TAL1, TP53INP1, WNK1, ZBTB4
hsa-miR-18a	AAGGUGC	RABGAP1, ZBTB4
hsa-miR-18a*	CUGCCCU	CITED2, DLC1, EFNA1, FZD4, ZBTB4
hsa-miR-19a	GUGCAAA	ABHD5, C10orf26, CEP350, DAAM1, DLC1, ELL2, ERBB3, FAM46C, HBP1, ID2, KIAA0831, KRAS, MBD4, PGM2L1, PIK3R3, PLCL2, PNRC1, RABGAP1, RCOR3, TP53INP1, WNK1, ZBTB4, ZFPM2
hsa-miR-19a*	GUUUUGC	CITED2, DLC1, FRMD4A, HOXC13, PIK3R1
hsa-miR-20a	AAAGUGC	AHNAK, C1orf63, C9orf5, CCNG2, CENPO, CNN1, CYBRD1, EPB41, ERBB3, FAM46C, FRMD4A, FZD4, HBP1, KIAA0831, MARCH8, MGEA5, MLL3, PGM2L1, PIK3R1, RRAGD, SH3BP5, SLC16A9, SLC40A1, TAL1, TP53INP1, WNK1, ZBTB4
hsa-miR-20a*	CUGCAUU	KIAA0831, MYST4, PAQR8, ZNF318
hsa-miR-19b-1	GUGCAAA	ABHD5, C10orf26, CEP350, DAAM1, DLC1, ELL2, ERBB3, FAM46C, HBP1, ID2, KIAA0831, KRAS, MBD4, PGM2L1, PIK3R3, PLCL2, PNRC1, RABGAP1, RCOR3, TP53INP1, WNK1, ZBTB4, ZFPM2
hsa-miR-19b-1*	GUUUUGC	CITED2, DLC1, FRMD4A, HOXC13, PIK3R1
hsa-miR-92a-1	AUUGCAC	ATP2B4, CDKN1C, CEP350, ING2, KIAA0831, MITF, PFKFB4, PIK3R3, SERTAD3, SYNJ1, ZFPM2
hsa-miR-92a-1*	GGUUGGG	
hsa-miR-106a	AAAGUGC	AHNAK, C1orf63, C9orf5, CCNG2, CENPO, CNN1, CYBRD1, EPB41, ERBB3, FAM46C, FRMD4A, FZD4, HBP1, KIAA0831, MARCH8, MGEA5, MLL3, PGM2L1, PIK3R1, RRAGD, SH3BP5, SLC16A9, SLC40A1, TAL1, TP53INP1, WNK1, ZBTB4
hsa-miR-20b	AAAGUGC	AHNAK, C1orf63, C9orf5, CCNG2, CENPO, CNN1, CYBRD1, EPB41, ERBB3, FAM46C, FRMD4A, FZD4, HBP1, KIAA0831, MARCH8, MGEA5, MLL3, PGM2L1, PIK3R1, RRAGD, SH3BP5, SLC16A9, SLC40A1, TAL1, TP53INP1, WNK1, ZBTB4
hsa-let-7a	GAGGUAG	ATP2B4, CBX2, DLC1, EPB41, FGD6, FZD4, GLRX, HIC2, KLHDC8B, LGR4, PGM2L1, SLC16A9, TRIB1
miR-21	AGCUUUAU	CCDC34, PIK3R1, ZFP36L2
miR-21*	AACACCA	PIK3R3, RNF43
hsa-miR-26b	UCAAGUA	CDKN1C, CEP350, FAM46C, MLL3, PGM2L1, PIK3R3, SLC25A37, THRAP3, TP53INP1, WNK1
hsa-miR-30b	GUAAACA	AHNAK, BCL6, C10orf26, CBX2, CEP350, CSNK1A1, ELL2, EPB41, ERMAP, FAM46C, FAM73B, FGD6, FRMD4A, GLCCI1, HIC2, KRAS, MARCH8, MLL3, PGM2L1, SLC36A1, STIM2, TP53INP1
hsa-miR-126	CGUACCG	
hsa-miR-146b-3p	GCCCUGU	KLHDC8B, PFKFB4, TFAP2B
hsa-miR-154	AGGUUUAU	
hsa-miR-154*	AUCAUAC	STIM2
hsa-miR-181a	ACAUUCA	AHNAK, BAG4, BMF, CEP350, EPB41, FAM46C, FAM73B, GLCCI1, HIC2, KRAS, MLL3, PIK3R3, PLCL2, RABGAP1, RNF43, SLC25A37, STIM2, WNK1, ZBTB4, ZNF292
hsa-miR-181a-2*	CCACUGA	KRAS, SYNJ1, TAL1
hsa-miR-223	GUCAGUU	C10orf26, ELL2, FAM46C, FRMD4A, MLL3, SETBP1, ZNF395
hsa-miR-223*	GUGUAUU	CAV2, CEP350, DLC1, TFAP2B
hsa-miR-379	GGUAGAC	
hsa-miR-379*	AUGUAAC	FRMD4A, HIC2, TFAP2B, ZNF292
hsa-miR-493	GAAGGUC	FAM102B, PIK3R1
hsa-miR-493*	UGUACAU	ATP2B4, CDKN1C, CITED2, CSNK1A1, FRMD4A, FZD4, HEMGN, HIC2, KIAA0831, PIK3R1, RXRA, WNK1
hsa-miR-590-5p	AGCUUUAU	CCDC34, PIK3R1, ZFP36L2

