

NORMAL ABL1 IS A TUMOR SUPPRESSOR AND THERAPEUTIC  
TARGET IN BCR-ABL1-POSITIVE LEUKEMIAS

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## ABSTRACT

### NORMAL ABL1 IS A TUMOR SUPPRESSOR AND THERAPEUTIC TARGET IN BCR-ABL1-POSITIVE LEUKEMIA

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BCR-ABL1 results from t(9;22)(q34;q11) reciprocal translocation resulting in BCR-ABL1 kinase expression, initiating chronic myeloid leukemia in chronic phase (CML-CP). At the initial stages of CML-CP both oncogenic BCR-ABL1 kinase and normal ABL1 kinase are expressed, however, loss of ABL1 kinase expression in CML-CP can result from an interstitial deletion in the normal chromosome 9 [del(9q34)] which may be combined with the transcriptional silencing of the alternative ABL1 promoter within the translocation eventually leading to disease progression and drug resistance. We found that BCR-ABL1 *Abli*<sup>-/-</sup> cells generated a CML-blast phase (BP)-like disease phenotype in NOD-SCID mice compared to the BCR-ABL1 *Abli*<sup>+/+</sup> cells. To determine the mechanisms responsible for blastic transformation of BCR-ABL1 *Abli*<sup>-/-</sup> cells, we examined the role of ABL1 in proliferation, differentiation, apoptosis, genomic instability, and stemness. The presence of ABL1 inhibited proliferation in BCR-ABL1 cells as BCR-ABL1 *Abli*<sup>-/-</sup> cells had higher clonogenic activity and proliferative rate compared to their wild-type counterparts. ABL1 is essential for myeloid differentiation since BCR-ABL1 *Abli*<sup>-/-</sup> cells showed an immature blast phenotype when stained with Wright-Giemsa and myeloid differentiation markers Gr-1 and CD11b. ABL1 promoted

apoptosis in response to genotoxic stress as revealed by reduced clonogenicity and expression of p53, phosphoserine-15 p53 and activated caspase 3 in BCR-ABL1 *Abl1* <sup>+/+</sup> compared to knock-out cells. Although the absence of ABL1 did not enhance ROS and oxidative DNA damage, it appears that an impaired DNA damage response may be responsible for higher chromosome numbers and an accumulation of high numbers of chromosomal aberrations in BCR-ABL1 *Abl1*<sup>-/-</sup> cells. We detected an expansion of Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup> leukemia stem cells (LSCs) in BCR-ABL1 *Abl1*<sup>-/-</sup> cells compared to BCR-ABL1 *Abl1*<sup>+/+</sup> or non-transformed counterparts; among the LSCs, there was a higher percentage of CD34<sup>-</sup>Flt3<sup>-</sup> long-term and CD34<sup>+</sup>Flt3<sup>-</sup> short-term stem cells. These results showed that ABL1 is involved in regulating the LSC compartment in BCR-ABL1 cells. DNA microarray analysis revealed changes in mRNA levels of several genes involved in proliferation, myeloid differentiation, apoptosis, DNA damage response and ‘stemness’ in BCR-ABL1 *Abl1*<sup>-/-</sup> cells in comparison to BCR-ABL1 *Abl1*<sup>+/+</sup> cells. Together, these results demonstrate a critical role of ABL1 as a tumor suppressor in BCR-ABL1-induced leukemia, prolonging survival in mice by suppressing proliferation and expansion of LSC, inducing myeloid differentiation, apoptosis and DNA damage response in BCR-ABL1 cells. Loss of ABL1 was also found to contribute to Imatinib resistance in BCR-ABL1 cells. Moreover, we hypothesized that enhancement of the tumor-suppressor function of ABL1 may have a significant impact on CML treatment. A small molecule activator of ABL1 kinase, 5-(1,3-diaryl-1H-pyrazol-4-yl)hydantoin (DPH), have been reported to interact with the myristoyl-binding site of ABL1 and destabilize the bent conformation of the  $\alpha$ 1 helix, thereby preventing the auto-inhibitory conformation. Western blot analysis revealed partially restored activation of ABL1 kinase when

Imatinib-treated cells were incubated with DPH. DPH along with Imatinib was found to inhibit viability of BCR-ABL1 *Abl1*<sup>+/+</sup> cells but not BCR-ABL1 *Abl1*<sup>-/-</sup> cells demonstrating its ABL1-specific mode of action. DPH when used in combination with tyrosine kinase inhibitors such as Imatinib and Ponatinib inhibited growth of CML CD34<sup>+</sup> cells, Philadelphia chromosome-positive B-Acute Lymphoblastic Leukemia (Ph+B-ALL) cells and relapsed Ph+B-ALL cells harboring T315I mutation without affecting normal counterparts. A similar inhibitory effect was observed when TEL-ABL1-expressing cell lines and NUP214-ABL1-expressing murine bone marrow cells were treated with DPH and Imatinib, as well as Acute Myeloid Leukemia (AML) cells expressing FLT3-ITD mutation when treated with DPH in combination with AC220 which is the FLT3-ITD inhibitor. In summary, ABL1 is a potential tumor-suppressor in BCR-ABL1-induced leukemia and stimulation of its function may play a significant role in the development of novel therapeutic strategies for CML and other Fusion Tyrosine Kinase (FTK)-mediated hematologic malignancies.

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# TABLE OF CONTENTS

	Page
ABSTRACT.....	iii
ACKNOWLEDGEMENTS.....	vi
DEDICATION.....	viii
LIST OF TABLES.....	xi
LIST OF FIGURES.....	xii
CHAPTER	
1. INTRODUCTION.....	1
CHRONIC MYELOID LEUKEMIA.....	1
BCR-ABL1-mediated transformation and disease progression.....	4
Treatment.....	9
ABL1 TYROSINE KINASE.....	12
<i>ABL1</i> fusion genes in hematologic malignancies.....	17
<i>ABL1</i> promoter methylation: Prognostic Significance.....	19
ABL1 as a tumor suppressor.....	21
2. ROLE OF NORMAL ABL1 AS A TUMOR SUPPRESSOR IN BCR-ABL1- MEDIATED LEUKEMIA	
Introduction.....	27
Materials and methods.....	27
Preliminary data.....	35

Results.....	38
Discussion.....	51
3. ABL1 REGULATES IMATINIB SENSITIVITY IN BCR-ABL1-MEDIATED LEUKEMIA AND IS A THERAPEUTIC TARGET IN BCR-ABL1- AND OTHER FTK-MEDIATED LEUKEMIAS	
Introduction.....	59
Materials and methods .....	60
Results.....	64
Discussion.....	71
4. CONCLUSION, SIGNIFICANCE AND FUTURE DIRECTIONS .....	75
REFERENCES.....	78

## LIST OF TABLES

Table	Page
1.1 Fusion partner genes of ABL1 and phenotypes of hematologic malignancies.....	19

## LIST OF FIGURES

Figure	Page
1.1 Reciprocal translocation between chromosomes 9 and 22 generate the Philadelphia Chromosome.....	3
1.2 ABL1 structure showing different functional domains.....	13
1.3 ABL1 and BCR-ABL1: Antagonistic effects.....	26
2.1 BCR-ABL1 induces CML-BP-like murine leukemia in the absence of ABL1 .....	37
2.2 ABL1 inhibits proliferation of BCR-ABL1-leukemia cells .....	40
2.3 ABL1 is required to induce myeloid differentiation of BCR-ABL1-leukemia cells.....	42
2.4 ABL1 plays an essential role in the induction of apoptosis in BCR-ABL1-leukemia cells in response to genotoxic agents.....	44
2.5 The lack of ABL1 causes an expansion of the LSCs but not HSCs.....	46
2.6 Loss of ABL1 causes accumulation of chromosomal aberrations in BCR-ABL1-leukemia cells.....	50
2.7 Normal ABL1 is a tumor suppressor in BCR-ABL1-mediated leukemia.....	57
3.1 ABL1 kinase regulates Imatinib sensitivity of BCR-ABL1 leukemia.....	65
3.2 Imatinib-resistant phenotype of BCR-ABL1 <i>Abi1</i> <sup>-/-</sup> leukemia cells.....	66
3.3 Activation of ABL1 enhances the efficacy of TKI by inhibiting clonogenicity and/or proliferation of CML-CP, Ph+B-ALL, AML-FLT3-ITD and cells expressing TEL-ABL1 and NUP214-ABL1 but not the normal counterparts.....	69

## CHAPTER 1

### CHRONIC MYELOID LEUKEMIA (CML)

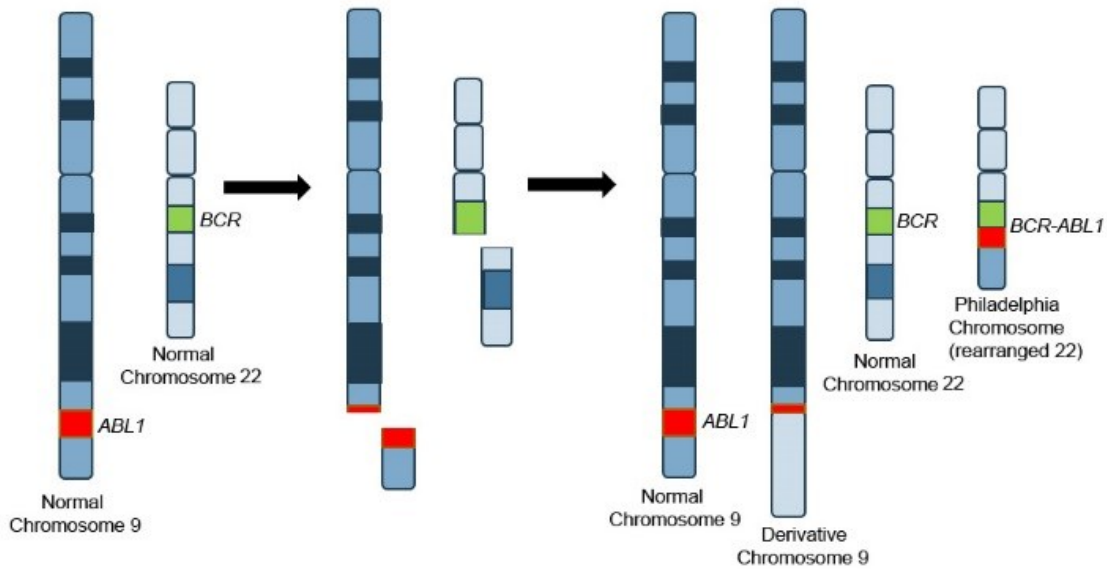
Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder of the hematopoietic stem cell which is consistently associated with an acquired genetic abnormality- the Philadelphia Chromosome (1-6). In 1960, Peter Nowell and David Hungerford associated an abnormally small chromosome with human granulocytic leukemia which was named 'Philadelphia Chromosome' from the place of its discovery. Using chromosome banding techniques, Janet Rowley in 1973 identified the Philadelphia chromosome to be a shortened chromosome 22 that results from a reciprocal translocation [t(9;22)(q34;q11)] between the long arms of chromosomes 9 and 22 (7,8). The chromosomal translocation which is the hallmark of CML, causes the *ABL1* gene on chromosome 9 to be juxtaposed to the *BCR* gene on chromosome 22 resulting in the *BCR-ABL1* fusion gene which is present in 95% of CML patients, and encodes the p<sup>210</sup>BCR-ABL1 chimeric protein (9-12).

CML has an incidence of one-two cases per 100,000 adults and accounts for 15% of newly diagnosed adult leukemias (10,13). CML is diagnosed mostly in the chronic phase (CP) through routine medical examinations and blood tests. The common symptoms at presentation include fatigue, weight loss, night sweat, left upper quadrant abdominal discomfort and early satiety which result from anemia and splenomegaly (10,13,14). CML has been characterized to be a biphasic and sometimes triphasic disease (14). The initial chronic phase has a duration of 3-5 years and is driven by expansion of myeloid progenitors and mature granulocytes in bone marrow, peripheral blood and extramedullary tissues (3,4,9,15,16). Most patients eventually progress to an aggressive

acute leukemia-like blast phase (BP) through a transitional accelerated phase (AP) . CML-AP which is not so well characterized, is associated with  $\geq 15\%$  immature blasts in the peripheral blood and a median survival time of 1-2 years (14,15,17). The terminal blast phase of CML is characterized by an arrest of hematopoietic differentiation in the myeloid or lymphoid lineages marked by the presence of  $\geq 30\%$  blasts in the bone marrow or peripheral blood . CML-BP has a poor prognosis with patients having a median survival of 3-12 months (4,10,15,17). Although BCR-ABL1 kinase is necessary and sufficient for malignant transformation as demonstrated through *in vitro* and *in vivo* models, the transition from CML-CP to CML-BP is driven by abnormal proliferation, defective adherence, suppression of apoptotic pathways and genomic instability. These are usually accompanied by several cytogenetic and molecular abnormalities some of which are trisomy of chromosome 8, isochromosome i(17q) which may account for the alterations in *p53* gene and duplication of Ph chromosome leading to increased BCR-ABL1 expression (4,10,16).

#### **t(9,22) Translocation: Molecular biology**

CML is the first human cancer to be consistently associated with a genetic abnormality -the Philadelphia chromosome, which encodes the constitutively active BCR-ABL1 fusion kinase, that drives the malignant transformation of a primitive hematopoietic stem cell (4,16). Although the etiology of CML is not clear, yet epidemiologic studies reveal exposure to ionizing radiation to be a risk factor for CML along with the physical arrangement of chromosomes 9 and 22 in human lymphocytes and CD34<sup>+</sup> cells which favor translocation between the two chromosomes (5,18).



**Figure 1.1. Reciprocal translocation between chromosomes 9 and 22 generate the ‘Philadelphia Chromosome’ containing the fusion *BCR-ABL1* oncogene.**

The breakpoint in the *ABL1* gene at 9q34 can occur anywhere over a large area of 300 kb between the two alternative first exons, usually 5’ of exon 2 (a2). In the *BCR* gene on chromosome 22 the breakpoints are located within a 5.8kb region spanning over 5 exons (e12-e16) which is called the major breakpoint cluster region (M-*bcr*) (**Fig 1.1**). Usually the break occurs in introns which are located either between exons e13 and e14 or between exons e14 and e15 (5,7,18). These variable *BCR* exons fuse to the *ABL1* exons (a2-a11) to form the e13a2 or e14a2 fusion transcripts which results in a 8.5kb mRNA which is translated to the 210kd p<sup>210</sup> BCR-ABL1 chimeric protein implicated in the pathogenesis of CML and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) (5,7,9,10). Another variant of the fusion transcript is formed when

the break occurs further upstream between *BCR* exons e2' and e2 called the minor breakpoint cluster region (m-bcr). This results in the e1a2 fusion transcript from which the 190kd p<sup>190</sup>BCR-ABL1 fusion protein is derived, implicated in Ph-positive ALL and rarely in CML patients (5,9). A third breakpoint has been identified in the *BCR* gene which is located between exons e19 and e20 ( $\mu$ -bcr) which when fused to the *ABL1* exons gives rise to the e19a2 transcript encoding the 230kd p<sup>230</sup>BCR-ABL1 protein which has been associated with some cases of Ph+ chronic neutrophilic leukemia (5,7,10).

### **BCR-ABL1-mediated transformation and disease progression**

Structure, Regulation and Localization: The reciprocal translocation between chromosomes 9 and 22 generates the *BCR-ABL1* gene which is translated to the p<sup>210</sup>BCR-ABL1 with constitutive tyrosine kinase activity in CML (19). This translocation results in the fusion of the truncated *BCR* gene to sequences upstream of the second exon of *ABL1* (20) which is believed to be responsible for the dysregulated kinase activity of BCR-ABL1 *in vitro* and *in vivo* (21). While the tyrosine kinase activity of normal ABL1 is tightly controlled by intra-molecular interactions, the aberrant kinase activity of BCR-ABL1 kinase necessary for malignant transformation results from the structural alterations which could either be the loss of the first exon and myristoylation signal of ABL1 or the fusion of amino-terminal (N-terminal) BCR sequences (22). BCR-ABL1 kinase contains several functional domains. The amino-terminus has the “Cap” region followed by SH3, SH2 and tyrosine kinase domains (23). The carboxy-terminal (C-terminal) part of ABL1 protein consists of the nuclear localization signals, DNA binding

domain and F-actin domain, the last of which is believed to play a role in transformation (24-26).

BCR has various functional domains but domain 1(amino acids 1-63) and domain 2 (amino acids 176-242) play key roles in the transforming function of the ABL1 kinase (27). BCR domain 1 in the N-terminal part is the coiled coil oligomerization domain which is responsible for the tetramerization of BCR-ABL1, that results in functional activation of the ABL1 kinase (27). While the presence of serine-threonine kinase domain in BCR accounts for activation of several signaling pathways by BCR-ABL1 (28), some other domains enable binding of adapter proteins like GRB2 and CRKL (29). Mutations in the coiled coil region of BCR inactivates the oligomerization function which impairs transforming activity of BCR-ABL1 protein in rat fibroblasts indicating that BCR-ABL1 oligomers play a crucial role in transforming function (27).

Wildtype ABL1 and BCR proteins can be both nuclear and cytoplasmic but the chimeric BCR-ABL1 is almost exclusively cytoplasmic (7,30-32).

Activation of signaling pathways: The various functional domains of the BCR-ABL1 oncoprotein along with its elevated tyrosine kinase activity enables binding and phosphorylation of several proteins leading to the activation of different mitogenic signaling pathways (11,18,22). Ectopic expression of BCR-ABL1 in growth factor-dependent cell lines has been found to induce growth factor-independence and confer protection to apoptosis by activating various signaling pathways (16). Some of the pathways activated by BCR-ABL1 which account for malignant transformation include Ras, MAPK, JNK/SAPK, PI-3K, STAT, NF- $\kappa$ B (33-41).

BCR-ABL1 like other receptor tyrosine kinases activates RAS signaling pathway by interaction with adaptor proteins like growth factor receptor-bound protein 2 (GRB2)/Gab2 complex, Crk-oncogene-like protein CrkL) and SRC homology 2-containing protein (SHC) (21,42,43). BCR-ABL1 bind to Grb2 by generating a binding site via autophosphorylation of tyrosine177 in BCR moiety. This is followed by Grb2-mediated conversion of GDP-bound Ras to GTP-bound state (44), which in turn binds to Raf; activation of Raf initiates the MAPK signaling pathway (45). Abrogation of the Ras signaling pathway was found to inhibit proliferation and induce sensitivity to apoptosis in BCR-ABL1-expressing cells (46-48).

Another signaling pathway which is key to BCR-ABL1-mediated transformation is the PI-3K/Akt dependent pathway (49). BCR-ABL1 activates PI-3K by interaction with its p85 regulatory subunit through docking proteins such as GRB2/Gab2 complex and c-CBL (50,51). This leads to activation of Akt kinase-dependent cascade (49) which modulates the localization and activity of target proteins such as BAD and MDM2 which result in inhibiting apoptosis and inducing p53 degradation respectively (52,53). The PI-3K/Akt pathway also down-regulates the expression and activity of the cyclin-dependent kinase (CDK) inhibitor p27 (54). Inhibition of the PI-3K/Akt pathway inhibited colony-formation in BCR-ABL1-expressing murine bone marrow cells and also inhibited leukemia development *in vivo* (49).

BCR-ABL1 activates the anti-apoptotic JAK and STAT signaling pathways in CML cell lines as well as in BCR-ABL1-expressing primary murine bone marrow (39,41). STAT5 is phosphorylated via the Src family protein hematopoietic cell kinase (Hck)

which is activated by BCR-ABL1. This facilitates nuclear translocation of STAT5 where it activates transcription of several target genes including the anti-apoptotic Bcl-X<sub>L</sub>, which confers protection from apoptosis to BCR-ABL1-positive cells; Imatinib treatment results in down-regulation of Bcl-X<sub>L</sub> levels thus sensitizing the cells to apoptosis (55).

Transcriptional activation of c-Myc in cells expressing BCR-ABL1 results in modulation of several target genes associated with cell cycle and apoptosis (56). Studies reveal, while Myc enhances BCR-ABL1-mediated transformation of fibroblast by three-fold, a dominant negative form impairs transformation of fibroblasts and murine bone marrow cells (57).

*Mechanism of Disease Progression:*

The generation of *BCR-ABL1* oncogene is the central pathogenetic event in CML and continued expression of p<sup>210</sup>BCR-ABL1 along with secondary molecular and cytogenetic changes are responsible for disease progression from CP to more advanced phases like AP or BP (3).

BCR-ABL1 overexpression: Continued expression and activity of BCR-ABL1 kinase is critical for CML maintenance as well as disease progression indicated by elevated levels of *BCR-ABL1* mRNA and protein in BP than in CP (58,59). Primitive CML CD34<sup>+</sup> progenitors have been found to have 200-fold higher BCR-ABL1 transcripts compared to more differentiated cells (60). Increased BCR-ABL1 expression results in additional genetic and epigenetic events which result in the molecular evolution of CML-BP leukemic stem cells (LSC) (61). In addition, BCR-ABL1 modulates growth factor

independence, resistance to apoptosis and clonogenicity in a dose-dependent manner (60,62).

Arrest of Differentiation: CML progression is marked by terminal differentiation block and clonal expansion of the granulocyte macrophage progenitor (GMP) pool (63,64).

With increased BCR-ABL1 dosage, modulation of activity of transcription factors like CEBP $\alpha$  and *Ikzf1* required for granulocyte differentiation and early lymphoid lineage commitment, impairs myeloid differentiation (65,66). Deletion/downregulation of both transcription factors have been associated with disease progression to CML-BP (64).

Genomic Instability and DNA repair: An aberrant DNA damage response causes genomic instability which facilitates CML progression (3,61). CML CD34<sup>+</sup> cells and BCR-ABL1-transformed cell lines have 2-6 times higher reactive oxygen species (ROS) compared to their normal counterparts (CML-BP>CML-CP>Normal) which results in higher levels of ROS-mediated oxidative DNA damage in the form of oxidized nucleobases and double strand breaks (DSB) in CML CD34<sup>+</sup> cells in comparison to normal counterparts (67-70). In addition to ROS, BCR-ABL1-expressing cells accumulate more DNA lesions due to ionizing radiation and genotoxic drugs, which cause chromosomal abnormalities (71,72). BCR-ABL1 also promotes unfaithful and/or inefficient repair of oxidized nucleobases and DSBs by modulating expression and activity of several key proteins like RAD51, DNA ligaseIII $\alpha$ , Werner etc.. Genomic instability not only contributes to disease progression but also TKI resistance by accumulation of point mutations and chromosomal aberrations like iso-chromosome 17q

associated with loss of *p53*, duplication of Ph, trisomy 8 along with other translocations, inversions etc. (61).

Decreased Apoptosis: In CML several signaling pathways contribute to apoptosis resistance (3). Some of the anti-apoptotic mechanisms in presence of *BCR-ABL1* oncogene include activation of PI-3K/Akt pathway which inactivates the pro-apoptotic protein BAD, elevated expression of BCL-2, activation of STAT5 resulting in upregulation of anti-apoptotic protein Bcl-X<sub>L</sub> levels and NF-κB-mediated protection from apoptosis (73-77).

### **Treatment**

Scientific discoveries led to the establishment of BCR-ABL1 as the critical pathogenetic event making it an ideal target for therapy (78). This led to the development of Imatinib Mesylate (IM), which is a BCR-ABL1 tyrosine kinase inhibitor, which revolutionized CML treatment and serves as frontline therapy (79). Splenic irradiation and cytotoxic drugs busulfan and hydroxyurea were the therapeutic options for CML before the discovery of Imatinib (80-82). Allogeneic stem cell transplantation is the only curative therapy in chronic compared to advanced phases because of higher rates of disease-free survival (81,82).

Radiation directed to the spleen or to the whole body was the standard therapy for 50 yrs in the early 1900s which resulted in a reduction of spleen size along with a decline in leukocyte count (82,83). This was followed by introduction of oral chemotherapeutic agents like busulfan and hydroxyurea which introduced hematologic remissions in 70-80% of patients with CML-CP (81). Although these agents were more efficient in

controlling CML than radiation therapy by prolonging survival yet they failed to induce Ph-negativity or prevent progression to terminal phases (81,83). This was followed by introduction of recombinant interferon- $\alpha$  (rIFN-  $\alpha$ ) for the treatment of CML which not only could induce complete hematologic response (CHR) rate of majority of CML-CP patients but could also induce complete cytogenetic response (CCyR) in a minority of patients (80). In spite of the survival advantage of rIFN-  $\alpha$  along with induction on Ph-negativity in some patients, discontinuing it had to be necessary for associated side-effects (81). Allogeneic stem cell transplantation remained to be the only curative therapy for CML (81,84). It was the treatment of choice for relatively younger patients in CML-CP due to the incidence of high disease-free survival rates and lower mortality and morbidity in these age groups (81,82). Lack of HLA-matched donors along with high rates of mortality and morbidity make a very small percentage of patients to be candidates for SCT (81). This led to the development of a selective inhibitor of the BCR-ABL1 kinase, STI571 (Gleevec®, Imatinib mesylate) which is a 2-phenylaminopyrimidine-based ATP competitive inhibitor. It binds to the ATP binding site of the inactive conformation of ABL1 kinase and inhibits proliferation along with abrogation of the BCR-ABL1 kinase-mediated downstream signaling pathways (79). Imatinib, in addition to BCR-ABL1 kinase also inhibits platelet-derived growth factor receptor (PDGFR), ARG and c-KIT. Imatinib induces CHR in 97% and CCyR in almost 86% patients in CML-CP and has become the frontline treatment for CML (85).

Despite significant hematologic and cytogenetic response, some patients mostly in advanced phase disease were found to experience Imatinib resistance which occurred *de*

*novo* or during treatment (86,87). An attempt to understand the underlying mechanism driving resistance revealed - point mutations in the ABL1 kinase domain which reactivate the BCR-ABL1 kinase, genomic amplification of *BCR-ABL1* with consequent increase in BCR-ABL1 levels, clonal evolution and a small sub-population of quiescent CML stem cells refractory to Imatinib, to be some of the major causes of resistance (86,87). This led to the development of the second-generation of tyrosine kinase inhibitors (TKI) Nilotinib, Dasatinib and Bosutinib which are more potent, well tolerated and are able to inhibit all BCR-ABL1 mutants except the T315I (78). The T315I mutation in BCR-ABL1 results in a change of threonine at position 315 to iso-leucine which impairs Imatinib-binding by hydrogen bond due to the absence of an oxygen atom in iso-leucine (88). The introduction of Ponatinib, a third generation TKI has been found to inhibit most of the Imatinib-resistant BCR-ABL1 mutations including T315I (89). In spite of the efficacy of available TKIs in CML treatment, minimal residual disease persists requiring continued treatment. Growing number of studies reveal that a small population of quiescent leukemia stem cells (LSC) are refractory to the inhibitory effects of TKIs due to the protective bone marrow microenvironment (90). This necessitates the development of agents which in combination with TKIs will inhibit the survival pathways of LSC and other targets, and be curative for CML (91). Many such non-TKI agents are being investigated for their potential application in CML therapy, some of which are JAK2 inhibitors, STAT3 inhibitors, mTORC inhibitor, Hedgehog and Wnt pathway inhibitors and HDAC and HSP90 inhibitors (91). These agents can be expected to enhance the inhibitory effect of TKIs in CML therapy

## **ABL1 TYROSINE KINASE**

ABL family of non-receptor tyrosine kinases are known to be conserved across evolution and consists of two paralogs, ABL1 (Abelson murine leukemia viral (v-abl) homolog 1 protein) and ABL2 (ARG, Abl-related gene tyrosine kinase). These kinases have been implicated in the regulation of cell proliferation, survival, cell adhesion and migration (92)

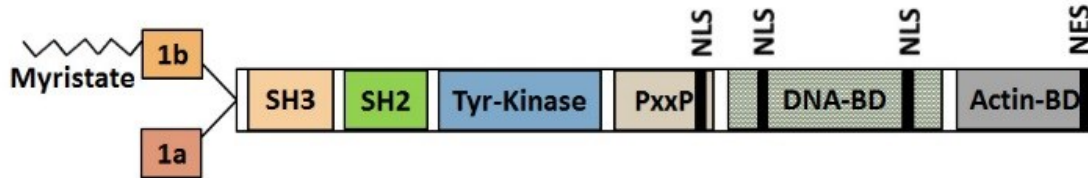
*Structure and Localization:* The ABL family of non-receptor tyrosine kinase consists of ABL1 and ABL2 which are localized on chromosomes 9 and 1 respectively (92). Both *ABL1* and *ABL2* genes encode 2 variant mRNA transcripts arising due to alternative splicing of the first exons, generating two proteins type 1a and 1b in humans with distinctly different amino-terminal sequences (93-95). The human ABL type 1b is 19 amino acids longer as it contains a consensus myristoylation signal at the amino-terminal glycine residue thus enabling membrane localization (31,96,97). ABL proteins contain a “cap” sequence, conserved Src-homology (SH) and kinase domains in their amino-termini but differ in the C-terminal domain (31).

The amino-terminal region of ABL1 contains three distinct functional domains:

- 1) a tyrosine kinase domain (SH1) that mediate catalytic functions,
- 2) a domain that binds tyrosine-phosphorylated peptides (SH2) and
- 3) a kinase inhibitory domain that also binds to proline-rich peptides (SH3) (31,98).

The long and unique C-terminal half contains F- and G-Actin binding domains, a single Nuclear Export Sequence (NES) and three Nuclear Localization Signals (NLS). In

addition, ABL1 also contains a bipartite DNA-binding domain and a proline-rich sequence which can bind SH3 domain-containing proteins (96,99) (**Fig 1.2**).



**Figure 1.2. ABL1 structure showing different functional domains (Tyr-Kinase: Tyrosine kinase, BD: Binding domain, NLS: Nuclear Localization Signal, NES: Nuclear Export Signal).**

The mammalian ABL1 localizes to several subcellular compartments including cytosol, nucleus, plasma membrane, endoplasmic reticulum, mitochondria, actin cytoskeleton and lipid rafts (100-110). The presence of three NLS and one NES accounts for different subcellular localization and the nuclear-cytoplasmic shuttling of ABL1. Much of the cytoplasmic ABL1 is bound to filamentous actin and plasma membrane, owing to its actin-binding domain and myristoylation signal, respectively (104,111). While ABL1 expression is mostly nuclear in fibroblasts (14,16,21), ABL1 is predominantly cytoplasmic in primary hematopoietic cell lines and neuronal cells (103,110).

Regulation/Activation: Structural and biochemical analyses reveal multiple autoinhibitory mechanisms of regulation of ABL1 kinase activity through intra-, as well as intermolecular interactions (92). The SH3 and SH2 domains of ABL1 contribute majorly to its autoinhibition. The SH3 domain binds the linker region that connects the SH2, the kinase domain and the small lobe of the kinase domain, while the SH2 forms a protein-

protein interface with the large lobe of the kinase domain. Together, these interactions impose a restriction on the tyrosine kinase domain (112,113). Deletion or mutation of the ABL1 SH3 and SH2 domains leads to increased tyrosine kinase activity *in vivo* (113-116). The myristoyl group in the amino-terminal end of ABL 1b isoform can bind to a deep hydrophobic pocket in the kinase domain thus adopting an auto-inhibited conformation which is further stabilized by the amino-terminal “cap” sequence. Forms of ABL 1b that lack the myristoyl group have constitutive tyrosine kinase activity (114). In addition, the ABL1 SH3 domain inhibits kinase activity by interaction with inhibitory proteins like Pag/MSP23. The tumor-suppressor protein Retinoblastoma (Rb) also inhibits ABL1 activity by binding to its ATP-binding lobe (117).

Disruption of the SH3 domain-mediated inhibitory interaction by proline-rich ligands like ST5, c-CBL, c-Jun and RFX1, that engage the SH3 domain, lead to activation of ABL1 activity (118-122). Similarly, several tyrosine-phosphorylated proteins like ABI-1, NCK, Paxillin, CBL,  $\gamma$ -PAK were found to bind the ABL1 SH2 domain *in vitro*, thus disrupting the SH2-kinase interface leading to kinase activation (115,118,123-125).

Autophosphorylation of two key regulatory tyrosine residues, Tyrosine-412 and Tyrosine-245 in the activation loop and mutation of the SH3 domain have also been found to stimulate the basal catalytic activity of ABL1 (126).

ABL1 kinase is activated by Epidermal Growth Factor (EGF) and Platelet-Derived Growth Factor (PDGF) stimulation. PLC- $\gamma$ 1 also activates the kinase by hydrolysis of phosphatidyl inositol biphosphate (PIP2) (127). Ionizing radiation (IR) activates ABL1

activity by Ataxia-Telangiectasia-mutated (ATM)-mediated phosphorylation of Serine465 and also by DNA-dependent protein kinase (DNA-PK) *in vitro* (117).

Function: ABL1 is implicated in many important cellular processes due to its multiple protein-protein and protein-DNA interactions along with its tyrosine kinase activity (117).

Cell growth and survival/apoptosis: ABL1 appears to be a positive and negative regulator of cell growth depending on its subcellular localization (92,99). Overexpression of ABL1 in fibroblasts inhibits cell growth and results in G1 arrest, an effect which requires nuclear localization of the protein (128,129). ABL1-mediated G1-growth arrest requires tumor suppressor proteins such as p53 and Rb as downstream effectors (129,130). Other reports contradict these findings by treating primary hematopoietic cells with ABL1-specific antisense oligodeoxynucleotides which downregulate *ABL1* mRNAs, leading to a reduction in the number of cells in S phase (131). This is consistent with the observation that *Abi1*-deficient fibroblasts experience a 4 hour delay in S phase entry in response to PDGF stimulation, compared to ABL1-reconstituted fibroblasts (132).

Several studies implicate ABL1 in regulating survival/apoptosis (92). B-cell lines derived from *Abi1*<sup>m1</sup> mice display increased sensitivity to apoptosis in absence of growth factor or by glucocorticoid treatment. This indicates an anti-apoptotic role of ABL1 under these specific conditions or a pro-apoptotic role of the ABL1 mutant (133). In contrast, other studies have indicated a pro-apoptotic role of ABL1. Yuan and coworkers have reported an apoptosis resistant phenotype of cells lacking ABL1 or expressing kinase-inactive mutant in response to IR. These cells display resistance to IR-induced loss of

clonogenic growth and apoptosis thus suggesting a pro-apoptotic function of ABL1 (117,134). p73 is a critical downstream effector of ABL1 activation following IR or cisplatin treatment which results in DNA damage-induced apoptosis. ABL1 increases the half-life of p73 resulting in its accumulation and also interacts with and phosphorylates p73 (101,135,136). Further, disruption of ABL1-p73 interaction fails to induce apoptosis in response to IR (135,136).

Cellular stress response: DNA damage and oxidative stress: The DNA binding domain of ABL1 which shares sequence similarity to HMG proteins enables it to be intimately associated with DNA replication, recombination and repair (collectively called DNA transactions) (117). Nuclear ABL1 undergoes robust activation in response to IR or other genotoxic agents through ATM as the upstream DNA damage response (DDR) regulator (117). Several studies indicate ABL1 to interact and/or phosphorylate a large number of proteins involved in DDR including RAD9, RAD51, RAD52, WRN, DNA-PK, DDB1, DDB2, ERCC3, ERCC6 and MLH1 (137-139). ABL1 has been shown to induce IR-induced G1 arrest by a p53-dependent mechanism (140). Another study shows that cisplatin-induced DNA damage activates apoptosis when ABL1 enhances the apoptotic function of p73 (141). An interesting hypothesis is that ABL1 acts as the decision maker in response to DNA damage whereby it induces growth arrest following moderate damage and induces apoptosis after severe damage (142).

Cytoplasmic ABL1 is activated 5-fold in response to oxidative stress which targets ABL1 to the mitochondria. The apoptotic response to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress is attenuated in *Abl1*-deficient fibroblasts due to the abrogation of cytochrome c release

(143). Another study reveals *Abl1*-deficient osteoblasts to exhibit hypersensitivity to oxidative stress with elevated induction of the anti-oxidant peroxiredoxin I and that PKC $\delta$  mediates the role of ABL1 in these events (144).

Actin-binding and cell adhesion: ABL1 kinase plays an important role in actin cytoskeletal reorganization as it can localize to sites of F-actin assembly upon growth factor stimulation where lateral ruffles are formed (145). ABL1 also plays a role in dorsal ruffle formation which is regulated by Rac (146). Increased ABL1 activity has been reported to reduce cell motility (147,148). The CRK family and CAS family of proteins are key regulators of cell adhesion and motility and ABL1-mediated phosphorylation of CRK disrupts its binding to CAS which leads to reduced cell migration (149,150). SRC-family kinases –mediated phosphorylation of ABL1 also regulates cell-migration (151).

### ***ABL1* fusion genes in hematologic malignancies**

Uncontrolled activation of tyrosine kinases (TKs) is considered a driving factor in the pathogenesis of many human malignant disorders. In leukemias, the formation of a fusion gene between a kinase and an independent, unrelated gene encodes a chimeric protein that consists of the TK Carboxy-terminal (C-terminal) region including the entire catalytic domain linked to the N-terminus of the partner gene. This leads to constitutive activation of the fusion tyrosine kinase (FTK) and potential malignant growth.

Constitutively activated mutants of the non-receptor tyrosine kinase ABL1 play a central role in the pathogenesis of clinically and morphologically distinct chronic myeloproliferative disorders (CMDs) but are also found in some cases of *de novo* acute

leukemia (152). Ligand-independent activation occurs as a consequence of the creation of FTKs by balanced reciprocal translocations or episomal amplification.

By far, the most frequent and best-studied *ABL1* fusion gene is *BCR-ABL1* in Philadelphia chromosome (Ph<sup>1</sup>), which is predominantly found in patients with chronic myelogenous leukemia (CML), and, to a lesser extent in pre-B-acute lymphocytic leukemia (ALL) and occasionally *de novo* acute myelogenous leukemia (AML) (152). The fusion between BCR exon 1 (e1) and ABL1 exon 2 (a2) (e1a2 fusion) encodes a chimeric protein of 190 kDa, referred to as p190<sup>BCR-ABL1</sup>. It is found in 50–70% of *BCR-ABL1*-positive ALL patients and very rarely in monocytosis-associated CML or *de novo* AML of M4/M5 phenotype. The e13a2 and e14a2 fusion transcripts (often referred to as b2a2 and b3a2, respectively) encode a p210<sup>BCR-ABL1</sup> fusion protein of 210 kDa in almost all CML cases and in about 30–50% of *BCR-ABL1*-Acute Lymphoblastic Leukemia (ALL) cases. The rare e19a2 fusion transcript encodes a p230<sup>BCR-ABL1</sup> fusion protein of 230 kDa. This fusion was associated with atypical and typical CML.

The second most frequent and biologically distinct *ABL1* fusion gene is *NUP214-ABL1*. Remarkably, this fusion is generated by circularization of the 500-kb genomic region from *ABL1* to *NUP214* and subsequent extrachromosomal (episomal) amplification (153). *NUP214-ABL1* fusion gene was found in approximately 4% of all cases of adult ALL.

Other *ABL1* fusion genes have been described but are uncommon. *EML1-ABL1* was found in a single female patient with T-ALL and a cryptic t(9;14)(q34;q32). The *ETV6-ABL1* fusion gene is the product of a t(9;12)(q34;p13) and is found in occasional patients with

acute leukemias or CMDs. Recently, *ZMIZ1* and *RCSD1* were identified as *ABL1* partners in single cases of B-ALL (152).

An overview of currently known *ABL1* fusion partners in hematological disorders is presented in **Table 1.1**. However, in addition to FTK these malignancies also express *ABL1* from the non-mutated allele.