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Table 4.4 **Target genes of down-regulated miRNAs.** Number of miRNAs targeting a certain gene (prediction frequency), fold change of target genes following Dasatinib treatment (fold change).

Gene Name	Full Name	Fold Change	Prediction Frequency
FRMD4A	FERM domain containing 4A	1,83	9
KIAA0831	KIAA0831	1,69	9
PGM2L1	phosphoglucomutase 2-like 1	1,80	9
FAM46C	family with sequence similarity 46, member C	1,65	8
TP53INP1	tumor protein p53 inducible nuclear protein 1	1,67	8
WNK1	WNK lysine deficient protein kinase 1	1,46	8
FZD4	frizzled homolog 4 (Drosophila)	1,89	7
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	1,66	7
ZBTB4	zinc finger and BTB domain containing 4	1,49	7
DLC1	deleted in liver cancer 1	1,81	6
EPB41	erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)	1,71	6
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	1,69	6
HBP1	HMG-box transcription factor 1	1,45	6
MLL3	myeloid/lymphoid or mixed-lineage leukemia 3	1,51	6
AHNAK	AHNAK nucleoprotein (desmoyokin)	1,56	5
CEP350	centrosomal protein 350kDa	1,65	5
PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)	3,13	5
MARCH8	membrane-associated ring finger (C3HC4) 8	1,68	5
SLC16A9	solute carrier family 16 (monocarboxylic acid transporters), member 9	1,90	5
TAL1	T-cell acute lymphocytic leukemia 1	1,56	4
C1orf63	chromosome 1 open reading frame 63	1,76	4
C9orf5	chromosome 9 open reading frame 5	1,61	4
CCNG2	cyclin G2	2,45	4
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	2,67	4
CNN1	calponin 1, basic, smooth muscle	1,63	4
CYBRD1	cytochrome b reductase 1	1,83	4
HIC2	hypermethylated in cancer 2	2,34	4
MGEA5	meningioma expressed antigen 5 (hyaluronidase)	1,93	4
RRAGD	RAS-related GTP binding D	1,55	4
SH3BP5	SH3-domain binding protein 5 (BTK-associated)	1,58	4
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	2,33	4
ATP2B4	ATPase, Ca ⁺⁺ transporting, plasma membrane 4	1,46	3
C10orf26	chromosome 10 open reading frame 26	1,49	3
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	3,66	3
ELL2	elongation factor, RNA polymerase II, 2	1,57	3
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1,72	3
RABGAP1	RAB GTPase activating protein 1	1,66	3
ZFPM2	zinc finger protein, multitype 2	1,67	3
ABHD5	abhydrolase domain containing 5	1,55	2
CBX2	chromobox homolog 2 (Pc class homolog, Drosophila)	1,66	2
CSNK1A1	casein kinase 1, alpha 1	1,57	2
DAAM1	dishevelled associated activator of morphogenesis 1	1,48	2
FGD6	FYVE, RhoGEF and PH domain containing 6	2,09	2
HOXC13	homeobox C13	2,25	2