**Table 1.1. Fusion partner genes of *ABL1* and phenotypes of hematologic malignancies**

<i>Fusion partner</i>	<i>Disease</i>
<i>BCR</i>	<i>CMD (CML), ALL, AML</i>
<i>ETV6 (TEL)</i>	<i>CMD, acute leukemias</i>
<i>EML1</i>	<i>T-ALL</i>
<i>NUP214</i>	<i>T-ALL</i>
<i>RCSD1</i>	<i>Pre-B-ALL</i>
<i>ZMIZ1</i>	<i>B-ALL</i>

#### ***ABL1* Promoter methylation: Prognostic significance**

DNA methylation of CpG islands and associated promoter regions is an effective mechanism of gene silencing. This results in inactivation of key tumor suppressor pathways, which drives tumorigenesis (154). In most cases of CML, the translocation breakpoint occurs in the region separating the two alternate first exons of *ABL1* which results in the exon 1a sequences and the associated promoter (Pa) to be retained in the Ph translocation (155,156). A lack of transcriptional activation of Pa promoter has been reported in some CML cell lines which is believed to be due to hypermethylation of the translocated Pa promoter in some cases of CML (156).

No *ABL1* Pa promoter methylation was detected in normal blood and bone marrow cells and also in cells from numerous AML cases, however *de novo* partial (15-20%) and high (>25%) methylation was more frequent in CML (157,158)-AP/BP (75-80% of cases) than CML-CP (45% of patients) implicating its role in CML malignant progression (156,157,159). Other cohorts of studies reported *ABL1* methylation in 26% and 77-81% of CML-CP cases (157,158,160). This discrepancy between *ABL1* methylation frequency in CML-CP may be due to heterogeneous patient population because partial methylation was mostly observed in CML-CP of long duration (>24 months after diagnosis) (157,161). Although CML-CP cells usually express p210BCR-ABL1 and p145ABL1 proteins (162,163), malignant progression to CML-BP is often associated with methylation of the *ABL1* Pa promoter embedded in the translocation (156,161,164) accompanied by methylation or genetic disruption of the normal *ABL1* allele in chromosome 9 not involved in the fusion (165,166), resulting in inhibition or complete loss of p145ABL1 protein expression (156). Interestingly, analysis of 11 CML-CP patients revealed that *ABL1* mRNA was expressed in 57-100% of BCR-ABL1 -positive CFU-GM colonies supporting the role of *ABL1* methylation in clonal evolution of some CML patients (161). Moreover, progressive silencing of the wild-type *ABL1* allele occurred also in CML cells not responding to imatinib (165). In conclusion, CML-CP patients usually express ABL1 kinase, but a cohort of CML-CP late patients and CML-AP/BP patients may not express or display diminished level of p145<sup>ABL1</sup>.

### **ABL1 as a tumor suppressor**

CML-CP cells usually express both BCR-ABL1 and ABL1 proteins (162,163). ABL1 and BCR-ABL1 proteins exert opposing effects on a myeloid cell. Different regulatory mechanisms along with sub-cellular localization may account for some of their antagonistic functions. Another probable hypothesis is that ABL1 and BCR-ABL1 can also compete for binding sites on the same protein or phosphorylate different sites on the same protein leading to opposing effects on the cell (32). Inactivation of tumor suppressor genes such as p53 and p16<sup>INK4a</sup> is one of the key mechanisms underlying CML progression to blast phase (167-169).

Several studies report that transcriptional silencing of the proximal promoter of *ABL1* due to progressive methylation leads to inhibition or complete loss of ABL1 kinase expression in advanced phases of CML (158,170). Diminished levels of ABL1 will impair the induction of growth arrest and apoptosis in response to genotoxic and oxidative stress, thereby enhancing genomic instability which has been associated with disease progression (3). These observations suggest a potential tumor-suppressor role of ABL1 in BCR-ABL1 mediated leukemia.

Structure: ABL1 is a 145kDa protein which is a member of the ABL family of non-receptor tyrosine kinases. ABL1 protein has two isoforms 1a and 1b (in humans) arising due to an alternative splicing of the first exon region; the 1b isoform is 19 amino acids longer due to a myristoylation signal in the N-terminus. The N-terminus of the protein also contains the tyrosine kinase domain, SH2 and SH3 domains (171-173). The C-

terminus is long and unique and crucial for ABL1 functions, containing three NLSs, a single NES, DNA-binding domain, HMG-like boxes and an actin-binding domain (25,174-176).

The Philadelphia chromosome is a shortened chromosome 22, resulting from a balanced reciprocal translocation that adds a 3' part of *ABL1* gene on chromosome 9q34 to the 5' segment of *BCR* gene on chromosome 22q11. Depending on the breakpoint, three fusion proteins are generated of which p210<sup>BCR-ABL1</sup> is found in 95% of CML patients (10). BCR-ABL1 contains several functional domains including the N-terminal "Cap" region, SH3, SH2 and tyrosine kinase domains along with DNA- and actin-binding domains, proline-rich motifs in the carboxy terminus which provide binding sites for CRK and GRB2 proteins (23,64). The BCR moiety also contains multiple domains some of which are the coiled-coil oligomerization domain, serine/threonine kinase domain, pleckstrin homology domain (64)

Subcellular Localization: The mammalian ABL1 kinase localizes to both the nucleus and the cytoplasm along with several other subcellular compartments like plasma membrane, mitochondria, endoplasmic reticulum, actin cytoskeleton and lipid rafts (100-110). The subcellular distribution of ABL1 in the nucleus and cytoplasm is due to the presence of the three NLSs and NES (92). In contrast to ABL1, BCR-ABL1 is almost exclusively localized in the cytoplasm (110).

Regulation/Activation: The low catalytic activity of ABL1 kinase stems from auto-inhibitory intra- as well as intermolecular interactions. The interactions between the SH3 and SH2 domains that bind to the distal face of the kinase region keeps the kinase in a

state of low catalytic activity (98,112,177). The interaction of ABL1 SH3 domain with proteins like Pag/Msp23 and the binding of Rb with the ATP-binding lobe of ABL1 are also kinase-inhibitory (117). ABL1 is activated by PLC- $\gamma$ -mediated hydrolysis of PIP2, growth factor (PDGF and EGF) stimulation and exposure of cells to genotoxic stress like IR and chemotherapeutic agents (32,178,179).

In contrast to the highly regulated ABL1 kinase activity, BCR-ABL1 has constitutive tyrosine kinase activity due to the coiled-coil oligomerization domain of the BCR moiety which permits dimerization of the BCR-ABL1 protein. This enables cross-phosphorylation of key tyrosine residues in the activation loop of the kinase domain, leading to its activation (31,98). Also the fusion of BCR sequences to the N-terminus of ABL1 disrupts the kinase-inhibitory interactions of the ABL1 SH3 domain, causing ABL1 kinase to become constitutively active (10,180).

*Functions:* While ABL1 has been implicated in a host of cellular processes including proliferation, survival/apoptosis, cell adhesion and migration (92), its transforming variant BCR-ABL1 regulates aberrant adhesion, apoptosis and proliferation of myeloid cells (9).

*Proliferation:* ABL1 exerts both positive and negative regulatory effect on cell proliferation depending on its subcellular localization (92). Cytoplasmic ABL1 reportedly promotes cell cycle progression in hematopoietic cells (131) and fibroblasts (in response to PDGF stimulation) (108). However, reports indicate overexpression of ABL1 to induce G1- arrest in fibroblasts (128,129). The growth inhibitory function of ABL1

requires its nuclear localization and the proteins p53 and Rb as downstream effectors (129,130).

Ectopic expression of BCR-ABL1 in multiple growth factor-dependent cell lines has resulted in constitutive activation of mitogenic signaling pathways, thereby inducing growth factor-independent proliferation and reduced susceptibility to apoptosis (16).

BCR-ABL1 exerts growth factor-independence via the cellular response of RAS and JAK/STAT pathways to growth factor induction (181,182).

*Adhesion:* ABL1 has been linked to the process of cellular adhesion as an increased ABL1 activity reduces cell motility (147,148). ABL1 may exert an inhibitory effect on fibroblast migration via modulation of Crk/CAS complexes at focal adhesions (183). In contrast, BCR-ABL1 induces increased cell motility, membrane ruffling and filopodia formation in both hematopoietic cells as well as fibroblasts (184). This may account for the premature bone marrow release of BCR-ABL1-positive progenitor cells in CML (3,30).

*Transcriptional Activity:* The carboxy-terminal fragment of ABL1 contains a DNA-binding domain which enhances the transcriptional activity of the tumor-suppressor p53 and also of Gal4/VP-16. The carboxy-terminal domain of RNA Polymerase II is also a substrate of ABL1, which is phosphorylated following DNA damage. These may suggest the role of ABL1 as a transcriptional co-activator. Gene transcription is also altered by BCR-ABL1-activated signaling pathways. The activation of c-Myc, c-Jun and STAT-5 is observed at the transcriptional level in BCR-ABL1-positive cells that drives malignant transformation (178).

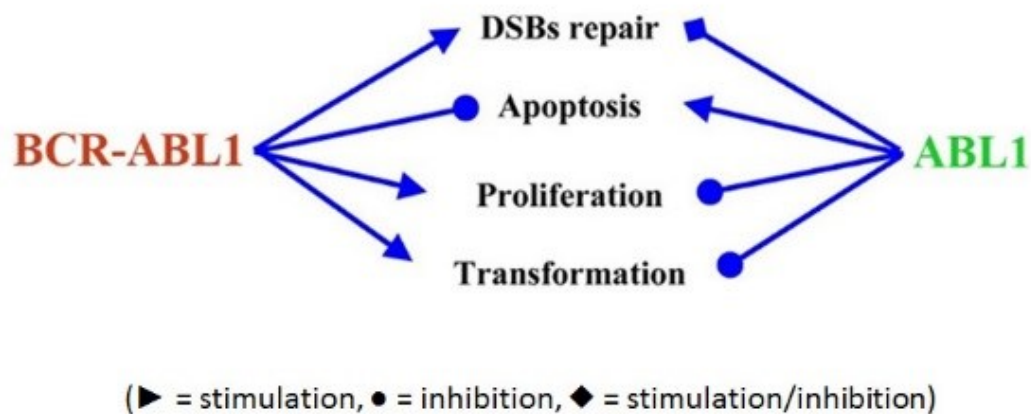
DNA Repair: ABL1 has been reported to play a distinct role in DNA repair (117). ABL1 interacts with multiple DNA damage repair proteins including RAD51, ATM and DNA-PK (139). ABL1 induces G1 arrest in response to IR which is believed to involve a role of p53 (129,130). In case of severe DNA damage, ABL1 induces apoptosis via the downstream effector p73 in response to genotoxic drugs such as cisplatin (142).

BCR-ABL1-transformed cells accumulate higher levels of irradiation-induced and drug-induced DNA damage along with higher levels of ROS-mediated oxidative DNA damage than normal cells (67,68,70-72). BCR-ABL1-mediated protection from apoptosis and/or deregulation of DNA damage response makes leukemia cells more tolerant to DNA damage (185). BCR-ABL1 oncoprotein promotes mutagenic nucleotide excision repair as well as unfaithful DNA double strand break repair and modulates DNA repair proteins such as downregulation of DNA-PKcs (67,68,186-189). BCR-ABL1 kinase enhances unfaithful repair of DNA double strand break which confers resistance to drug therapy which in turn contributes to genomic instability (30).

Apoptosis: ABL1 modulates apoptosis and several studies indicate a pro-apoptotic role of ABL1 kinase when overexpressed in fibroblast (129,190). Wang *et al* reported cisplatin-induced DNA damage activates apoptosis by p73 as the downstream effector of ABL1 kinase (141). ABL1 kinase is also activated in response to oxidative stress indicated by an attenuated pro-apoptotic response to H<sub>2</sub>O<sub>2</sub> in fibroblasts (191).

BCR-ABL1 expression conferring protection from apoptosis in growth factor-dependent murine and human cell lines in response to apoptotic stimuli such as growth factor withdrawal, irradiation of cytotoxic agents (182,192,193). The upregulation of

anti-apoptotic BCL-2 and BCL-X<sub>L</sub> proteins through the RAS (194) and STAT5 or PI-3K (75,195) pathways respectively, is one of the mechanisms of protection from apoptosis. The activation of PI-3K/AKT-dependent pathway by BCR-ABL1 results in inactivation of the pro-apoptotic protein BAD which is another mechanism contributing to apoptosis resistance in BCR-ABL1-expressing cells (73).



**Figure 1.3. ABL1 and BCR-ABL1: Antagonistic biological effects.**

Thus, opposing regulatory effects of the two proteins, ABL1 and BCR-ABL1, on cell proliferation, survival/apoptosis, DNA repair and cell adhesion suggest a critical role of ABL1 in BCR-ABL1-transformed cells. Finally, BCR-ABL1 initiates malignant transformation, but ABL1 may prevent transformation (11,196). We hypothesize that ABL1 is a potential tumor suppressor in leukemogenesis mediated by BCR-ABL1 and other FTKs containing the ABL kinase (see Table 1.1).

## CHAPTER 2

# ROLE OF NORMAL ABL1 AS A TUMOR SUPPRESSOR IN BCR-ABL1-MEDIATED LEUKEMIA

### Introduction

BCR-ABL1 and ABL1 can exert opposite effects on a variety of cellular functions (32). Previous findings from our laboratory showed that BCR-ABL1 protects leukemia cells from genotoxic and oxidative stress by stimulation of DNA repair (67,186,197-199), whereas ABL1 may inhibit or stimulate DNA repair (134,137,200-203). BCR-ABL1 inhibits DNA damage-induced apoptosis (204,205), however, ABL1 kinase may facilitate apoptosis (135,136,206-208). Thus, pro-apoptotic function of ABL1 may counteract anti-apoptotic activity of BCR-ABL1 in response to genotoxic stress. Activation of the nuclear ABL1 may inhibit cell proliferation, whereas cytoplasmic BCR-ABL1 stimulates cell cycle progression (30,209). In addition, ABL1 inhibits, whereas BCR-ABL1 stimulates cell migration (92). Finally, BCR-ABL1 initiates malignant transformation, but ABL1 may prevent transformation (11,196). We hypothesize that ABL1 plays an important tumor suppressor role in leukemogenesis mediated by BCR-ABL1.

### Materials and Methods

**Cells:** *Abl1* +/- transgenic mice kindly provided by Dr. A. J. Koleske (Department of Molecular Biophysics and Biochemistry, Yale University) were bred to obtain +/+ and -/- littermates. Bone marrow cells (BMC) were harvested from tibia and femur by flushing with culture medium. Mononuclear cells were obtained after density separation by

Lympholyte-M (Cedarlane, Hornby, ON, Canada). Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS) in presence of SCF and IL-3 at concentrations necessary to maintain proliferation of cells. Cells were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C.

**Retroviral Infection:** Bosc23 (American Type Culture Collection, Manassas, VA, USA) cells were transiently transfected with pMIG-BCR-ABL1-IRES-GFP retroviral construct and 36 hours post-transfection viral supernatant was collected, filtered through a 0.45µm filter and concentrated by centrifugation at 20,000 rpm, at 14°C for two hours. Target cells were infected with a 1:1 mixture of concentrated viral supernatant and fresh medium, twice a day for two days in the presence of 4µg/ml Polybrene (Sigma). Green fluorescent protein (GFP)-positive cells were isolated by fluorescence activated cell sorting (FACS) and analyzed for BCR-ABL1 expression by Western Blot. GFP-positive BCR-ABL1 *Abl1*<sup>-/-</sup> cells, thus obtained were infected with pMSCV-retroviral construct encoding yellow fluorescent protein (YFP)-ABL1 fusion protein and GFP/YFP-positive cells were isolated by FACS and cultured in medium with growth factors.

**Leukemogenesis in NOD-SCID Mice:** 10<sup>3</sup>, 10<sup>4</sup> and 10<sup>5</sup> BCR-ABL1 *Abl1*<sup>-/-</sup>, BCR-ABL1 *Abl1*<sup>+/+</sup> and BCR-ABL1 *Abl1*<sup>-/-</sup> cells restored with YFP-ABL1 were injected into the tail vein of sub-lethally irradiated (300 rads) outbred NOD-SCID mice. Mice were monitored for leukemia development and terminally ill mice were sacrificed. Leukemia development was confirmed on necropsy. Median survival time was calculated using the log-rank test. Tissue sections of spleen from leukemia-sick mice were stained with hematoxylin and eosin.

**Colony formation assay:** GFP-positive BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells were isolated by flow cytometry and maintained in presence of SCF and IL-3 at concentrations required to maintain proliferation. Colony formation was studied by plating freshly infected BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells (10<sup>4</sup>/ml) Methocult H4100 semisolid medium (StemCell Technologies) in the absence of growth factor; colonies were scored after 7 days. This was repeated every two weeks until colonies stopped growing. 10<sup>4</sup>/ml growth factor-independent GFP-positive BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells and GFP/YFP-double positive BCR-ABL1 *Abl1*<sup>-/-</sup> cells restored with YFP-ABL1 were plated in the absence of growth factors and colonies were scored after 7 days.

**Competitive growth assay:** A mixture of 10% GFP-positive BCR-ABL1 *Abl1*<sup>-/-</sup> and 90% GFP/YFP-positive BCR-ABL1 *Abl1*<sup>-/-</sup> cells restored with YFP-ABL1 were maintained in IMDM supplemented with FBS and SCF and IL-3 *in vitro* and also simultaneously injected into NOD-SCID mice. After a period of 5 weeks the resultant cell mixture was analyzed by flow cytometry to determine the percentage of GFP and GFP/YFP double positive cells *in vitro*. Bone marrow was harvested from NOD-SCID mice and mononuclear cells were isolated as described previously. Bone marrow (BM)-derived cells were analyzed to determine the percentage GFP and GFP-YFP double positive cells *in vivo* by flow cytometry.

**Wright-Giemsa staining:** BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells growing in suspension were cultured in IMDM supplemented with 10% FBS in the presence of SCF and IL-3 or G-CSF (10ng/ml) (PeproTech) for 7 days. Cells from each group were

washed in 1X Phosphate Buffered Saline (PBS), resuspended in 1X PBS supplemented with 1% Bovine serum albumin (BSA) and centrifuged in a Cytospin II centrifuge (Shandon Inc., Pittsburgh, PA). Cells were fixed in methanol for 1 minute and dried. Cells were stained with 1-2 ml of Wright Giemsa stain (Sigma) for one minute and equal volume of deionized water was added. After 3 minutes slides were rinsed with deionized water and allowed to air dry.

**Drug resistance assay:** Cisplatin (Platinol-AQ; Bristol-Myers Squibb Co., Princeton, N.J.), Mitomycin C (Sigma Chemical Co., St. Louis, Mo.), etoposide (Ben Venue Labs, Inc.), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Sigma) and H<sub>2</sub>O<sub>2</sub> (Rite Aid Corporation) were added to BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells (103/ml) growing in semisolid methylcellulose medium at indicated concentrations. Colonies were scored after 7 days.

**Protein expression:** Total cell lysates were obtained from BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells cultured in the presence of 1.5µg/ml of cisplatin for 0-, 12- and 24 hour(s). Cells were lysed by adding 1X SDS Laemmli sample buffer (100mM Tris HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) to the cell pellet and boiling for 3 minutes at 100°C, followed by sonication for 10 seconds on ice. Lysates were resolved by SDS-PAGE and examined by Western Blot analysis using anti-ABL1, anti-actin, anti-GFP, anti-p73, anti-p53, anti-Phospho-Serine15-p53, anti-activated caspase 3, and anti-tubulin as indicated. All antibodies were obtained from Santa Cruz Biotechnology except anti-Phospho-Serine15-p53 which was obtained from Cell Signaling and anti-ABL1 from

Calbiochem. IRDye800 or 680 secondary antibodies were used to develop the blots in the Odyssey Imaging system (Licor).