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ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	2,38	2
KLHDC8B	kelch domain containing 8B	1,61	2
MBD4	methyl-CpG binding domain protein 4	1,54	2
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	1,99	2
PLCL2	phospholipase C-like 2	1,69	2
PNRC1	proline-rich nuclear receptor coactivator 1	1,94	2
RCOR3	REST corepressor 3	1,60	2
STIM2	stromal interaction molecule 2	1,53	2
TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta)	1,68	2

Table 4.5 **Target genes of up-regulated miRNAs.** Number of miRNAs targeting a certain gene (prediction frequency), fold change of target genes following Dasatinib treatment (fold change).

Gene Name	Full Name	Fold Change	Prediction Frequency
ETV1	ets variant gene 1	-3,45	3
CHSY1	carbohydrate (chondroitin) synthase 1	-1,70	2
ENC1	ectodermal-neural cortex (with BTB-like domain)	-2,15	2
EPAS1	endothelial PAS domain protein 1	-1,98	2
FAM60A	family with sequence similarity 60, member A	-1,99	2
HCCS	holocytochrome c synthase (cytochrome c heme-lyase)	-1,68	2
IGF1	insulin-like growth factor 1 (somatomedin C)	-2,85	2
KSR1	kinase suppressor of ras 1	-1,98	2
PTP4A1	protein tyrosine phosphatase type IVA, member 1	-1,48	2
RAN	RAN, member RAS oncogene family	-2,03	2
SOCS1	suppressor of cytokine signaling 1	-7,20	2
SPRED2	sprouty-related, EVH1 domain containing 2	-1,69	2
ZC3H12C	zinc finger CCCH-type containing 12C	-2,00	2

5. Discussion

5.1. Identification of novel interactors of BCR-ABL

Translocation of c-ABL located on chromosome 9 to the breakpoint-cluster region (*BCR*) on chromosome 22 generates the fusion oncogene *BCR-ABL*. It produces the chimeric, constitutively active, tyrosine kinase p210, whose presence is sufficient for malignant transformation of hematopoietic cells in culture^{295, 296} and causes a CML-like MPD in mice^{6, 297}.

BCR-ABL exerts its oncogenic function by acting upstream of several important cellular signalling pathways. It contains multiple distinct sites and protein domains, which constitute the link to those pathways. In this study we were addressing the role the BCR-ABL SH3 domain plays in BCR-ABL signalling.

Our interest in the SH3 domain originated from a recent report that demonstrated that BCR-ABL dependent tyrosine-phosphorylation of the GRB2 carboxy-terminal SH3 domain prevents it from interacting with the SH3 ligand SOS²⁷⁹. Based on the extensive structural and sequence homologies shared by both SH3 domains we were wondering if phosphorylation of the BCR-ABL SH3 domain is affecting its binding pattern in a likewise manner.

Under normal circumstances SH3 domains are specialized in the recognition of peptides displaying a polyproline type II helix conformation. We speculated that phosphorylation of Tyr134 of the BCR-ABL SH3 domain interferes with this type of interactions, at the same time generating a docking site for the recruitment of SH2 domain harbouring proteins. In order to validate this hypothesis we applied a peptide pull-down strategy, the peptide sequences corresponding to the region of interest of the BCR-ABL SH3 domain and the GRB2 carboxy-terminal SH3 domain.

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The SH2 domain containing proteins PLCG1 and SHP2 (PTPN11) were found to bind specifically to the Tyr134 (BCR-ABL) and Tyr209 (GRB2) phosphopeptides, but not to the unphosphorylated control peptides. We subsequently confirmed the specific interaction of PLCG1 and SHP2 with the tyrosine-phosphorylated BCR-ABL SH3 domain by applying a co-immunoprecipitation approach. PLCG1 and SHP2 association with p210 was significantly reduced in the Y134F BCR-ABL mutants compared to the wild-type constructs, underscoring the importance of phosphorylated Y134 in this context.