**Immunostaining of bone marrow-derived murine hematopoietic stem cells:** BM-derived cells were isolated from *Abl1*<sup>+/+</sup> and *Abl1*<sup>-/-</sup> mice and retrovirally transduced with pMIG-BCR-ABL1-IRES-GFP. Cells were stained after 0 week in culture and 2- and 8 weeks post-retroviral infection to isolate hematopoietic stem ( $\text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+$ ) cells. Cells from each sample were washed and resuspended in 1X PBS supplemented with 1% BSA. Cells were stained with Fc Block for 10 mins at room temperature followed by incubation with APC or PerCP-Cy5.5-conjugated anti-lineage cocktail (CD3e, CD11b, CD45R/B220, Ly-76, Ly-6G, and Ly6C), PE-conjugated anti-c-Kit (CD117), and PE-Cy7-conjugated anti-Sca-1 (LY6A/E) antibodies (BD PharMingen, San Diego, CA) for 30 minutes at 37°C. Cells were washed and resuspended in PBS and analyzed by FACS.

The  $\text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+$  population of the long-term cultured (8 weeks) BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells and non-transformed *Abl1*<sup>-/-</sup> and *Abl1*<sup>+/+</sup> cells were additionally stained with APC-H7-conjugated anti-CD34 antibody (BD PharMingen, San Diego, CA) to distinguish long-term ( $\text{CD34}^- \text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+$ : LT) and short-term ( $\text{CD34}^+ \text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+$ : ST) stem cells for 30 minutes at 37°C. Cells were washed and resuspended in PBS and analyzed by FACS.

To isolate quiescent stem cells, BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells and non-transformed *Abl1*<sup>-/-</sup> and *Abl1*<sup>+/+</sup> cells were washed with 1X PBS two times, and cultured overnight in serum-free and growth factor-free medium supplemented with 0.1% BSA. After 16 hours, cells were washed and resuspended in PBS and stained with

2  $\mu$ M Cell Proliferation Dye (CPD, eFluor670; eBiosciences) for 15 minutes. Cells were washed and maintained in culture medium for 4 days and a portion of cells were incubated with demecolcine solution (Sigma, St. Louis, MO) to be used as positive control. All cells were stained with anti-lineage, anti-c-Kit (CD117), and anti-Sca-1 (LY6A/E) antibodies (as described previously) and for 30 minutes at 37°C. Cells were washed and resuspended in PBS and subsequently analyzed by FACS.

BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells were cultured in IMDM supplemented with 10% FBS in the presence of SCF and IL-3 or G-CSF (10ng/ml) for 7 days.  $5 \times 10^5$  cells from each sample were washed with PBS and resuspended in 1X PBS supplemented with 1% BSA. Cells were incubated with Fc Block for 10 mins at room temperature followed by incubation with PE-conjugated CD11b and APC-conjugated Ly6G/C antibodies (BD PharMingen, San Diego, CA) for 30 mins at 37°C. Cells were washed and resuspended in ice-cold 1X PBS for analysis by FACS.

**ROS Assays:** Cells were incubated with redox-sensitive fluorochromes such as dihydroethidium (DHE) that detects cellular  $\cdot\text{O}_2^-$  and Redox Sensor Red (CC1) that detects  $\cdot\text{O}_2^-/\text{H}_2\text{O}_2$  (Molecular Probes, Eugene, OR), to determine the levels of intracellular ROS. Briefly,  $2 \times 10^5$  cells/mL of culture medium were incubated with 5  $\mu$ M DHE and CC1 for 15 minutes in 37°C. Next, samples were washed, resuspended in phosphate-buffered saline (PBS), and analyzed using BD FACSCanto (BD Biosciences, San Jose, CA).

**Oxidative DNA damage:** Cytospins were prepared from cells growing in suspension as described previously. Cells were fixed with 4% formaldehyde for 20 minutes, washed

and permeabilized with 0.02% Triton X-100 and then blocked with 2% BSA for 45 minutes. Cells were stained with anti- $\gamma$  phosphorylated-H2AX (Millipore) and anti-8-oxoguanine (8-oxoG) (Chemicon, Temecula, CA) antibodies followed by goat-anti-mouse secondary antibody conjugated to Alexa Fluor 594 (Millipore). Negative controls were performed without staining with the primary antibody. DNA was counterstained with the DNA fluorochrome 4', 6'-diamidino-2-phenylindole (DAPI). Samples were mounted using an anti-fade reagent (Slow fade) and visualized using an inverted Olympus IX70 fluorescence microscope (Olympus America, Melville, NY) equipped with a 100  $\times$ /1.35 numeric aperture UPlan Apo objective and Cooke Sensicam QE camera (Cooke, Auburn Hills, MI). A series of 3-dimensional images of each individual picture (cell) was stored in SlideBook software version 3.0.1 (Intelligent Imaging Innovations, Denver, CO). Deconvolution was applied to increase the resolution and contrast of the images. A collection of 3-dimensional images describing individual cells was converted to one 2-dimensional picture. At least 30 individual cells were analyzed per experimental group. Pictures were prepared using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA).

**SKY Analysis:** Metaphase spreads from cells were prepared by standard procedures and analyzed by Dr. Christina Richardson, using the Applied Spectral Imaging software. Results indicate consistent chromosomal aberrations detected by SKY (number of metaphases displaying the chromosomal aberrations), and Statistical analyses were performed using the unpaired Student's t test.

**Genome-wide expression array:**  $10^6$  BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells were cultured in suspension in absence of SCF+IL-3 and G-CSF. RNA was prepared using an RNeasy kit (Qiagen). RNA processing and microarray analysis were carried out at the Penn Microarray Facility, University of Pennsylvania School of Medicine. RNA samples were analyzed on Affymetrix GeneChip Mouse Gene 1.0 ST arrays using standard Affymetrix protocols. The raw data CEL files were normalized using Partek Genomic Suite software (<http://www.partek.com/partekgs>) by Dr. John Tobias, Penn Molecular Profiling Facility, University of Pennsylvania.

One-way analysis of variance (ANOVA) was performed on the imported, normalized and summarized microarray data to find differently expressed genes between cell subpopulations. Lists of differentially expressed genes were created using a false discovery rate (FDR) limit of 0.05 and degree of fold change of 1.5 (upregulated or down-regulated). To determine which functional groups of genes were differentially expressed we performed Gene Ontology ANOVA (GO ANOVA). Partek GS utilizes the Gene Ontology (GO) database to map genes to standardized functional groupings. The ANOVA model was configured to contrast the two groups in order to determine the significance and average fold change of each GO category across all comparator groups. The average fold-change is plotted on the y-axis, and corresponding genes associated with each of the biological process on the x-axis with the respective FDR (as described in (210)).

**Statistical Analysis:** Results are represented as mean  $\pm$  standard deviation (SD).

Statistical analyses were performed using the unpaired Student's t test, *P* values less than or equal to 0.05 were considered statistically significant.

Dr. Mateusz Koptyra, Dr. Grazyna Hoser, Dr. Tomasz Stoklosa, Dr. Mariusz Wasik and Dr. Christine Richardson contributed to some of the aforementioned experiments.

### **Preliminary data**

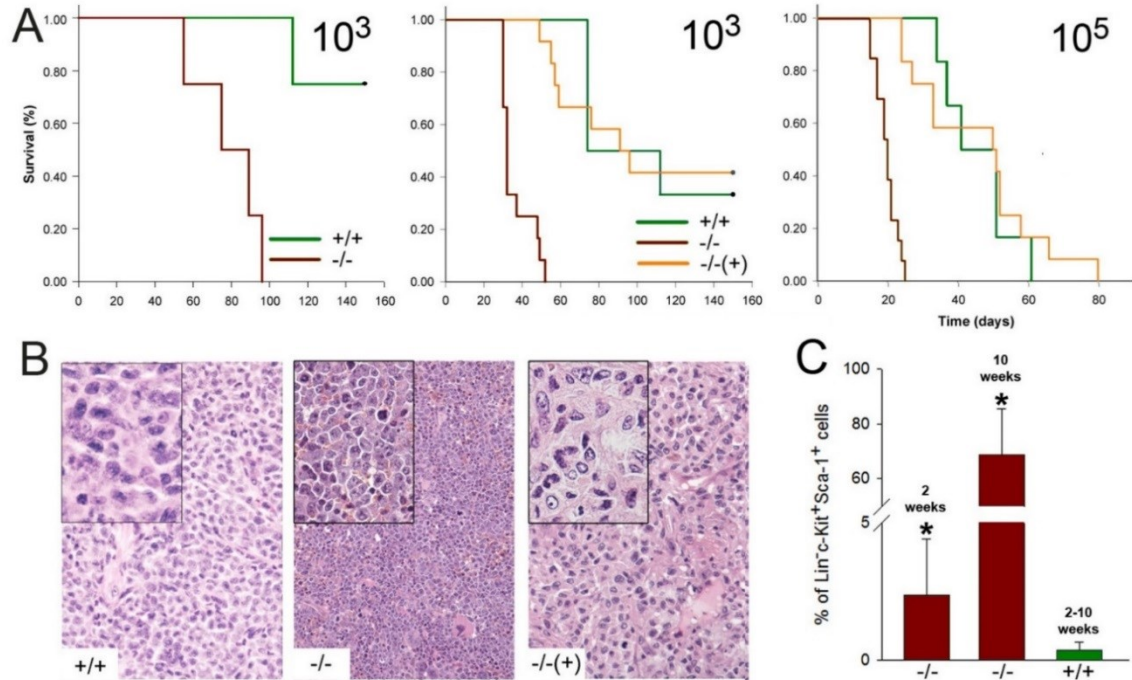
#### *Leukemogenesis in mice*

The role of functional loss of ABL1 in BCR-ABL1-mediated leukemogenesis was studied by leukemogenesis in mice. 100% (5/5) of mice injected with BCR-ABL1 *Abl1*<sup>-/-</sup> cells developed and died of leukemia with a Median Survival Time (MST) of 78 days whereas mice injected with BCR-ABL1 *Abl1*<sup>+/+</sup> cells had MST of 140 days and 20% lethality, displaying no signs of leukemia on necropsy (**left panel**). Mice injected with 10<sup>3</sup> growth factor-independent BCR-ABL1 *Abl1*<sup>-/-</sup> cells developed and died of leukemia with 100% (5/5) lethality and a MST of 32 days whereas expression of ABL1 in BCR-ABL1 cells impaired leukemia development in mice which had 35% lethality and a MST of 74 days. Reconstitution of wild-type ABL1 expression was found to delay leukemia development and prolongs survival in mice with 45% lethality and MST of 59 days (**middle panel**). When mice were injected with a higher dose of growth factor-independent BCR-ABL1 *Abl1*<sup>-/-</sup> cells (10<sup>5</sup>) there was a significant difference in survival time with a MST of 20 days and 100% lethality and 51 days when injected with BCR-ABL1 *Abl1*<sup>+/+</sup> and 50

days for BCR-ABL1 *Abl1*<sup>-/-</sup> cells with reconstituted ABL1 expression (**right panel**) (**Fig 2.1A**).

Next, tissue sections obtained from spleens of mice injected with BCR-ABL1 *Abl1*<sup>-/-</sup>, BCR-ABL1 *Abl1*<sup>+/+</sup> and BCR-ABL1 *Abl1*<sup>-/-</sup> cells reconstituted with YFP-ABL1 were analyzed by immunohistochemistry. Splenocytes showed moderate to strong differentiation in the presence of ABL1 (**Fig 2.1B, left panel**) with large, multi-lobed nuclei whereas BCR-ABL1 *Abl1*<sup>-/-</sup> cells (**Fig 2.1B, middle panel**) showed an arrest of differentiation with small and rounded nuclei. The defect in cell differentiation was however restored in splenocytes from mice injected with BCR-ABL1 *Abl1*<sup>-/-</sup> cells when ABL1 expression was reconstituted (**Fig 2.1B, right panel**).

ABL1-mediated regulation of leukemic stem cells (LSC) *in vivo* was examined. While BMC obtained from mice injected with BCR-ABL1 *Abl1*<sup>+/+</sup> cells 2-10 weeks post-retroviral transduction displayed  $(0.37 \pm 0.28)$  % GFP<sup>+</sup>Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup> LSC, mice injected with BCR-ABL1 *Abl1*<sup>-/-</sup> cells 2 weeks post-transduction had  $(2.38 \pm 2.02)$  % LSC and there was a marked expansion of the LSC population in growth factor-independent BCR-ABL1 *Abl1*<sup>-/-</sup> cells 10 weeks post-transduction  $(68.68 \pm 16.89)$  % (**Fig 2.1C**).



**Figure 2.1. BCR-ABL1 induces CML-BP-like murine leukemia in the absence of ABL1.** BCR-ABL1 *Abl1* <sup>-/-</sup> [<sup>-/-</sup>], BCR-ABL1 *Abl1* <sup>+/+</sup> [<sup>+/+</sup>] and BCR-ABL1 *Abl1* <sup>-/-</sup> leukemia cells reconstituted with YFP-ABL1 [<sup>-/-</sup>(+)] were analyzed. (A) Survival of NOD-SCID mice (5 mice per group) injected intravenously with  $10^3$  and  $10^5$  cells (described in IM-sensitivity Cancer Research);  $P < 0.001$ , +/+ versus -/- and -/-(+); Kaplan Meier analysis, (B) Representative hematoxylin-eosin stained-histograms of spleen sections from leukemia-sick NOD-SCID mice; magnification 20X, inset 40X, (C) Mean percentage  $\pm$  SD of stem (Lin<sup>-</sup>Kit<sup>+</sup>Sca-1<sup>+</sup>) cell sub-populations in bone marrow cells isolated from NOD-SCID mice transplanted with -/- and +/+ cells 2-10 weeks post-retroviral transduction; \* $P < 0.001$ .

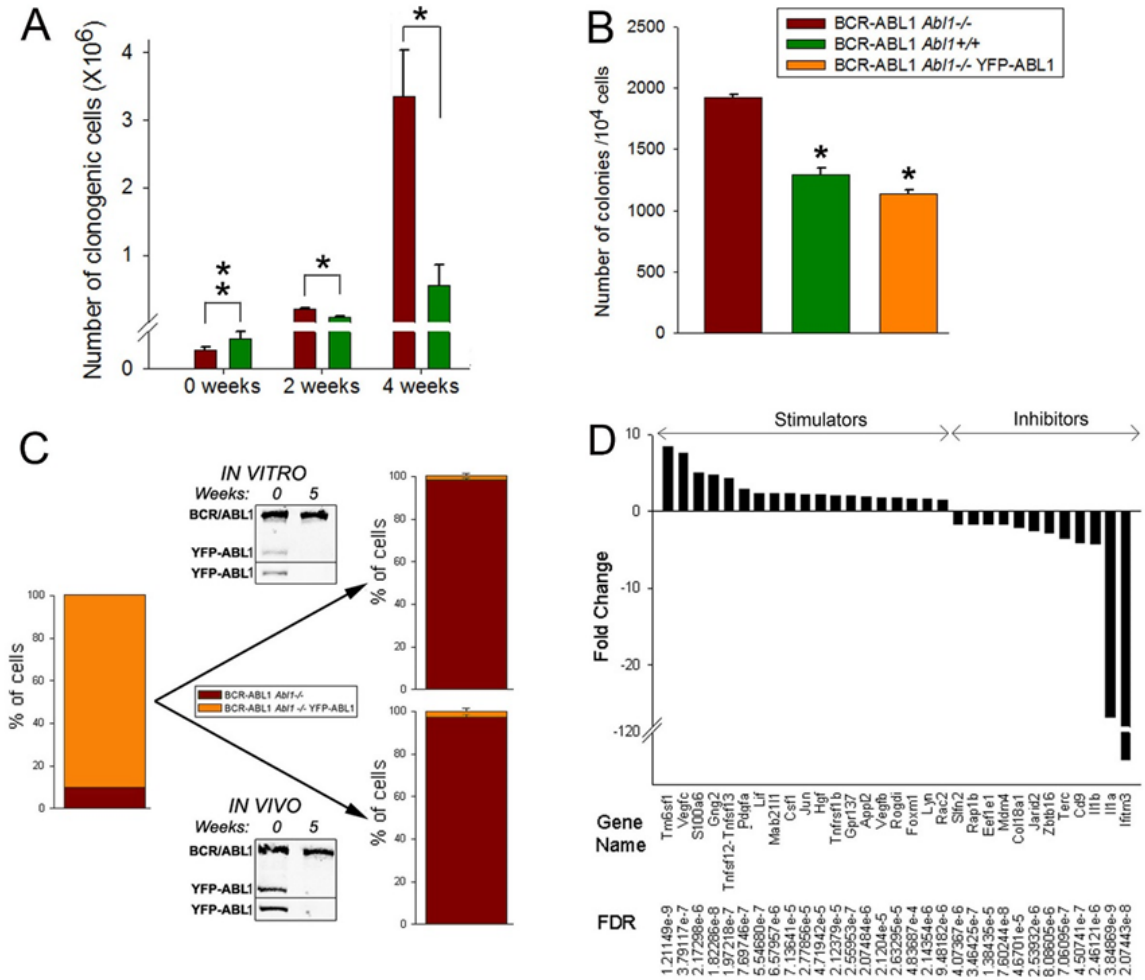
## Results

### Role of ABL1 in cell proliferation

Based on mice survival studies, ABL1 was found to have an important role in leukemia progression which prompted us to investigate the various biological processes that can be potentially regulated in BCR-ABL1-positive cells. Effect of ABL1 in proliferation of BCR-ABL1-expressing cells was examined. Initially, at 0 week after FACS, BCR-ABL1 *Abli*<sup>+/+</sup> group had 1.5-fold higher number of clonogenic cells ( $6600.00 \pm 1437.92$ ) based on total number of cells in suspension and number of colonies formed compared to the knock-out cells ( $4145.00 \pm 534.26$ ). In the course of 2 weeks, BCR-ABL1 *Abli*<sup>-/-</sup> group ( $198357.14 \pm 22814.44$ ) had more than 2-fold higher number of clonogenic cells than BCR-ABL1 *Abli*<sup>+/+</sup> ( $77091.51 \pm 17977.99$ ). However, after 4 weeks, total clonogenic cells were 6-fold higher in BCR-ABL1 *Abli*<sup>-/-</sup> BMC ( $3351894.00 \pm 681059.24$ ) compared to wild-type counterparts ( $552082.91 \pm 312817.64$ ) based on total cell count obtained by trypan blue exclusion and colony-formation assays (**Fig 2.2A**). Growth factor independent BCR-ABL1 *Abli*<sup>-/-</sup> cells formed on an average  $1922.00 \pm 25.46$  colonies which was 1.5-fold higher compared to the wild-type cells which formed an average of  $1296.00 \pm 50.9$  colonies. ABL1 reconstitution was found to inhibit colony formation by about 2-fold compared to knock-out cells ( $1139.88 \pm 29.88$ ) (**Fig 2.2B**). Competition assay was performed with a mixture of GFP-positive BCR-ABL1 *Abli*<sup>-/-</sup> and GFP/YFP-positive BCR-ABL1 *Abli*<sup>-/-</sup>-YFP-ABL1 cells in order to examine proliferation rate *in vitro* and *in vivo*.  $10^6$  cells containing 90% BCR-ABL1 *Abli*<sup>-/-</sup>-YFP-ABL1 and 10% BCR-ABL1 *Abli*<sup>-/-</sup> were passaged for 5 weeks in liquid culture and

simultaneously injected into NOD-SCID mice. After 5 weeks in culture the cell mixture was analyzed by flow cytometry to determine the ratio of GFP versus GFP/YFP-positive cells which was found to be 95% and 5% respectively. A loss of YFP-ABL1 expression was confirmed by Western analysis of these cells. A similar result was obtained when BMC harvested from transplanted mice were scored for GFP and GFP/YFP expression. The *in vivo* cell mixture contained 95% GFP-positive cells and 5% GFP/YFP-positive cells. Western analysis of the resultant cell mixture confirmed the expression of BCR-ABL1 and the loss of YFP-ABL1 expression *in vivo* (**Fig 2.2C**).