Finally, in GST-pull-downs, using GST-SH3 fusion proteins as bait, we observed a dramatic decrease of PLCG1 association with the BCR-ABL SH3 domain after the introduction of the Y134F mutation.

Both proteins foster mitogenic signalling and have been implicated in proliferation and tumorigenesis^{298, 299}. We find it therefore tempting to speculate that PLCG1 and SHP2 are participating in BCR-ABL mediated transformation of hematopoietic cells and leukemogenesis.

5.1.1. PLCG1

In the phospholipase C- γ subfamily (PLCG1 and PLCG2 in mammals), the X and Y catalytic lipase domains are separated by a SH region containing two SH2 domains, an SH3 domain and a split PH domain (Fig. 5.1). PLCG1 is a multidomain protein containing two SH2 domains and one SH3 domain between the catalytic lipase domains.

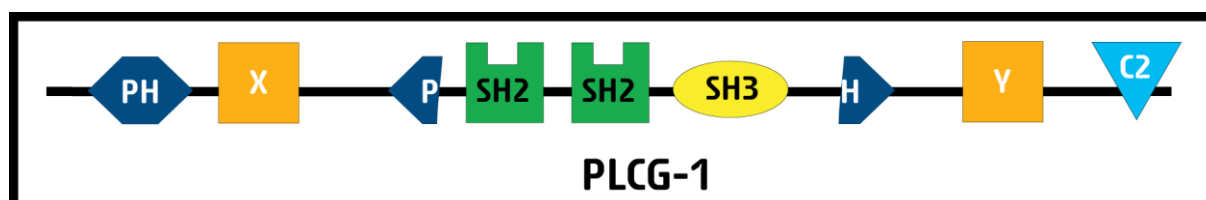


Figure 5.1 Domain structure of PLCG1.

The SH2 domains of PLCG1 have been implicated in the association between PLCG1 and activated receptor tyrosine kinases. Following recruitment to

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autophosphorylated receptor tyrosine kinases (e.g. PDGFR) PLCG1 becomes phosphorylated and hence activated³⁰⁰⁻³⁰². The lipase activity of PLCG1 is then causing the generation of the two intracellular second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) by cleavage of phosphatidylinositol-4,5-bisphosphate, which in turn promote the activation of protein kinase C (PKC) and the release of Ca^{2+} from intracellular store^{303, 304}.

The individual roles of the two SH2 domains of PLCG1 were investigated by functional inactivation of each domain and expression of the mutant proteins in a PLCG1-deficient fibroblast cell line²⁸⁹. While the amino-terminal SH2 domain was required for PDGF-induced enzyme activation, the carboxy-terminal SH2 domain was not. In addition, it has been proposed that the SH2 domains are involved in PLCG1 autoinhibition, functioning as an intramolecular “cap” to occlude the active site in the absence of activating events³⁰⁵.

It is the SH3 domain rather than the catalytic domain of PLCG1 that has been reported to be essential for cellular proliferation and growth factor induced mitogenesis. The SH3 domain interacts with SOS, which leads to the activation of RAS and subsequently of the ERK-MAPK pathway³⁰⁶. Additionally, the SH3 domain serves as a GEF (guanine nucleotide exchange factor) for Dynamin and PIKE, thereby potentiating ERK and PI3K activity, respectively^{307, 308}. On the other hand, negative regulation of PLCG1 is mainly mediated by the interaction of the PLCG1 SH3 domain with CBL and the interaction of GRB2 with tyrosine phosphorylated PLCG1^{309, 310}.

Homozygous disruption of the PLCG1 gene was shown to cause an embryonic lethal phenotype owing to growth attenuation³¹¹. Furthermore, PLCG1 was shown to participate in tumorigenesis. The expression level of PLCG1 was found to be elevated in cancer tissues compared to normal tissues^{312, 313}. Interestingly, overexpression of the PLCG1 SH2-SH2-SH3 domain in rat 3Y1 cells was reported to be sufficient to induce cellular transformation and to cause tumor mass generation when transplanted into nude mice³¹⁴.