In order to validate our findings, a genome-wide expression array of BCR-ABL1-transformed *Abl1*<sup>-/-</sup> and *Abl1*<sup>+/+</sup> BMCs, was performed. The entire array of genes was screened for GO:0008283 to identify genes associated with ‘Cellular Proliferation’ as indicated in the Gene Ontology (GO) database for ‘Biological process’. Of the 133 genes regulating cell proliferation, 31 were differentially expressed on average by  $\geq 1.5$ -fold with a statistically significant false discovery rate (FDR) of  $< 0.001$  in BCR-ABL1 *Abl1*<sup>-/-</sup> cells when compared to BCR-ABL1 *Abl1*<sup>+/+</sup> cells. *Jun*, *Pdgfa*, *Pdgfb*, *Vegfc*, *Rac2* and *Tnfrsf1b* are some of the positive modulators and *Mdm4*, *Coll8a1*, *Cd9*, *Il1a*, *Il1b* and *Ifitm3* are some of the negative regulators of proliferation (**Fig 2.2D**).



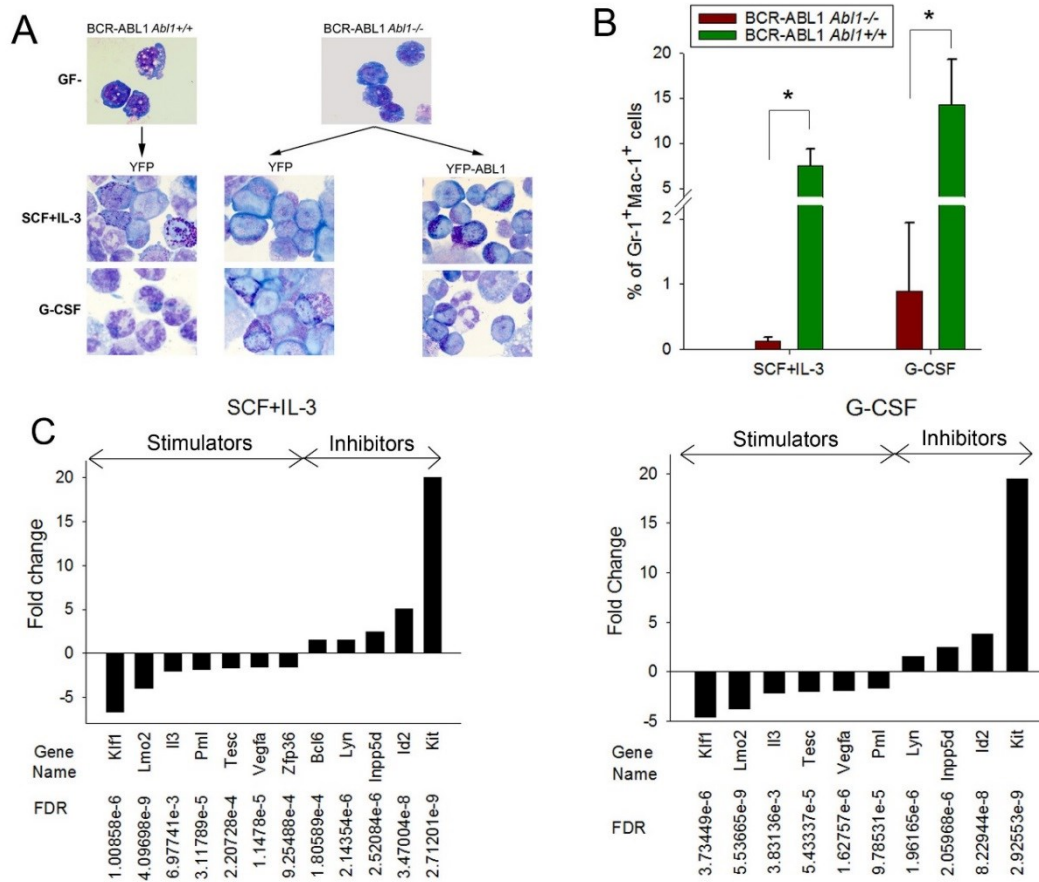
**Figure 2.2. ABL1 inhibits proliferation of BCR-ABL1-leukemia cells.** (A) Estimated total number of BCR-ABL1 *Abi1*<sup>-/-</sup> and BCR-ABL1 *Abi1*<sup>+/+</sup> cells with clonogenic potential from an initial 10<sup>6</sup> cells cultured *in vitro*. Results represent mean number of clonogenic cells  $\pm$  SD; \*\* $P$ <0.05, \* $P$ <0.001 in comparison to BCR-ABL1 *Abi1*<sup>-/-</sup> cells. (B) Mean number of colonies  $\pm$  SD from BCR-ABL1 *Abi1*<sup>-/-</sup>, BCR-ABL1 *Abi1*<sup>+/+</sup> and BCR-ABL1 *Abi1*<sup>-/-</sup> leukemia cells reconstituted with YFP-ABL1; \* $P$ <0.001 in comparison to BCR-ABL1 *Abi1*<sup>-/-</sup> cells, (C) Competition experiment illustrating the expansion of BCR-ABL1 *Abi1*<sup>-/-</sup> and BCR-ABL1 *Abi1*<sup>-/-</sup> leukemia cells reconstituted with YFP-ABL1 *in vitro* and *in vivo*. The initial cell mixture comprised of 90% BCR-ABL1 *Abi1*<sup>-/-</sup> leukemia cells reconstituted with YFP-ABL1 (GFP+YFP+) and 10% BCR-ABL1 *Abi1*<sup>-/-</sup> (GFP+) leukemia cells. Over a period of 5 weeks the resultant mixture contained more than 95% BCR-ABL1 *Abi1*<sup>-/-</sup> leukemia cells both *in vitro* and *in vivo*. Western blot analysis with the use of anti-ABL1 and anti-GFP antibodies (upper and lower boxes in each panel reveals expression of BCR-ABL1 as well as YFP-ABL1 protein in the initial cell mixture (week 0) and after 5 weeks (week 5) *in vitro* and *in vivo*, (D) Gene ontology expression analysis of genes regulating proliferation (GO CELL PROLIFERATION GO:0008283) in BCR-ABL1 *Abi1*<sup>-/-</sup> versus BCR-ABL1 *Abi1*<sup>+/+</sup>

leukemia cells. Results represent statistically significant (FDR<0.05) fold changes (>1.5) of expression of indicated genes.

### Role of ABL1 in myeloid cell differentiation

Wright-Giemsa stained images of *Abl1*<sup>-/-</sup> and *Abl1*<sup>+/+</sup> cells expressing BCR-ABL1 cultured in growth factor-free medium show immature myeloid precursors, which are small, with deep blue cytoplasm, and scant to no cytoplasmic granulation. In the presence of IL-3 and SCF both BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells showed myeloid precursors, having deep blue cytoplasm. But BCR-ABL1 *Abl1*<sup>+/+</sup> BMC showed more mature myeloid cells displaying abundance of cytoplasmic granules and many mature forms of neutrophils. Reconstitution of YFP-ABL1 in BCR-ABL1 *Abl1*<sup>-/-</sup> cells showed comparatively more mature myeloid cells. BCR-ABL1 *Abl1*<sup>+/+</sup> marrow in presence of G-CSF showed predominantly mature neutrophils with very minimal myeloid precursors while BCR-ABL1 *Abl1*<sup>-/-</sup> BMC display mostly myeloid precursors having pale blue cytoplasm, abundant cytoplasmic granules, with minimal mature neutrophils. Interestingly, when YFP-ABL1 expression was restored, BMC treated with GCSF showed more mature myeloid cells thus restoring the effect of ABL1 in inducing myeloid differentiation (**Fig 2.3A**).

To confirm the aforementioned finding, FACS analysis revealed an average of only  $0.1333 \pm 0.0577$  % Gr-1/Mac-1-positive cells in BCR-ABL1 *Abl1*<sup>-/-</sup> while the wild-type counterparts had significantly higher number of Gr-1/Mac-1-positive cells with an average of  $7.4667 \pm 1.8502$  % double-positive cells in presence of IL-3 and SCF.



**Figure 2.3. ABL1 is required to induce myeloid differentiation of BCR-ABL1-leukemia cells.** (A) Wright-Giemsa staining of BCR-ABL1 *Abi1*<sup>+/+</sup> and BCR-ABL1 *Abi1*<sup>-/-</sup> leukemia cells expressing YFP or YFP-ABL1 as indicated. Cells were maintained in growth factor-free (GF-) medium (upper panels) and in the presence of SCF+IL-3 (middle panels) or G-CSF (lower panels). Representative images are shown; magnification 100X, (B) Mean percentages of Gr-1/Mac-1-double positive cells + SD in BCR-ABL1 *Abi1*<sup>-/-</sup> and BCR-ABL1 *Abi1*<sup>+/+</sup> leukemia cells maintained in the presence of SCF+IL-3 or G-CSF; \**P*<0.002, (C) Gene Ontology (GO) expression analysis of genes regulating myeloid differentiation (GO myeloid cell differentiation GO:0030099) in BCR-ABL1 *Abi1*<sup>-/-</sup> versus BCR-ABL1 *Abi1*<sup>+/+</sup> leukemia cells maintained with SCF+IL-3 (left panel) or G-CSF (right panel). Results represent statistically significant (FDR<0.05) fold changes (>1.5) of expression of indicated genes.

In presence of G-CSF, BCR-ABL1 *Abi1*<sup>-/-</sup> cells had only  $0.9000 \pm 1.0392$  % Gr-1/Mac-1-positive cells whereas BCR-ABL1 *Abi1*<sup>+/+</sup> group had  $14.2667 \pm 4.9903$  % Gr-1/Mac-1-positive cells (**Fig 2.3B**).

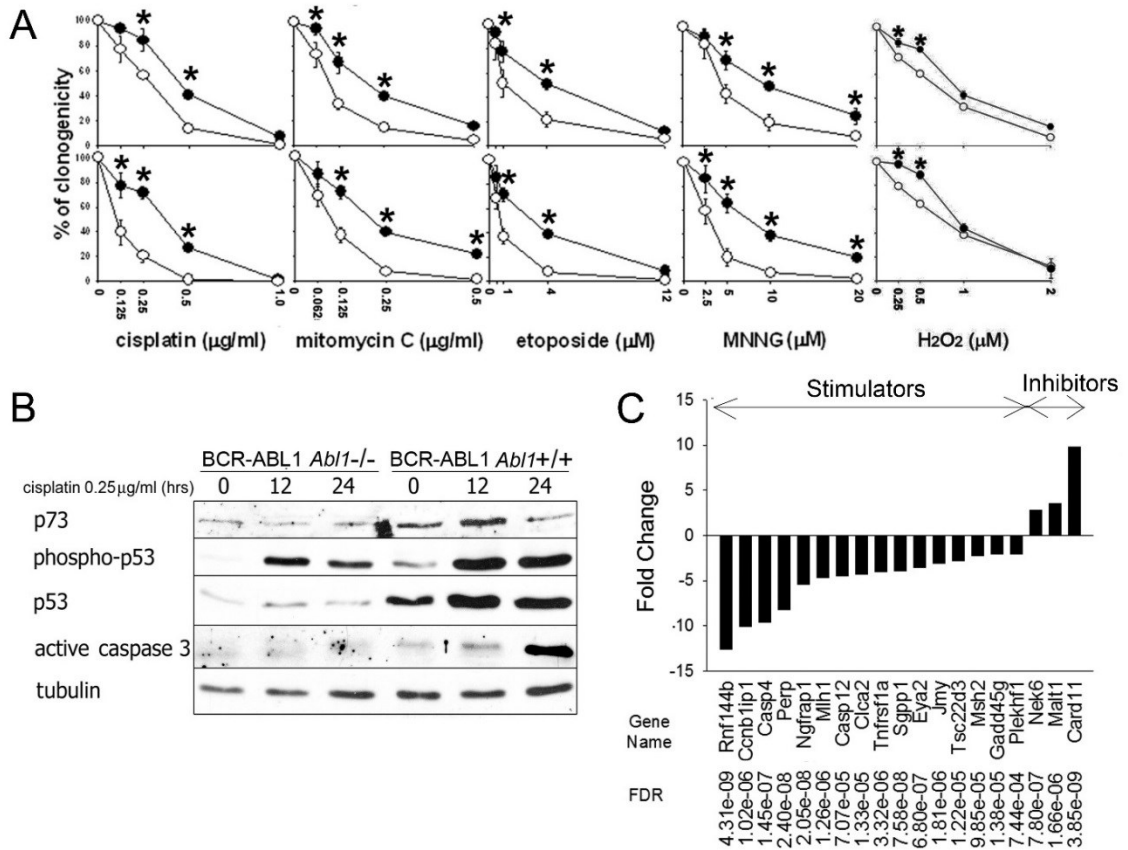
Genome-wide expression array was analyzed to confirm our findings. The array of genes represented was screened for GO:0030099 to identify genes associated with 'Myeloid Cell Differentiation' as indicated in the GO database. 35 genes regulating myeloid cell differentiation were represented in the array of which 11 were differentially expressed on average by  $\geq 1.5$ -fold with a statistically significant FDR of  $< 0.001$  in BCR-ABL1 *Abl1*<sup>-/-</sup> cells when compared to BCR-ABL1 *Abl1*<sup>+/+</sup> cells. *Klf1*, *Lmo2*, *Il3*, *Pml* and *Zfp36* are positive regulators of myeloid cell differentiation and are downregulated in BCR-ABL1 *Abl1*<sup>-/-</sup> cells whereas *Bcl6*, *Lyn*, *Id2* and *Kit* inhibit myeloid differentiation and are upregulated in absence of ABL1 compared to BCR-ABL1 *Abl1*<sup>+/+</sup> cells (**Fig 2.3C**).

*Role of ABL1 in genotoxic stress-induced apoptosis*

ABL1 has been reported to induce apoptosis in response to DNA damage (141) which prompted us to determine its role in regulating apoptosis in cells expressing BCR-ABL1. BCR-ABL1 *Abl1*<sup>+/+</sup> cells were found to be more sensitive under genotoxic stress compared to the knock-out cells, indicating a role of ABL1 in the induction of apoptosis in BCR-ABL1 cells (**Fig 2.4A**).

Further, to identify the key proteins modulated by ABL1 in order to induce apoptosis, Western Blot analysis showed that BCR-ABL1 *Abl1*<sup>-/-</sup> leukemia cells failed to induce expression of p73, p53 and activated caspase 3, whereas BCR-ABL1 *Abl1*<sup>+/+</sup> cells showed induction of p73 expression which peaked at 12 hours post-cisplatin treatment. The wild-type cells had elevated levels of p53 and phospho-Serine15-p53 along with

activation of caspase 3, 24 hours post-cisplatin treatment in comparison to BCR-ABL1 *Abl1*<sup>-/-</sup> leukemia cells which did not express these modulators of apoptosis (**Fig 2.4B**).



**Figure 2.4. ABL1 plays an essential role in the induction of apoptosis in BCR-ABL1-leukemia cells in response to genotoxic agents.** (A) Percentage clonogenicity  $\pm$  SD of BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells after treatment with indicated DNA damage-inducing agents in comparison to untreated cells; \* $P < 0.05$ , (B) Western blot analysis of p73, p53, phospho-Serine15 of p53 (pS15-p53) and activated caspase 3 in BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells treated with cisplatin (0.25 µg/ml); tubulin served as loading control, (C) Gene ontology expression analysis of genes regulating apoptosis (GO APOPTOSIS GO:0006915) in BCR-ABL1 *Abl1*<sup>-/-</sup> versus BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells. Results represent statistically significant (FDR < 0.05) fold changes (>2.0) of expression of indicated genes.

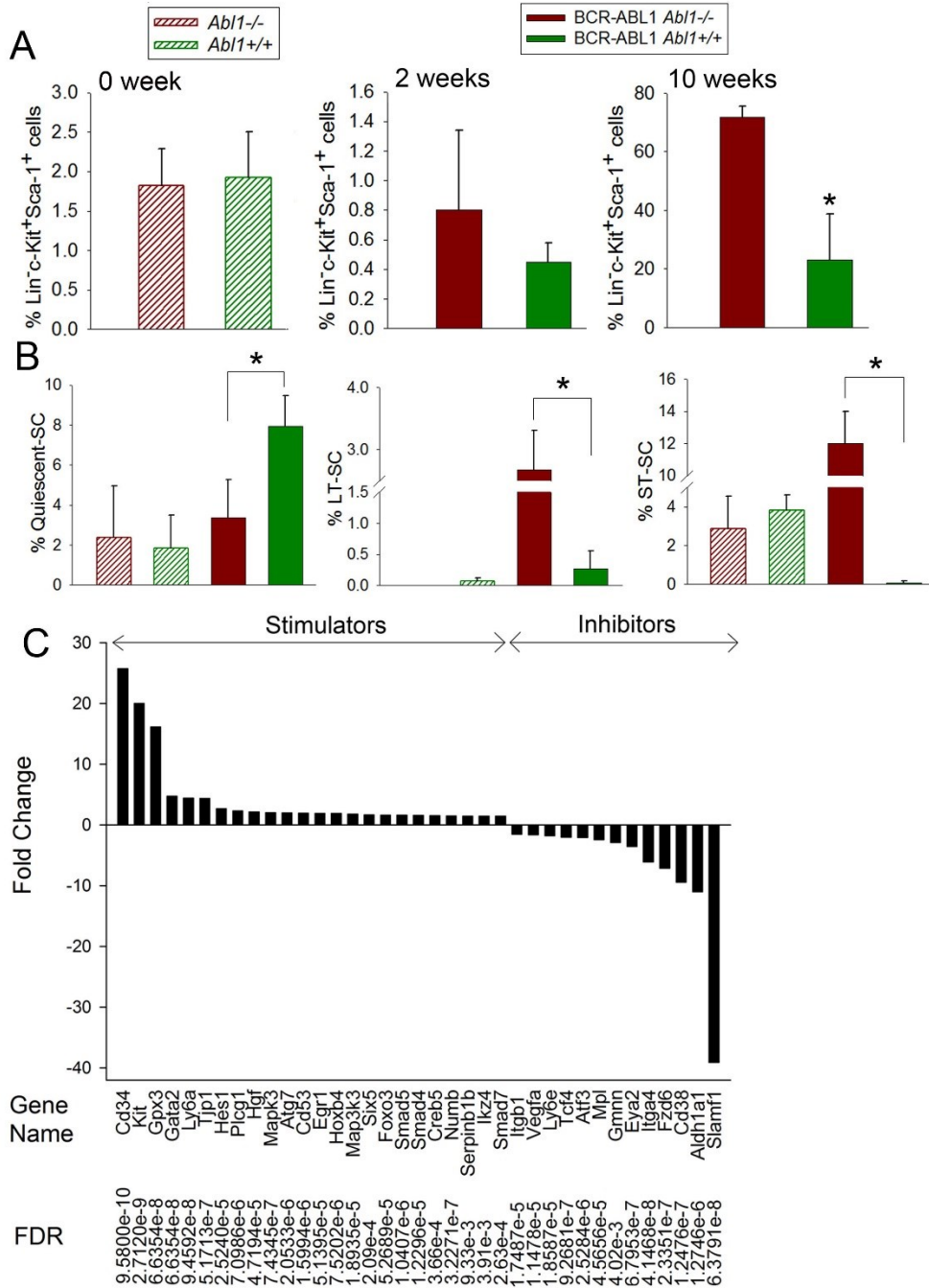
Apoptosis in BCR-ABL1 expressing cells could be affected by 455 genes represented in the array as indicated by GO Apoptosis GO:0006915; expression levels of 19 of these

genes are changed on average by  $\geq 2$ -fold with a statistically significant FDR of  $\leq 0.001$ . Several pro-apoptotic genes including *Rnfl44b*, *Ccnblip1*, *Casp4*, *Casp12*, *Eya2* and *Gadd45g* were down-regulated and three anti-apoptotic genes *Nek6*, *Malt1* and *Card11* were upregulated in BCR-ABL1 *Abl1*<sup>-/-</sup> cells compared to their wildtype counterparts (**Fig 2.4C**).

#### Role of ABL1 in stem cell regulation

A growing number of studies point to the fact that a primitive population of leukemic stem cells (LSC) is responsible for CML disease progression and therapeutic resistance (211). We sought to determine how ABL1 loss-of-function affects the accumulation of stem cells in non-transformed and BCR-ABL1-transformed cells. There was no significant difference in the percentage of LSC in *Abl1*<sup>-/-</sup> ( $1.82 \pm 0.47$ ) and *Abl1*<sup>+/+</sup> ( $1.92 \pm 0.58$ ) bone marrow cells. Although the percentage of LSC seem to be higher in BCR-ABL1 *Abl1*<sup>-/-</sup> ( $0.80 \pm 0.54$ )% than in wild-type ( $0.45 \pm 0.13$ )% bone marrow cells, the difference is not statistically significant. However, when cultured long-term for 10 weeks, the percentage of LSC in BCR-ABL1 *Abl1*<sup>-/-</sup> cells ( $71.67 \pm 3.73$ )% is more than 3-fold higher than that of the wild-type counterparts ( $23.05 \pm 15.70$ )% showing that lack of ABL1 results in an expansion of LSC in BCR-ABL1-positive cells (**Fig 2.5A**).

The SC of long term cultured (10 weeks) non-transformed and BCR-ABL1-transformed cells were further sorted to quantify quiescent (eFluor670<sup>hi</sup>), LT (CD34<sup>+</sup>lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup>) and ST (CD34<sup>+</sup>lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup>) sub-populations. The cells were then stained



**Figure 2.5. The lack of ABL1 causes an expansion of the LSCs but not HSCs.**

(A) Mean percentage  $\pm$  SD of hematopoietic stem (Lin<sup>-</sup>Kit<sup>+</sup>Sca-1<sup>+</sup>) cell sub-populations in freshly isolated *Abi1*<sup>-/-</sup> and *Abi1*<sup>+/+</sup> bone marrow cells (left panel) and 2- (middle panel) and 10- (right panel) weeks post-retroviral transduction (BCR-ABL1 *Abi1*<sup>-/-</sup> and BCR-ABL1 *Abi1*<sup>+/+</sup> leukemia cells); \**P*<0.001, (B) Mean percentage  $\pm$  SD of quiescent- (CPD<sup>max</sup> Lin<sup>-</sup>Kit<sup>+</sup>Sca-1<sup>+</sup>), long-term (LT, Lin<sup>-</sup>Kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>+</sup>) and short-term (ST, Lin<sup>-</sup>Kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>+</sup>) HSCs/LSCs in *Abi1*<sup>-/-</sup> and *Abi1*<sup>+/+</sup> bone marrow cells,

and in BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells; \**P*<0.002, (C) Statistically significant (FDR<0.05) fold changes (>1.45) of expression of indicated genes regulating stem cell characteristics.

with the Cell Proliferation Dye, eFluor670 in order to isolate quiescent stem cells. This fluorescent dye binds to any cellular protein containing primary amines, and as cells divide, the dye is distributed equally between daughter cells that can be measured as successive halving of the fluorescence intensity of the dye. The quiescent cells have maximum fluorescence intensity since those cells do not divide. We detected no significant difference in the percentage of quiescent cells in long term cultured non-transformed *Abl1*<sup>-/-</sup> ( $2.36 \pm 2.59$ )% and *Abl1*<sup>+/+</sup> ( $1.83 \pm 1.65$ )% cells. Interestingly, we detected a 2-fold increase in the percentage of quiescent cells in BCR-ABL1 *Abl1*<sup>+/+</sup> ( $7.95 \pm 1.50$ )% group compared to knock-out leukemia cells ( $3.35 \pm 1.91$ )% (**Fig 2.5B**).

Further we quantified LT- and ST-stem cells, but did not detect any significant difference in the numbers of LT- and ST-SC in GFP-positive non-transformed *Abl1*<sup>-/-</sup> [LT-SC: ( $0.00 \pm 0.00$ )%; ST-SC: ( $2.87 \pm 1.69$ )%] and *Abl1*<sup>+/+</sup> (LT-SC:  $0.067 \pm 0.06$ ; ST-SC:  $3.83 \pm 0.78$ ) cells. However, there was an almost 10-fold increase in LT-SC in BCR-ABL1 *Abl1*<sup>-/-</sup> ( $2.68 \pm 0.63$ )% cells compared to wild-type ( $0.27 \pm 0.29$ )% counterparts. A dramatic increase of more than 50-fold was detected in ST-SC in BCR-ABL1 *Abl1*<sup>-/-</sup> ( $12.00 \pm 1.99$ )% cells versus BCR-ABL1 *Abl1*<sup>+/+</sup> ( $0.07 \pm 0.12$ )% cells (**Fig 2.5B**).

Genome-wide array reveals differential expression of several genes potentially involved in regulating stem cell-like characteristics. Expression levels of 35 genes represented in the array were altered on average by  $\geq 1.45$ -fold with a statistically significant FDR of  $\leq$

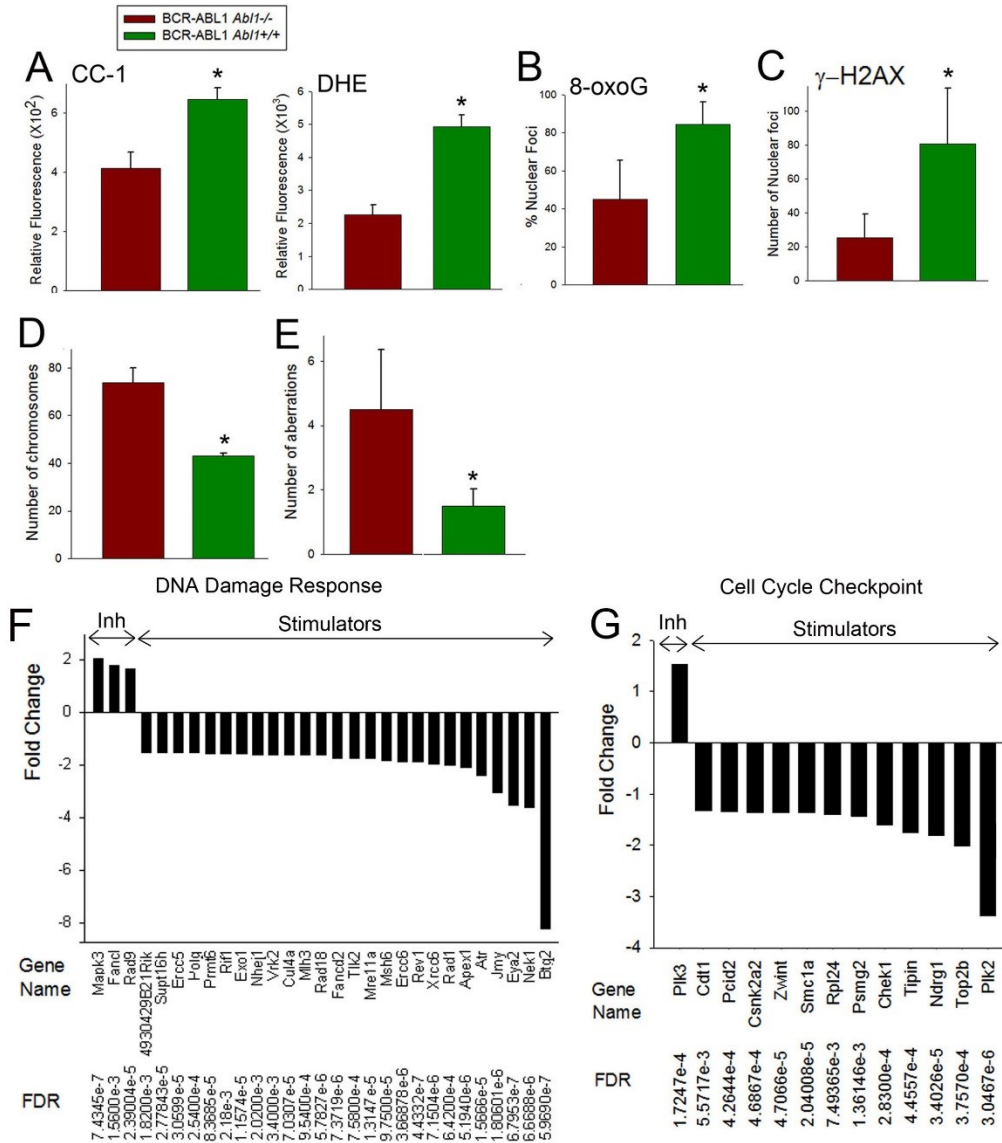
0.9%. Several genes positively regulating 'stemness' including *Cd34*, *Kit*, *Ly6a*, *Hoxb*, *FoxO3*, *Ikz4*, *Smad5* and *Smad4* were upregulated and genes inhibiting 'stemness' like *Ly6e*, *Fzld6*, *Eya2* and *Slamf1* were upregulated in BCR-ABL1 *Abl1*<sup>-/-</sup> cells compared to their wildtype leukemia cells (**Fig 2.5C**).

*Role of ABL1 in regulation of DNA damage response*

ABL1 is known to play a key role in DNA repair (117), hence we investigated the role of ABL1 in regulation of ROS accumulation and consequent ROS-mediated oxidative DNA damage and repair and their effect on genomic instability in BCR-ABL1-positive cells. In order to detect ROS levels, BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells were incubated with RedoxSensor Red CC-1 dye which detects superoxide anion ( $\cdot\text{O}_2^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and DHE which detects superoxide anion ( $\cdot\text{O}_2^-$ ) and subsequently analyzed by flow cytometry. We detected a 1.5 fold and 2.5-fold increase in ROS levels when incubated with RedoxSensor Red CC-1 and DHE respectively in BCR-ABL1 *Abl1*<sup>+/+</sup> cells (CC1:  $644.33 \pm 41.10$ , DHE:  $4936.67 \pm 354.81$ ) compared to BCR-ABL1 transformed *Abl1*<sup>-/-</sup> cells (CC1:  $412.67 \pm 54.65$ , DHE:  $2254.00 \pm 303.54$ ) (**Fig.2.6A**).

Next we studied the effect of elevated intracellular ROS levels on ROS-mediated DNA lesions such as 8-oxoG and DNA double strand breaks (DSB) (determined by  $\gamma$ -H2AX). BCR-ABL1 *Abl1*<sup>+/+</sup> cells ( $84.55 \pm 11.77$ ) displayed 2-fold more DNA lesions in the form of 8-oxoG in accordance with high levels of accumulated ROS in these cells in comparison to BCR-ABL1 *Abl1*<sup>-/-</sup> leukemia cells ( $45.14 \pm 20.27$ ) (**Fig.2.6B**). BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells were analyzed and 3 –fold higher number

of DSBs were detected in BCR-ABL1 *Abl1*<sup>+/+</sup> cells ( $80.64 \pm 33.08$ ) as indicated by a higher number of  $\gamma$ -H2AX foci in these cells compared to knock-out cells ( $25.50 \pm 13.79$ ) (**Fig.2.6C**). Higher levels of ROS and ROS-mediated oxidative DNA damage encouraged us to look at chromosomal abnormalities in BCR-ABL1-transformed *Abl1*<sup>-/-</sup> and *Abl1*<sup>+/+</sup> cells. SKY analysis of chromosomal aberrations was performed in which all the chromosomes can be simultaneously visualized in different fluorescent colours in order to detect translocations, deletions and other structural aberrations. We detected an almost 2-fold higher chromosome numbers as indicated by duplication of multiple chromosomes in BCR-ABL1 *Abl1*<sup>-/-</sup> cells ( $73.75 \pm 6.21$ ) in comparison to BCR-ABL1 *Abl1*<sup>+/+</sup> cells ( $43.00 \pm 1.05$ ) (**Fig.2.6D**). Chromosomal aberrations were found to be 2.5-fold higher in BCR-ABL1 *Abl1*<sup>-/-</sup> cells ( $4.50 \pm 1.85$ ) in comparison to BCR-ABL1 *Abl1*<sup>+/+</sup> cells ( $1.50 \pm 0.53$ ) (**Fig.2.6E**).



**Figure 2.6. Loss of ABL1 causes accumulation of chromosomal aberrations in BCR-ABL1-leukemia cells** (A) ROS were measured with CC-1 (left panel) and DHE (right panel) in BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells; \**P*<0.001 in comparison to BCR-ABL1 *Abl1*<sup>-/-</sup> cells (B) Mean percentage ± SD of 8-oxoG foci in BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells assessed by immunofluorescence in DAPI-counterstained nuclei; \**P*<0.001 in comparison to BCR-ABL1 *Abl1*<sup>-/-</sup> cells (C) Mean number ± SD of γ-H2AX foci in BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells assessed by immunofluorescence in DAPI-counterstained nuclei; \**P*<0.001 in comparison to BCR-ABL1 *Abl1*<sup>-/-</sup> cells, Quantification of SKY analysis of BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells based on the number of aberrations (D) and number of chromosomes (E), Results represent mean ± SD; \**P*<0.001 in comparison to BCR-ABL1 *Abl1*<sup>-/-</sup> cells,

Gene Ontology (GO) expression analysis of genes regulating DNA damage response (GO DNA damage response GO:0006974) (F) and cell cycle checkpoint response (G) in BCR-ABL1 *Abl1*<sup>-/-</sup> versus BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells. Results represent statistically significant (FDR<0.05) fold changes (>1.5) of expression of indicated genes.

We found 31 genes potentially involved in resistance to ‘DNA damage response’ identified by GO:0006974 to be aberrantly regulated on average by  $\geq 1.5$ -fold with a statistically significant FDR <0.2%. The majority of downregulated gene products are involved in DNA repair, for example *Ercc5*, *Polg*, *Exo1*, *Nhej1*, *Rad18*, *Mre11*, *Msh6*, *Xrcc6* and *Atr*. The expression of the three genes *MapK3*, *Fancl* and *Rad9* have been upregulated in BCR-ABL1 *Abl1*<sup>-/-</sup> compared to BCR-ABL1 *Abl1*<sup>+/+</sup> cells (**Fig.2.6F**). Analysis of the genome-wide expression array demonstrated altered expression of genes regulating ‘Cell cycle checkpoint’, ‘meiotic replication checkpoint’, ‘mitotic replication checkpoint’ and several other associated processes. We found a total of 65 genes regulating cell cycle checkpoint and associated processes, out of which 15 genes were aberrantly expressed on an average by  $\geq 1.3$  fold with a FDR < 2%. Most of these genes such as *Rad17*, *Pcid*, *Csnk2a2*, *Rpl22*, *Top2b* and *Plk2* implicated in cell cycle checkpoint-associated processes were downregulated in BCR-ABL1 *Abl1*<sup>-/-</sup> cells compared to wildtype leukemia cells (**Fig.2.6G**).

## Discussion

Preliminary mice survival studies indicate a role of ABL1 in BCR-ABL1-mediated leukemogenesis. Loss of ABL1 has been found to be associated with accelerated leukemia development and reduced survival of mice which suggests ABL1 to exert an opposing effect on BCR-ABL1-mediated malignant transformation. Reconstitution of

ABL1 kinase was found to prolong survival in mice, further confirming the onco-suppressive activity of ABL1. Although the effect of kinase-inactive mutant reconstitution has not been studied with respect to leukemia development in mice, we speculate that ABL1 activity in opposing malignant transformation *in vivo* may be kinase-dependent. This is due to the fact that some of the key biological functions of ABL1 that may be responsible such as induction of apoptosis and DNA damage repair are kinase-dependent (117). Although ABL1 shares sequence similarity with Src family of kinases yet it has a long and unique C-terminal domain which accounts for its DNA- and actin-binding activities and nuclear-cytoplasmic shuttling (92). Additionally, mice expressing a mutant C-terminal domain have similar phenotypic defects as *Abl1*-deficient mice (117). Thus, ABL1 has both kinase-dependent as well as kinase-independent functions.

To elucidate the role of ABL1 in inhibiting BCR-ABL1-mediated malignant transformation the effect of loss of ABL1 on proliferation of BCR-ABL1-expressing cells was studied. The fact that ABL1 expression is required for proliferation of freshly transduced BCR-ABL1-expressing BMC is supported by the findings of Rosti et al. They showed that inhibition of ABL1 at the mRNA level inhibited cell cycle progression to S-phase which inhibited *in vitro* myeloid colony growth of CD34+ cells CML-CP (212). We detected continuous BCR-ABL1 expression to reduce this dependency on ABL1 for proliferation as the cells become “oncogene addicted”, an observation which also finds support from the fact that inhibition of *ABL1* mRNA had no effect on the clonogenic growth of cells from advanced phase CML (212). On achieving growth factor

independence, the functional loss of ABL1 resulted in aberrant proliferation of BCR-ABL1 leukemia cells and expression or reconstitution of ABL1 stalled clonogenic growth. Thus, ABL1 negatively regulates proliferation of BCR-ABL1-transformed cells. Subcellular localization of ABL1 modulates its regulation of cell proliferation; while cytoplasmic ABL1 has been associated with induction of proliferation, nuclear ABL1 predominantly induces growth arrest and apoptosis (212). Hence, subcellular localization of ABL1 may account for ABL1-mediated regulation of BCR-ABL1 leukemia cell proliferation, although previous reports suggest cytoplasmic localization of ABL1 in primary hematopoietic cells (92). Genome-wide array confirmed this phenotype as detected by upregulation of genes which positively modulate proliferation.

Myeloid differentiation was inhibited due to loss of ABL1 in BCR-ABL1-transformed cells. Regulation of myeloid differentiation by ABL1 is kinase dependent since reconstitution of wild-type but not kinase-inactive ABL1 (previous findings from our laboratory) restored the defect in myeloid differentiation of BCR-ABL1 *Abl1*<sup>-/-</sup> cells. While BCR-ABL1 causes differentiation arrest (64), driving CML progression to blast phase which is marked by accumulation of immature blasts (213), other studies have associated ABL1 with B-cell development due to the phenotypic defects in ABL1-deficient mice and also by effect of pharmacologic inhibition of ABL1 (214). ABL1 has been reported to have a role in normal myelopoiesis (215) as well as in myeloid colony formation of CML-CP cells (212). These studies support our finding that ABL1 is necessary for myeloid differentiation of BCR-ABL1-positive cells.

We detected ABL1-induced apoptosis in BCR-ABL1-expressing cells in response to genotoxic stress in presence of ABL1 while the *Abl1*-deficient cells display a more resistant phenotype. Downstream targets of ABL1-induced apoptosis were identified by Western blot analysis showing induction of p73, phospho-serine315-p53, p53 and caspase 3 expression upon cisplatin treatment. While ABL1 can induce apoptosis by p53 induction, several studies have reported a p53-independent pathway for induction of apoptosis which involves p73 as the downstream effector (101). This study corroborates our finding that ABL1 kinase promotes genotoxic stress-induced apoptosis via a p73-dependent pathway in cells expressing BCR-ABL1. Microarray analysis confirmed the apoptosis-resistant phenotype of the BCR-ABL1 *Abl1*<sup>-/-</sup> cells by upregulation of several anti-apoptotic genes.