In a recent publication by Plattner et al. a link between the endogenous c-ABL tyrosine kinase and PLCG1 signalling was described³¹⁵. According to the authors PLCG1 is acting upstream of c-ABL in PDGF induced chemotaxis and is required for

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the PDGFR mediated activation of c-ABL. It was proposed that phosphatidylinositol-4,5-bisphosphate (PIP₂) is inhibiting c-ABL and that hydrolysis of PIP₂ by activated PLCG1 is releasing this restraint. They showed that c-ABL and PLCG1 form a complex *in vivo*, probably involving SH2-phosphotyrosine binding. A negative feedback loop was established where activated c-ABL negatively regulates PLCG1 lipase activity through inhibitory tyrosine-phosphorylation. As a consequence PIP₂ levels are again increasing and interfering with c-ABL activity.

Based on these facts it is conceivable that the SH2 domain mediated interaction of PLCG1 with the SH3 domain of Y134 phosphorylated BCR-ABL is adding a further boost to mitogenic signalling in K562 cells. Similar to the recruitment of PLCG1 to autophosphorylated RTKs, it appears plausible that in K562 cells, the PLCG1 SH2 domains additionally recognize tyrosine phosphorylated BCR-ABL. Whether this interaction resembles the c-ABL – PLCG1 interaction, involving PLCG1 inhibition and PIP₂ increase remains to be seen. However, regardless of the lipase activity, proliferation and survival pathways are anyway mainly activated via the PLCG1 SH3 domain, leading to the activation of the mitogenic kinases ERK and PI3K. Moreover, considering the constitutive active nature of the BCR-ABL tyrosine kinase, negative regulation by PIP₂ as observed for c-ABL can be neglected.

5.1.2. SHP2

The Src homology-2 (SH2) domain containing phosphatases (Shps) are a small, highly conserved subfamily of protein-tyrosine phosphatases. There exist two vertebrate Shps – SHP1 and SHP2. They have two tandemly arranged amino-terminal SH2 domains (N-SH2 and C-SH2), a classic protein-tyrosine phosphatase (PTP) domain that displays absolute specificity for hydrolyzing phosphotyrosine, and a carboxy-terminal tail (C-tail) (Fig. 5.2).

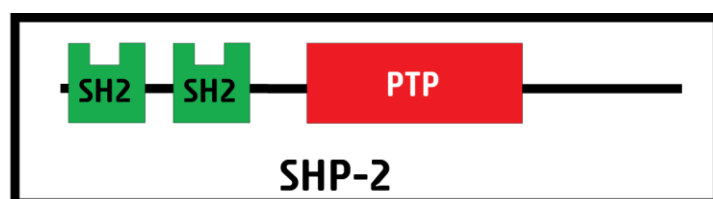


Figure 5.2 Domain structure of SHP2.

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In the basal state the PTP domain is maintained in an inactive conformation by an intramolecular interaction involving the catalytic cleft of the PTP domain and the “back-side-loop” (the side opposing the phosphotyrosine binding surface) of the N-SH2 domain^{50, 316}. This culminates in mutual allosteric inhibition, with the N-SH2 domain suppressing PTP activity and the PTP domain contorting the phospho-tyrosyl binding pocket of the N-SH2 domain on the opposite surface of the N-SH2-PTP binding interface. The phospho-tyrosyl binding pocket of the C-SH2 domain is unperturbed and it was postulated that it is surveying the cell for appropriate phosphopeptides⁵⁰. C-SH2 domain mediated binding of bisphosphorylated ligands is thought to increase the local phosphotyrosine concentration, thereby alleviating N-SH2 inhibition by the PTP domain and enabling phosphatase activation⁵⁰. Alternatively, presence of high-affinity ligands for the N-SH2 domain or intramolecular interaction of the SH2 domains with the tyrosine phosphorylated C-tail was proposed to have the same effect.