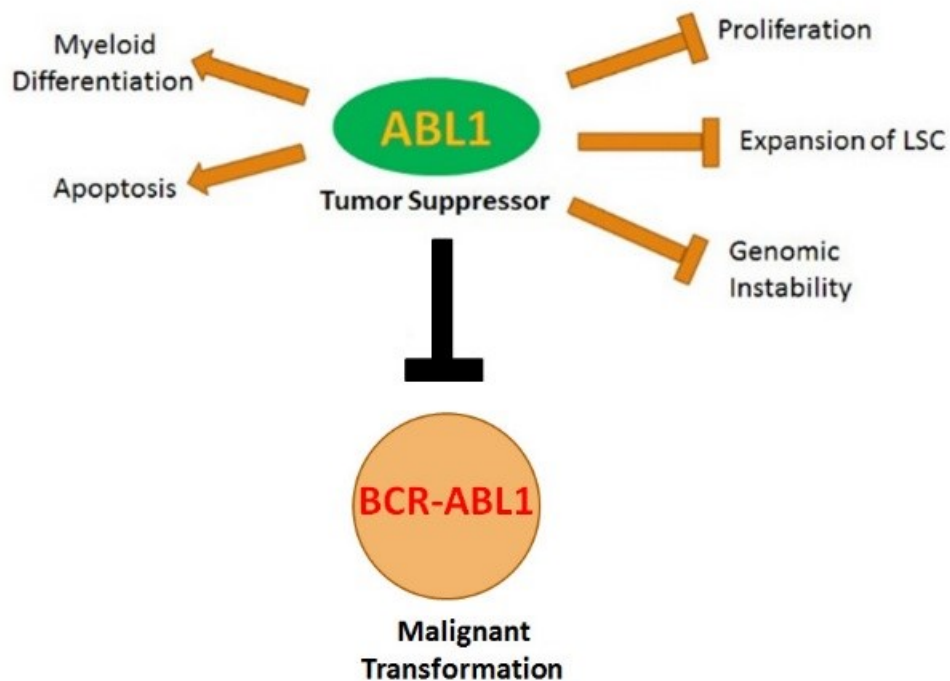
Studies with CML mouse models showed BCR-ABL1-expressing Lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> cells to function as leukemic stem cells (216). Although ABL1 does not seem to have a role in stem cell expansion in non-transformed or freshly-transduced BCR-ABL1-expressing *Abl1*<sup>-/-</sup> or *Abl1*<sup>+/+</sup> cells, the loss of ABL1 promoted LSC expansion in growth factor-independent leukemia cells. ABL1 did not seem to affect the quiescent stem cell subpopulation in non-transformed cells although presence of ABL1 caused an upregulation of quiescent leukemic stem cells. Continuous expression of BCR-ABL1 may be responsible for the accumulation of immature cells in the absence of ABL1, since BCR-ABL1 affects myeloid differentiation in the advanced phases (3). The fact that we found inhibition of proliferation in BCR-ABL1 *Abl1*<sup>+/+</sup> cells could be partly responsible for the accumulation of quiescent cells as opposed to the rapidly dividing BCR-ABL1

*Abl1*<sup>-/-</sup> cells. We found that lack of ABL1 not only promotes expansion of long-term LSC in BCR-ABL1-transformed bone marrow but it also causes a dramatic increase in the short-term LSC sub-population in growth factor-independent BCR-ABL1-expressing cells. This finding partly explains our previous observations of abnormal cell proliferation and arrested myeloid differentiation in absence of ABL1, which can be attributed to accumulation of cycling, short-term leukemic stem cells which may be capable of driving disease progression to an acute leukemia-like phase as evident from leukemogenesis in mice. These findings were also confirmed by microarray data showing upregulation of genes such as *c-kit*, *Ly6a*, *Cd34* and *Smad* family, all of which are involved in promoting stem cell-like characteristics in cells that lack ABL1 expression.

Endogenous DNA damage arises from Reactive Oxygen Species (ROS) such as superoxide anion ( $\bullet\text{O}_2^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (61). We observed accumulation of higher levels of intracellular ROS in the form of superoxide anion ( $\bullet\text{O}_2^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in presence of ABL1 in comparison to *Abl1*-deficient cells.  $\text{CD34}^+$  CML-CP and BCR-ABL1-transformed cell lines accumulate 2-6 times more ROS than normal cells (67,68,70). Detection of higher levels of ROS in BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells are also consistent with the findings showing that ABL kinases target catalase on the two tyrosine residues that results in its ubiquitination and to a consequent proteasomal degradation (217). *Abl1*-deficient cells also display a higher level of expression of the antioxidant protein peroxiredoxin I (Prx1) (218). Depending on the oxidative level in the cell, ABL1/ARG also phosphorylates and activates glutathione peroxidase1 on tyrosine-96 (219). In summary, ABL1 activation has a negative effect on

enzymes involved in the antioxidant defence. Genomic instability results from impaired response to increased DNA damage which in CML cells can be regulated by BCR-ABL1-kinase or may be kinase independent (61). On the other hand, ABL1 plays a key role in DNA repair and has been speculated to be the decision maker in response to DNA damage by inducing growth arrest in case of moderate damage and activating programmed cell death in response to severe DNA damage (117). In accordance with high levels of ROS, we detected enhanced ROS-induced DNA lesions such as 8-oxoG and DNA double strand breaks(DSBs; determined by  $\gamma$ -H2AX foci) in BCR-ABL1 *Abl1*<sup>+/+</sup> cells. CML cells are known to generate 3-8 time more oxidized nucleobases and 4-8-fold higher number of DSBs (67,68,70). Although *Abl1*-deficient cells had lower ROS levels and displayed less DNA lesions compared to the *Abl1*-expressing cells, yet they presented widespread chromosomal aberrations and a 2-fold higher number of chromosomes. This suggests an additional aberrant cellular response resulting in genomic instability, apart from the presence of BCR-ABL1. Microarray data reveal down-regulation of many genes involved in various DNA damage repair pathways such as *Ercc5*, *Ercc6*, *Mlh3*, *Msh6*, *Mre11*, *Rad18*, *Atr* etc. ABL1 is known to interact and/or phosphorylate several key proteins involved in various DNA damage repair pathways (117). Hence, loss of functional ABL1 results in an impaired cellular DNA damage response coupled with BCR-ABL1 expression which is reported to facilitate unfaithful DNA repair (61). Moreover, deregulated expression of genes involved in cell-cycle checkpoint, meiotic and mitotic recombination checkpoint suggests a plausible explanation for the higher number of chromosomes in absence of ABL1. Genomic

instability in absence of ABL1 in BCR-ABL1-expressing cells may contribute to disease progression as well as therapeutic resistance. It can be postulated that accumulation of chromosomal aberrations can be one of the mechanisms driving accelerated leukemia development in mice, that is phenotypically similar to CML-BP when NOD/SCID mice are injected with BCR-ABL1 *Abl1*<sup>-/-</sup> cells in comparison to BCR-ABL1 *Abl1*<sup>+/+</sup> cells. The phenotype of BCR-ABL1-positive *Abl1*-deficient cells make them ideal candidates to induce a CML-BP-like disease as indicated by leukemogenesis in mice.



**Figure 2.7. Normal ABL1 is a tumor suppressor in BCR-ABL1-mediated leukemia.**

In conclusion, ABL1 regulates several cellular processes including but not limited to proliferation, myeloid cell differentiation, apoptosis, stem cell expansion, intracellular ROS production as well as DNA damage response and repair in BCR-ABL1-transformed

cells. We have also established how BCR-ABL1-positive *Abl1*-deficient cells display aberrant proliferation, differentiation arrest, resistance to apoptosis, expansion of stem cell pool and impaired DNA damage repair, thus facilitating disease progression to an acute-leukemia like phase. Our findings provide strong evidence supporting the idea that normal ABL1 is a tumor-suppressor in BCR-ABL1-positive leukemia.

## CHAPTER 3

### **ABL1 REGULATES IMATINIB SENSITIVITY IN BCR-ABL1-MEDIATED LEUKEMIA AND IS A THERAPEUTIC TARGET IN BCR-ABL1- AND OTHER FUSION TYROSINE KINASE-MEDIATED LEUKEMIAS**

*De novo* DNA methylation of ten genes including *ABL1*, leading to transcriptional silencing of the promoters have been associated with clinical progression and Imatinib resistance in CML (158). CML-CP patients usually express ABL1 kinase, but a cohort of CML-CP (late) patients and CML-AP/BP patients may not express or display diminished level of p145ABL1. Thus, aberrant *ABL1* promoter methylation along with other allele-specific changes in patients with therapy-resistant disease results in a progressive increase in *BCR-ABL1* expression relative to normal *ABL1* but not *BCR* expression (165). Based on these observations, we hypothesize that ABL1 has a potential role in regulating sensitivity to tyrosine kinase inhibitors (TKI) such as Imatinib in CML.

The functional loss of ABL1 activity accelerated leukemia development in NOD/SCID mice. We have established the role of ABL1 as a tumor suppressor in BCR-ABL1-mediated leukemia. We hypothesize that strategies aimed to stimulate ABL1 kinase activity will enhance the tumor-suppressor role of ABL1 which when combined with TKI therapy will have a synergistic anti-CML effect .

As mentioned previously, ABL1 kinase activity is tightly regulated by intramolecular interactions whereby the N-terminal myristoyl group binds to the myristoyl-binding site in the C-terminus. This facilitates bending of the  $\alpha 1$  helix which

enables docking of the SH2 domain onto the kinase domain thereby forming a closed, inhibitory conformation of the SH2, kinase and SH3 domains. Yang *et al* identified a small molecule ABL1 activator, 5-(1,3-diaryl-1H-pyrazol-4-yl)hydantoin (DPH), which is cell permeable and binds to the myristoyl-binding site of ABL1. Structural analyses demonstrate that DPH binding displaces the myristate moiety from the myristoyl-binding site thereby preventing formation of the auto-inhibitory bent conformation of the  $\alpha 1$  helix by steric hindrance. This conformational change induces ABL1 kinase activation and consequent phosphorylation of two key tyrosine residues (Y245 and Y412). Moreover, they demonstrate DPH-induced N-terminal ABL1 kinase activation *in vitro* and cellular activity of DPH by stimulating ABL1 and its downstream substrate CRK phosphorylation. DPH-mediated endogenous ABL1 activation provided a strategy to examine the pathophysiological effects of ABL1 kinase stimulation in BCR-ABL1- and other fusion tyrosine kinase-expressing cells (220). We hypothesize that stimulation of ABL1 activity by DPH will enhance the inhibitory effect of TKIs in inhibiting CML cells more potently.

### **Materials and Methods**

**Cells:** *Abl1*<sup>+/+</sup> and *Abl1*<sup>-/-</sup> bone marrow-derived cells were retrovirally transduced with pMIG-IRES-BCR-ABL1-GFP as described in Chapter 2. LAMA84R, KCL22 cells, and green fluorescent protein (GFP<sup>+</sup>) BCR-ABL1-positive *Abl1*<sup>-/-</sup> leukemia cells were then retrovirally transduced with pMSCV retroviral construct encoding YFP-ABL1 fusion protein or kinase-dead YFP-ABL1 (K290R) mutant (kindly obtained from Dr. Koleske,

Yale University, New Haven, CT, USA). GFP, yellow fluorescent protein (YFP), and YFP/GFP-positive cells were sorted and expanded in growth factor-free conditions.

Patient cells were obtained from anonymous donors and healthy donor samples were purchased from Cambrex BioScience and maintained in StemSpan H3000 medium (StemCell Technologies) supplemented with 200 pg/ml SCF, 200 pg/ml GM-CSF, 1 ng/ml G-CSF, 50 pg/ml LIF, 200 pg/ml MIP-1 $\alpha$  and 1 ng/ml IL-6 (StemCell Technologies). Lin<sup>-</sup>CD34<sup>+</sup> cells were obtained from Ficoll separated samples by magnetic sorting using the EasySep negative selection human progenitor cell enrichment cocktail followed by human CD34-positive selection cocktail (StemCell Technologies). Philadelphia chromosome positive B-ALL primary mouse xenografts at diagnosis and at relapse (harboring T315I mutation) were obtained from Dr. Markus Muschen, UCSF and maintained in RPMI supplemented with 10% FBS. BaF3 cell lines expressing TEL-ABL1 were maintained in IMDM supplemented with 10% FBS and SCF in concentration necessary to maintain proliferation. Bone marrow-derived cells expressing NUP214-ABL1 were obtained from Dr. Oliver Hantschel, Lausanne; they were cultured in RPMI medium supplemented with 20% FBS. Also Lin<sup>-</sup>CD34<sup>-</sup> Acute myeloid leukemia (AML) xenografts harboring the fms-like tyrosine kinase 3 (FLT3) gene with a gain-of-function internal tandem duplication (ITD) mutation were obtained from Dr. Peter Valent, Medical University of Vienna. These cells were maintained in StemSpan H3000 medium supplemented with 200 pg/ml SCF, 200 pg/ml GM-CSF, 1 ng/ml G-CSF, 50 pg/ml LIF, 200 pg/ml MIP-1 $\alpha$  and 1 ng/ml IL-6 (PeproTech). CML-BP cell lines LAMA84R and

KCL22 were kindly provided by Dr. Ravi Bhatia, City of Hope, which were maintained in RPMI medium supplemented with 10% FBS.

**Sensitivity to Imatinib:** BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells, LAMA84R, KCL22 cells and BCR-ABL1 *Abl1*<sup>-/-</sup> leukemia cells expressing YFP-ABL1 or YFP-ABL1 (K290R) mutant, were treated with imatinib (Novartis Pharma, AG, Basel, Switzerland) for 48 hours and evaluated by clonogenic assay by plating 10<sup>4</sup> cells/ml in presence of Imatinib (concentration as indicated) as described in Chapter 2-Materials and Methods.

**Imatinib retention:** Radiolabeled drug uptake was carried out by Dr. Tomasz Stoklosa, Medical University of Warsaw, using <sup>14</sup>C-labeled imatinib (Novartis Pharma, Basel, Switzerland). Briefly, 2 × 10<sup>6</sup> cells were incubated with 1.6 μmol/L <sup>14</sup>C-labeled Imatinib (3,052 MBq/mg) at 37°C for 2 hours. After incubation, the cells were washed twice with ice-cold PBS and incubated in culture medium at 37°C for another 15 minutes. Cell pellet was then solubilized in 50 μL of distilled water and radioactivity was counted using β-counter (Perkin Elmer).

**Protein expression:** Total cell lysates obtained from BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells, were analyzed by Western blotting using primary antibodies recognizing ABL1, Abcb1, CHIP, and tubulin (Calbiochem); phospho-tyrosine (4G10) (Upstate), Bag1, Cbl, and GFP (Santa Cruz Biotechnology); Oct-1 (Novus Biologicals); Abcg2, HSP90, and cathepsin B (Abcam Inc.) and Hsc70 (Enzo Life Sciences International, Inc.) as described previously in Chapter 2-Materials and Methods. Total cell lysates obtained from Bosc23 cells overexpressing ABL1 treated with DMSO, 1 μM

Imatinib, 10  $\mu$ M DPH and a combination of imatinib and DPH and analyzed by Western blotting using primary antibodies recognizing ABL1 (Calbiochem), phosphotyrosine245-ABL (Cell signaling) and tubulin (Calbiochem); as described previously.

**Genome-wide expression array:** Genome-wide expression array was performed as described previously in Chapter 2- Materials and Methods.

**Small-molecule activator treatment:** Cells grown in suspension were treated with 1 $\mu$ M Imatinib and 10 $\mu$ M DPH (obtained from Sigma Aldrich) in combination and individually at 0, 24 and 48 hours. Cells treated with DMSO served as control for the experiment. Viable cells were then counted by Trypan blue exclusion and CD34<sup>+</sup> cells from CML patients and healthy donors were plated in Methocult semi-solid medium as described previously; colonies were scored after 7 days. Ph+B-ALL murine xenografts at diagnosis and at relapse (harboring a T315I mutation) were treated in a similar way but with DPH and Imatinib or Ponatinib (Sellekchen) (12.5 nM) (Ph+B-ALL-T315I) in combination and individually. Similarly, BaF3-TEL-ABL1 cell lines and BMC expressing NUP214-ABL1 were incubated with DPH and Imatinib (1 $\mu$ M for BaF3-TEL-ABL1, 0.0625 $\mu$ M for NUP214-ABL1-positive BMC) in combination and individually. AML cells expressing FLT3-ITD mutant kinase were incubated with DPH and FLT3-inhibitor AC220 (Quizartinib; Sellekchen) (10nM) in combination and individually. Cells were treated at 0, 24 and 48 hours with TKI followed by DPH in a sequential manner and counted after 72 hours by Trypan blue exclusion .

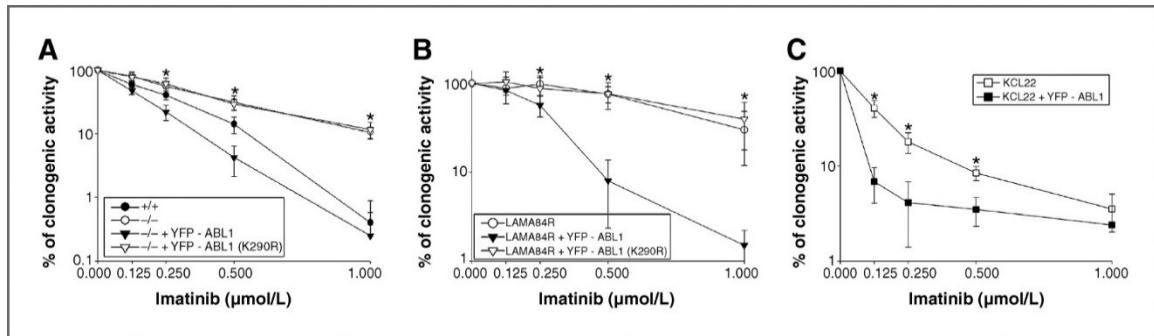
## Results

### Regulation of Imatinib-sensitivity by ABL1

In collaboration with Dr. Elisabeth Nacheva we reported that approximately 10% of CML-CP patients who failed to achieve complete cytogenetic remission (CCyR) within 12 months of TKI treatment acquired a cryptic deletion in 9q34 region in normal chromosome 9 [del(9q34)] resulting in the loss of remaining normal *ABL1* allele (221). The study also reported that several cell lines established from patients in CML-BP lack normal ABL1 expression on non-rearranged chromosome 9. This prompted us to study the role of ABL1 in regulating Imatinib sensitivity of BCR-ABL1-positive cells.

We found that BCR-ABL1 *Abl1*<sup>+/+</sup> cells were more sensitive to Imatinib compared to BCR-ABL1 *Abl1*<sup>-/-</sup> cells that lack functional ABL1 protein. Reconstitution of wildtype YFP-ABL1 expression but not kinase-inactive YFP-ABL1(K290M) mutant in BCR-ABL1 *Abl1*<sup>-/-</sup> cells restored Imatinib-sensitivity in BCR-ABL1 *Abl1*<sup>-/-</sup> cells (**Fig 3.1A**).

We also tested two different cell lines LAMA84R and KCL22 established from CML-BP patients for Imatinib sensitivity after restoring ABL1 expression. We found that restoration of YFP-ABL1 and not mutant YFP-ABL1(K290M) expression increased the sensitivity of the cells to Imatinib (**Fig 3.1B, C**).