Protein-tyrosine phosphatases (PTPs) are conventionally thought to qualify as prime suspects for tumor suppressors by opposing protein-tyrosine kinase (PTKs) activities and attenuating pro-growth signalling. Surprisingly, only few phosphatase genes have been unequivocally identified as tumor suppressors. Be that as it may, SHP2 (encoded by *PTPN11*) was found to be a bona fide proto-oncogene. Somatic gain-of-function mutations in *PTPN11* occur in juvenile myelomonocytic leukemia (JMML) and, more rarely, in adult and solid tumors³¹⁷⁻³¹⁹. Germ line mutations of *PTPN11* on the other hand cause Noonan syndrome and the Noonan-like LEOPARD syndrome^{320, 321}. In both cases the mutation clusters occur in the N-SH2 and PTP domains, where they affect residues that are participating in basal inhibition and are heavily buried in the closed form. The integrity of the autoinhibitory cleft structure is corrupted, causing constitutive phosphatase activity and retained N-SH2 domain capacity to bind tyrosine phosphorylated proteins⁵⁰.

As a result SHP2 is constitutively activated, resulting in the continuing activation of the downstream RAS/ERK-MAPK pathway.

Enhanced RAS/ERK pathway activation is probably the key feature of mutant SHP2 evoked pathogenesis. In most RTK signalling pathways, SHP2 is required for enhanced and sustained activation of the ERK MAPK pathway^{322, 323}. Cells that are

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lacking functional SHP2 usually exhibit defective RAS activation, placing SHP2 upstream of RAS^{324, 325}.

There exist different models of SHP2 mediated positive RAS/ERK signalling. One possibility is SHP2 induced dephosphorylation of RASGAP binding sites on RTKs, thereby interfering with RASGAP mediated suppression of RAS^{326, 327}. However, this model cannot explain the mechanism of SHP2 action in pathways where SHP2 is recruited to phosphorylated adaptor proteins rather than to RTKs. It is thought that the interaction between scaffolding adaptors and SHP2 is crucial to target SHP2 into the vicinity of its substrates. In an alternative model, this might allow the direct or indirect dephosphorylation of SFK inhibitory tyrosines, considering that expression of a GAB1-SHP2 fusion protein results in enhanced SRC activity and that SRC inhibitors prevent the fusion protein from activating ERK³²⁸.

Recent studies of oncogenic SHP2 mutants raise the possibility that the transcription factor ICSBP (IRF8) may be a key target that indirectly controls RAS pathway activation by regulating NF1 levels³²⁹. SHP2 was shown to catalyze ICSBP dephosphorylation, rendering it unable to promote NF1 gene transcription, resulting in decreased NF1 levels and hyperactivation of the RAS/ERK pathway in myeloid progenitor cells³²⁹. Support for this model comes from the observation that ICSBP^{-/-} mice develop an MPD similar to that seen in mice expressing leukemogenic SHP2 mutants⁶⁵.

Ultimately, SHP2 might contribute to RAS/ERK activation by dephosphorylation of SPROUTY proteins, proteins that in their tyrosyl-phosphorylated form have been implicated in local sequestration of RAS³³⁰.

SHP2 is highly expressed in hematopoietic cells and plays a critical role in hematopoietic cell development and function³³¹⁻³³³. SHP2 is also known to form a stable protein complex with p210 and to be heavily phosphorylated within this complex³³⁴⁻³³⁶. At least in part this interaction occurs through the BCR-ABL autophosphorylation site Y177, which was previously shown to be recognized by GRB2. As described in the introduction, GRB2 is subsequently recruiting GAB2, which in turn is binding PI3K and SHP2.

Growth factor-independent colony formation evoked by BCR-ABL is attenuated in GAB2^{-/-} BM cells. Consistent with these findings, GAB2 is a crucial determinant of the

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severity of BCR-ABL evoked leukemia, the PI3K and SHP2 binding sites being required for myeloid and lymphoid transformation⁵¹.

More importantly, SHP2 itself was found to be required for BCR-ABL mediated hematopoietic cell transformation³³⁷. A knock-out of *PTPN11* in hematopoietic cells compromised the leukemic potential of BCR-ABL due to proteasome mediated degradation of BCR-ABL and decreased oncogenic signalling³³⁷. SHP2 was shown to positively regulate the stability of BCR-ABL through the interaction with heat shock protein 90 (HSP90). The role of SHP2 in the stability of p210 is independent of its catalytic activity. Overexpression of catalytically-inactive SHP2 did not lead to p210 degradation but it nevertheless attenuated cell survival and enhanced serum starvation-induced apoptosis³³⁷. SHP2 therefore appears to also function in BCR-ABL downstream oncogenic signalling.

Mirroring the proposed interaction between PLCG1 and BCR-ABL, the SH2 domain mediated recruitment of SHP2 to the Y134 phosphorylated SH3 domain of BCR-ABL might confer a further enhancement to leukemic signalling.

However, SHP2 was already found to be associated with BCR-ABL via GAB2 at phosphorylated Y177 in the BCR region of the fusion protein. Also, bone marrow myeloid progenitors from GAB2 defective mice were shown to be resistant to transformation by BCR-ABL⁵¹, an effect that at least in part was attributed to decreased SHP2 association. These findings are rather arguing against a possible redundancy of Y177 (BCR) and Y134 (ABL) in SHP2 recruitment and signalling.

On the other hand, our co-immunoprecipitation experiments clearly demonstrate that mutation of Y134 to phenylalanine is dramatically reducing the percentage of BCR-ABL associated SHP2, underlining the weightiness of this interaction site.

5.1.3. Conclusions

Although we assume that only a small portion of the p210 molecules is actually phosphorylated in the SH3 domain and that in addition PLCG1 and SHP2 must compete for these few SH2 domain binding sites, the oligomeric nature of BCR-ABL might compensate for this. It is conceivable that BCR-ABL oligomerization allows the simultaneous and spatially collocated engagement of the “complexed” SH3 domains

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with canonical ligands via its PxxP binding site and with PLCG1 and SHP2 via phosphorylated Y134.

In conclusion, to our knowledge this is the first study showing a phosphotyrosine dependent interaction between a SH2 domain and a SH3 domain. Our findings emphasize the complexity of the molecular basis of CML and suggest that even well characterized protein modules can have concealed features that go beyond their conventional *modus operandi*.

To further characterize the SH3 domain mediated interaction of BCR-ABL with PLCG1 and SHP2 the functional relevance of this interaction will have to be addressed. For a start, transient transfection of HEK cells with either BCR-ABL wild-type constructs or BCR-ABL Y134F constructs and subsequent functional studies could shed some light on the underlying mechanisms. It is conceivable that for example differences in the transforming potential, the proliferation rate, the apoptotic rate, etc., will be observed. Also, one might look for changes at the level of the effector proteins in the signalling pathways downstream of PLCG1 and SHP2 (e.g. ERK phosphorylation, PKC and PI3K activity...).

Moreover, it will be interesting to see whether and how the phosphorylation patterns of SHP2, PLCG1 and BCR-ABL differ in BCR-ABL WT and BCR-ABL Y134F transfected cells and if overexpression of phosphatase dead SHP2, harboring the C459S mutation, affects BCR-ABL tyrosine-phosphorylation.

Finally, more information about PLCG1 and SHP2 in CML could be gained by characterizing the respective interactomes by performing tandem affinity purifications (TAPs).

5.2. MicroRNA deregulation in BCR-ABL suppressed cells

MiR-21 is a known oncogenic miRNA that was shown to be overexpressed in a variety of human malignancies ²³¹. Known miR-21 target genes include tumor suppressor genes like *PTEN*, *TPM1*, *PDCD4*, or *SERPINB5* ^{264, 266-269, 274}.

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Accordingly, elevated miR-21 levels are usually connected to enhanced cell survival and metastasis.

We have previously found miR-21 expression to be ~3.5 fold decreased following Dasatinib treatment of K562 cells on a protein microarray, raising the question of whether miRNAs in general and miR-21 in particular are involved in the manifestation of CML.

We now confirmed by miR-21 specific miR-qRT-PCR that inhibition of the BCR-ABL tyrosine kinase in K562 cells by either Dasatinib or Nilotinib treatment is indeed ensued by miR-21 downregulation.

To address the physiological role of miRNAs in BCR-ABL mediated leukemogenesis on a more extended level we looked for Dasatinib and Nilotinib induced alterations in the expression levels of all human miRNAs in K562 cells performing a miRNA-based microarray chip assay.

We found 31 miRNAs to be differentially expressed depending on whether the cells were subjected to drug treatment or not. Among these candidates are several miRNAs that have already been shown to possess either tumor-suppressive or oncogenic potential.

Most prominently, downregulation of miR-21 after drug treatment was confirmed by the miRNA microarray data. After four hour drug treatment, the amount of miR-21* strands was reduced to ~50%.

In general most downregulated miRNAs of our dataset were only detected at the star strand (miRNA*) level. Considering that miRNA* strands are generally present in much lower numbers and have a higher turnover rate than their complementary counterparts, the mature miRNA strands, it is only logical that reduced transcriptional activity will initially have a more dramatic effect on the miRNA* population.

Although drug treatment for four hours is apparently often not sufficient to reduce mature miRNA levels to a detectable degree, harvesting at a later time point might distort the results. K562 cells rely on the presence of catalytically active BCR-ABL, which is why the longer the drug mediated interference with the tyrosine kinase activity takes, the faster the apoptotic rate of the affected cells becomes. As apoptosis is accompanied by the activation of various cell death associated pathways, it would no longer be permissible to draw a direct connection between miRNA deregulation and BCR-ABL inhibition.

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In accordance with earlier reports that implicated transcriptional activation of the *miR-17-92* polycistronic cluster in oncogenesis we found members and paralogs of this miRNA family to be negatively regulated by both Dasatinib and Nilotinib treatment. Venturini et al. have shown that overexpression of a variant of miR-17-92 in K562 cells induces increased cell proliferation and antagonizes anti-c-MYC RNAi mediated proliferation arrest²⁴⁹. The authors postulated the existence of a BCR-ABL – c-MYC – miR-17-92 signalling pathway where miR-17-92 downstream of c-MYC positively regulates cell proliferation and survival.

We additionally identified several miRNAs that were upregulated in Dasatinib and Nilotinib treated cells. With the exception of miR-155 these miRNAs have so far no assigned role in tumorigenesis. As for miR-155, it is of notice that it was expressed at significantly higher levels in drug treated cells compared to the untreated samples. Based on previous reports, suggesting that miR-155 is acting as an oncogene, one would rather expect its upregulation in CML.

As a first step in the analysis of the relationship between candidate miRNA expression and BCR-ABL in CML, we used the web based miRNA target gene prediction tool at 'www.targetscan.org' to look for overlapping hits in the microarray dataset of genes that were found to be deregulated by Dasatinib. We found several potential miRNA target genes whose expression levels are inversely correlated with the levels of their targeting miRNA following drug treatment. However further studies will be required to verify some of this target genes and to elucidate their role in leukemogenesis.

In summary, the identification of these 31 differentially expressed miRNAs could represent a further step toward the identification of gene regulatory networks involved in CML pathogenesis.

In order to validate some of the potential miRNA target genes, qRT-PCR experiments, targeting the respective mRNAs, will have to be performed, comparing the mRNA expression levels in dependence of BCR-ABL activity or in the presence of respective miRNA antagomirs or mimics. To prove the translational repression by a specific miRNA, it will ultimately be necessary to generate 3'-UTR-reporter constructs, where the 3'UTR of the suspected target gene is cloned downstream of a

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luciferase reporter gene. Transient transfection of the reporter construct and the corresponding miRNA mimic in cells with a low endogenous expression for this specific miRNA, followed by subsequent monitoring of the reporter activity allows the confirmation of a miRNA – target gene relationship.

Alternatively, if antibodies for the gene product of a certain target gene are available, one could determine by immunoblotting if treatment with a specific miRNA mimic or antagomir is showing an effect at the protein level.

Additionally, treatment of K562 cells with specific miRNA mimics or inhibitors, combined with microarray-based gene expression profiling, would enable us to further characterize the role of a certain miRNA in CML.

Finally, deconvolution of the large and complex dataset could be accomplished by the bioinformatic analysis of miRNA – target genes networks, thereby entitling us to discern relevant connections and pathways that might be vital to BCR-ABL mediated leukemogenesis.

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