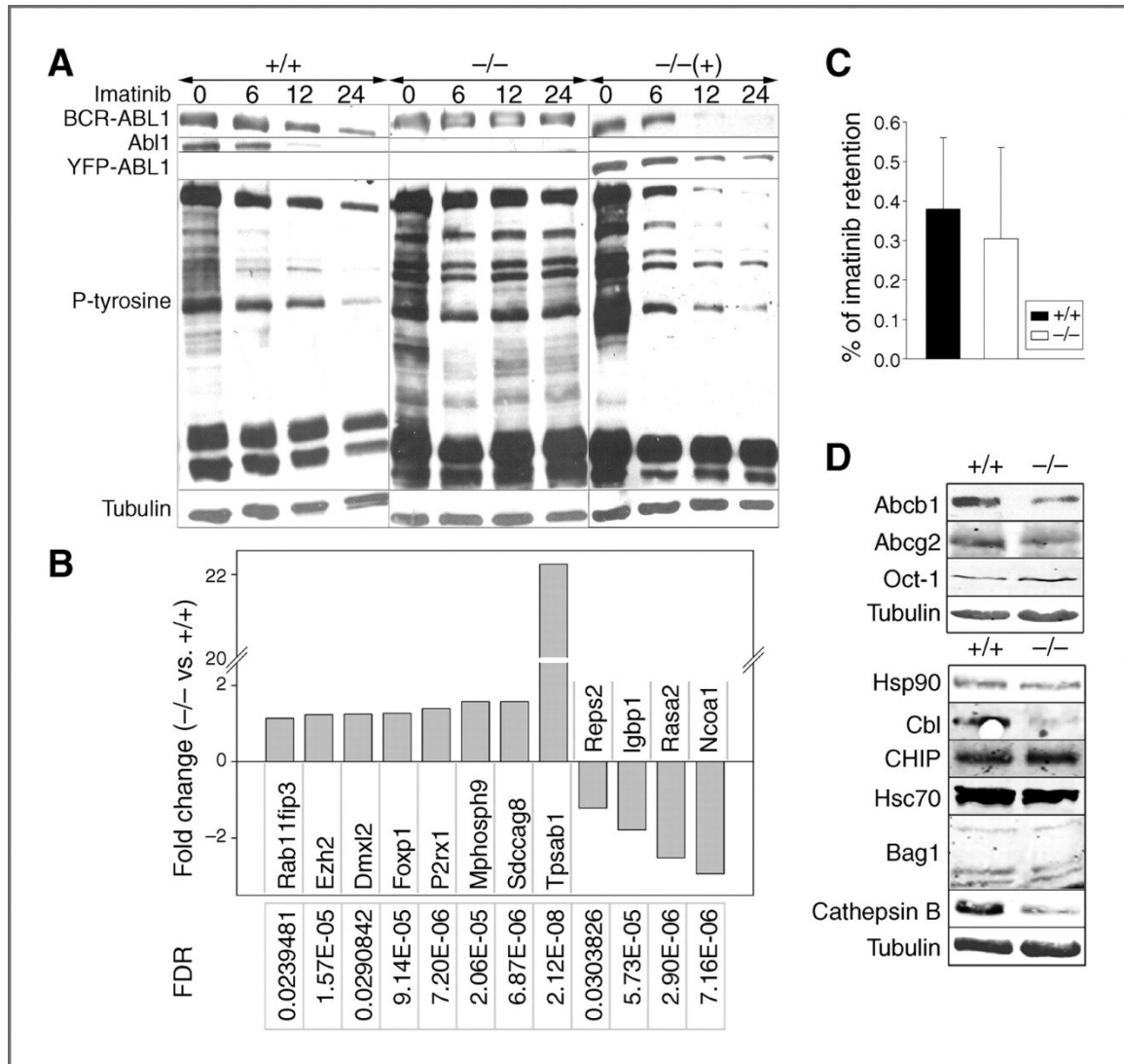


**Figure 3.1 . ABL1 kinase regulates imatinib sensitivity of BCR-ABL1 leukemia.** (A) BCR-ABL1 *Abla*<sup>-/-</sup> leukemia cells (-/-), BCR-ABL1 *Abla*<sup>+/+</sup> leukemia cells (+/+), and BCR-ABL1 *Abla*<sup>-/-</sup> leukemia cells reconstituted with YFP-ABL1 and YFP-ABL1(K290R), (B) LAMA84R cells and these transfected with YFP-ABL1 and YFP-ABL1(K290R), and (C) KCL22 cells and these transfected with YFP-ABL1 were incubated with imatinib and clonogenic cells were counted. Results represent mean percentages  $\pm$  SD.

Western analysis revealed BCR-ABL1 *Abla*<sup>-/-</sup> cells to display reduced capability to abrogate BCR-ABL1-mediated tyrosine phosphorylation by Imatinib in comparison to BCR-ABL1-transformed wildtype cells or *Abla*<sup>-/-</sup> leukemia cells with restored ABL1 expression (**Fig 3.2A**).

Genome-wide expression array reveals an Imatinib resistant molecular signature of BCR-ABL1 *Abla*<sup>-/-</sup> cells by aberrant expression of genes associated with Imatinib resistance in CD34<sup>+</sup> cells from therapy-resistant CML patients (**Fig 3.2B**).

We did not detect any difference in intracellular retention of Imatinib in BCR-ABL1 *Abla*<sup>-/-</sup> versus BCR-ABL1 *Abla*<sup>+/+</sup> cells when <sup>14</sup>C-labeled Imatinib uptake was measured by a  $\beta$ -counter (**Fig 3.2C**).



**Figure 3.2 . Imatinib-resistant phenotype of BCR-ABL1 *Abl1*<sup>-/-</sup> leukemia cells.** BCR-ABL1 *Abl1*<sup>-/-</sup> leukemia cells (-/-), BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells (+/+), and BCR-ABL1 *Abl1*<sup>-/-</sup> leukemia cells reconstituted with YFP-ABL1 [-/- (+)] were used, (A) Western analysis of the total cell lysates from cells incubated with 1  $\mu\text{mol/L}$  Imatinib for 0, 6, 12, and 24 hours, (B) statistically significant (FDR < 0.05) fold-changes (>1) of the expression of indicated genes in BCR-ABL1 *Abl1*<sup>-/-</sup> versus BCR-ABL1 *Abl1*<sup>+/+</sup> samples. (C) intracellular retention of Imatinib; results represent mean percentages  $\pm$  SD of total  $^{14}\text{C}$ -imatinib, (D) Western blot analysis of total cell lysates to detect Imatinib transporters (top) and proteins involved in BCR-ABL1 degradation (bottom).

We studied the relative expression of drug exporters ABCB1, ABCG2 and drug importer OCT1 in BCR-ABL1 *Abl1*<sup>-/-</sup> versus BCR-ABL1 *Abl1*<sup>+/+</sup> cells. Western analysis of total cell lysates reveal that loss of ABL1 did not affect the expression of these proteins. Next we sought to look at the role of ABL1 in altering the expression levels of proteins involved in BCR-ABL1 stability and degradation. Western analysis indicate a more than 10-fold downregulation of Cbl E3 ligase and a more than 3-fold downregulation of cathepsin B. However ABL1 did not affect the expression levels of the chaperone protein Hsp90 or other proteins involve in BCR-ABL1 degradation such as Hsc70, Bag1 and E3 ligase CHIP. Tubulin served as the loading control (**Fig 3.2D**).

#### *ABL1 as a therapeutic target*

We postulate that stimulation of ABL1 activity may enhance the effect of TKIs in CML treatment. Western Blot analysis was used to detect the expression of ABL1 and phosphorylated-Tyrosine245-ABL1 upon DPH treatment;  $\beta$ -actin served as a loading control. We detected partial activation of ABL1 as indicated by phosphorylation of Tyrosine245 (Y245) in DMSO-treated cells. There was at least a 2-fold increase in the expression of phospho-Y245-ABL1 in cells incubated with DPH in correlation with the findings of Yang *et al*, followed by an abrogation of ABL1 kinase activation in Imatinib-treated cells since Imatinib inhibits ABL1 kinase. However, DPH was able to restore partial activation of ABL1 in presence of Imatinib (**Fig 3.3A**).

Next, we examined the effect of Imatinib in combination with DPH on BCR-ABL1 *Abl1*<sup>-/-</sup> and BCR-ABL1 *Abl1*<sup>+/+</sup> cells. We found that treatment of 0.125 $\mu$ M Imatinib in combination ( $18.62 \pm 8.93$ )% with 10 $\mu$ M DPH for 3 consecutive days reduced the

viability of BCR-ABL1 *Abl1*<sup>+/+</sup> leukemia cells significantly than treatment with Imatinib ( $43.68 \pm 5.88$ )% or DPH ( $77.21 \pm 9.32$ )% alone. However, BCR-ABL1 *Abl1*<sup>-/-</sup> leukemia cells were not found to be sensitive to the combination treatment [IM: ( $113.92 \pm 34.43$ )%, DPH: ( $105.87 \pm 47.12$ )%, IM+DPH: ( $86.16 \pm 24.14$ )%] (**Fig 3.3B**).

We examined if DPH exerts a synergistic effect on tyrosine kinase inhibitors (TKI) activity in primary CD34<sup>+</sup> cells from patients in CML-CP. We tested the potential inhibitory effect of 1 $\mu$ M Imatinib and 10 $\mu$ M DPH on primary CML-CP CD34<sup>+</sup> cells and found that the combinatory effect of DPH and Imatinib ( $30.12 \pm 5.44$ )% was higher in inhibiting viability as well as clonogenic growth of CML-CP CD34<sup>+</sup> cells by 2-fold than with either agent alone [IM: ( $55.31 \pm 8.60$ )%; DPH: ( $103.73 \pm 30.30$ )%] (**Fig 3.3C**). We also treated Philadelphia chromosome-positive B cell-acute lymphoblastic leukemia (Ph+B-ALL) xenografts at diagnosis (**Fig 3.3D**) and those at relapse harboring an Imatinib-resistant T315I mutation (**Fig 3.3E**) with 10 $\mu$ M DPH in combination with Imatinib (1 $\mu$ M) and Ponatinib (12.5nM)



treatment with DMSO (control) or indicated concentrations of DPH and/or Imatinib consecutively for 72 hrs *in vitro*; \* $P < 0.001$ , (C) Percentage of viable cells  $\pm$  SD from 3 Philadelphia Chromosome-positive B-ALL patients at diagnosis on treatment with DMSO (control) or indicated concentrations of DPH and/or Imatinib consecutively for 72 hrs *in vitro*; \* $P < 0.05$ , (D) Percentage of viable cells  $\pm$  SD from 3 Philadelphia Chromosome-positive B-ALL patients harboring a T315I mutation (right panel) on treatment with DMSO (control) or indicated concentrations of DPH and/or Ponatinib consecutively for 72 hrs *in vitro*; \* $P < 0.05$ , (E) Percentage clonogenicity  $\pm$  SD of cells from 3 healthy donors after treatment with DMSO (control) or indicated concentrations of DPH and/or Imatinib consecutively for 72 hrs *in vitro*; \* $P < 0.001$ .

respectively for 72 hours. 10-fold reduction in viability of both cell types was observed when treated with DPH and the respective TKIs [(8.33  $\pm$  14.43)%; T315I: (3.70  $\pm$  6.42)] than with DPH [(69.44  $\pm$  33.68)%; T315I: (47.22  $\pm$  20.97)% or TKI [(88.89  $\pm$  19.25)%; T315I: (50.93  $\pm$  26.40)%] alone.

In an attempt to broaden the application of this potent combination of DPH with TKI we studied the effect of this combinatory treatment on other types of leukemias harboring different fusion tyrosine kinases such TEL-ABL1 and NUP214-ABL1 and also fms-like tyrosine kinase 3 (FLT3) gene with a gain-of-function internal tandem duplication (ITD) mutation. When TEL-ABL1-positive BaF3 cell lines were treated with 1  $\mu$ M Imatinib and 10  $\mu$ M DPH, there was a 50% reduction in viability (16.00  $\pm$  4.83) compared to either agents alone [IM: (33.73  $\pm$  3.70)%; DPH: (81.08  $\pm$  14.73)%] (**Fig 3.3F**). NUP214-ABL1-positive murine bone marrow cells were found to be 2-fold more sensitive to the treatment of Imatinib and DPH (19.20  $\pm$  2.71)% in combination than with either agent alone [IM: (33.56  $\pm$  1.46)%; DPH: (102.12  $\pm$  13.70)%] (**Fig 3.3G**). We also found a similar effect on primary AML cells harboring the FLT3-ITD mutation when treated with the 10nM- FLT3 inhibitor AC220 in combination with 10  $\mu$ M DPH (60.81  $\pm$  1.11)%. The combination inhibited viability of cells by 50% compared to either AC220 (103.09  $\pm$  9.13)% or DPH (91.18  $\pm$  14.75)% alone (**Fig 3.3H**). Finally DPH in combination with

Imatinib showed no cytotoxicity in normal cells as the results displayed no inhibition on the growth of CD34<sup>+</sup> cells obtained from healthy donors [IM: (182.25 ± 26.44)%; DPH: (141.32 ± 140.52)%; IM+DPH: (133.84 ± 142.44)%] (**Fig 3.31**).

### Discussion

The role of ABL1 in regulating Imatinib sensitivity has been demonstrated by the cryptic deletion in normal chromosome 9 [del(9q34)] resulting in the loss of normal *ABL1* gene expression in 3/21 patients who failed to achieve complete cytogenetic response (CCyR) following 12 months of TKI therapy. Loss of *ABL1* was accompanied by other genetic alterations including acquiring a second 'Ph' in the *Abli*-deficient clone. This observed loss of ABL1 results in genomic instability and karyotype evolution, which leads to disease progression and TKI-resistance (221).

Our findings demonstrate BCR-ABL1-positive cells expressing ABL1 to be more sensitive to Imatinib compared to *Abli*<sup>-/-</sup> cells, indicating that ABL1 regulates Imatinib sensitivity in these cells. Further, the restoration of anti-leukemic effect of Imatinib by kinase-active YFP-ABL1 but not kinase-inactive YFP-ABL1(K290M) in BCR-ABL1-*Abli*<sup>-/-</sup> cells proves ABL1 kinase to play a key role in modulating TKI-sensitivity. A similar effect was observed in the drug-resistant CML-BP cell line LAMA84R. In addition to BCR-ABL1 overexpression and elevated levels of drug-transporters, loss of ABL1 expression also contributes to drug resistance in LAMA84R (222). We observed restoration of anti-leukemic effect of Imatinib upon reconstitution of ABL1 expression. Overexpression of kinase-active ABL1 in another CML-BP cell line KCL22 with low levels of endogenous ABL1 expression increased Imatinib sensitivity in these cells,

suggesting that relative increase in BCR-ABL1:ABL1 ratio may also reduce the efficacy of Imatinib (170). These observations clearly indicate that ABL1 exerts a significant impact on anti-CML effect of Imatinib. This effect could partly be due to induction of apoptosis following nuclear and mitochondrial localization of ABL1 upon dissociation from 14-3-3 as a consequence of Imatinib-induced BCR-ABL1 inhibition (142,223). So far we established that ABL1 kinase modulates cellular response to Imatinib. The various functional domains of the BCR-ABL1 oncoprotein along with its elevated tyrosine kinase activity enables binding and phosphorylation of several proteins leading to the activation of different mitogenic signaling pathways (11,18,22). Imatinib displayed reduced capability to inhibit BCR-ABL1-mediated tyrosine phosphorylation in BCR-ABL1 *Abl1*<sup>-/-</sup> compared to wild-type counterparts. To elucidate how ABL1 regulates TKI-sensitivity, we analyzed a genome-wide expression array which reveals an Imatinib-resistant molecular signature of BCR-ABL1 *Abl1*<sup>-/-</sup> cells revealed by aberrant expression of genes associated with Imatinib resistance in therapy-resistant patients. Intracellular retention of Imatinib and deregulated expression of drug transporters, which otherwise contribute to Imatinib resistance were unaffected by loss of ABL1. Downregulation of BCR-ABL1 in Imatinib-treated CD34<sup>+</sup> CML-CP cells reportedly regulates drug sensitivity (224). Significant downregulation of proteins involved in BCR-ABL1 degradation in absence of ABL1 may contribute to decreased Imatinib sensitivity by increasing BCR-ABL1 stability.

So far our results indicate that loss of ABL1 accelerates malignant transformation in BCR-ABL1 cells and contributes to TKI resistance. In order to further establish the

clinical significance of ABL1 expression and/or activity, we stimulated ABL1 kinase with the cell permeable activator DPH, and examined its potency in combination with Imatinib and other FTK-inhibitors on BCR-ABL1-and other FTK-mediated leukemias. Phosphorylation of tyrosine245 indicated DPH-mediated stimulation of ABL1 kinase, which confirmed the findings of Yang *et al.* In addition, DPH restored the inhibitory effect of Imatinib on ABL1 kinase activation. This could be due to the fact that 1 $\mu$ M Imatinib, although inhibits cytoplasmic BCR-ABL1 is sub-optimal for inhibition of nuclear and cytoplasmic ABL1 (225,226). DPH in combination with Imatinib was found to inhibit viability of BCR-ABL1 *Abli*<sup>+/+</sup> cells but not BCR-ABL1 *Abli*<sup>-/-</sup> leukemia cells, indicating an ABL1-specific mode of action. The combination of DPH and Imatinib inhibited clonogenic growth of CML-CD34<sup>+</sup> cells but not those from healthy donors which indicates that DPH has no cytotoxic effect on normal cells and that it specifically targets CML cells. We observed a similar effect on Philadelphia-positive B-ALL xenografts at diagnosis and at relapse (harboring the T315I). The potency of the combination in comparison to either agents alone could be due to sequential effect of Imatinib-mediated inhibition of BCR-ABL1 which promotes release of ABL1 from 14-3-3 proteins and subsequent DPH-mediated activation and nuclear localization of ABL1 kinase thereby restoring its pro-apoptotic role (142,223). In order to broaden the scope of this combination therapy we tested other leukemias expressing activated ABL1 fusion tyrosine kinases such as TEL-ABL1-expressing cell lines as well as bone marrow cells expressing NUP214-ABL1. The use of both agents potently inhibited viability of these cells indicating a critical role of normal ABL1 activity, possibly as a tumor suppressor.

Interestingly, we also observed an inhibitory effect of DPH and FLT3-inhibitor AC220 on AML cells expressing FLT3-ITD mutant kinase which suggests a potential application of this treatment beyond ABL1-associated hematological malignancies.

## CHAPTER 4

### CONCLUSION, FUTURE DIRECTIONS AND SIGNIFICANCE

Based on the functions of ABL1 in opposing malignant transformation driven by BCR-ABL1 oncogenic kinase, coupled with epigenetic silencing of ABL1 promoter in advanced phase CML, we hypothesized that ABL1 is a tumor suppressor in BCR-ABL1-mediated leukemia. Our preliminary findings showed BCR-ABL1-positive *Abl1*-deficient cells accelerated leukemia development in mice. Restored ABL1 expression in these cells was found to prolong survival. To elucidate further the role of ABL1 in BCR-ABL1-leukemogenesis, we studied the effect of loss of ABL1 expression on cell proliferation, myeloid cell differentiation, induction of apoptosis, regulation of stem cell characteristics, genomic instability and tyrosine kinase inhibitor resistance. Loss of ABL1 in BCR-ABL1-transformed leukemia cells resulted in aberrant cell proliferation, arrested myeloid differentiation, resistance to genotoxic stress-induced apoptosis, enhanced genomic instability and decreased sensitivity to Imatinib. The molecular signature of BCR-ABL1-positive *Abl1*-knockout cells, as revealed by a genome-wide expression array shows deregulated expression of several genes associated with these processes, thereby confirming our observations.

Thus, our work clearly establishes the role of ABL1 as a tumor suppressor in BCR-ABL1-leukemias. The fact that ABL1 plays a ‘tumor-suppressor role’ in CML is novel since it has been reported to be ‘proto-oncogene’. The various functional domains along with different sub-cellular localizations may allow ABL1 kinase to interact with a

host of proteins and consequently modulate several cellular processes which may account for its dual function. ABL1 can modulate cellular processes in opposite ways based on its sub-cellular localization. We can gain more insight into how ABL1 regulates different cellular processes by determining which of its functions are kinase dependent or independent. ABL1 kinase has a long unique carboxy terminus which contains several functional domains that regulate ABL1 activity, interactions with different proteins, DNA and its localization. Moreover, the microarray data can also be used to identify pathways that are directly or indirectly regulated by ABL1 in BCR-ABL1-positive cells so that its role as a potential therapeutic target can be explored. Moreover, clinical significance of the role of ABL1 is due to the fact that in patients harboring the resistance-inducing T315I or other BCR-ABL1 mutations, TKI therapy would inhibit normal ABL1 kinase and not the oncogenic BCR-ABL1, thereby accelerating disease progression.

Based on our previous findings, we also showed that stimulation of ABL1 activity can enhance the anti-CML effect of Imatinib. We used a small molecular activator of ABL1, DPH, in combination with different tyrosine kinase inhibitors to treat CML, Ph+B-ALL, AML cells and cells expressing TEL-ABL1 and NUP214-ABL1. Stimulation of ABL1 activity was found to exert a synergistic effect on the inhibitory effect of TKIs on different hematological malignancies without any noticeable cytotoxicity on normal cells. This suggests that ABL1 can be a potential tumor-suppressor and therapeutic target not only in CML but also other fusion tyrosine kinase-mediated leukemias. In order to test the viability of the combination therapy, pharmacokinetic assays need to be conducted for *in vivo* studies. Another limitation of

this potential treatment regime is the fact that ABL1 expression is often transcriptionally silenced in advanced phase CML due to promoter methylation or genetic deletion. All the studies conducted by us *in vitro* are on CM-CP cells when both normal ABL1 and oncogenic BCR-ABL1 kinases are expressed, and not on CML-BP cells. Use of a demethylating agent in order to reverse the epigenetic silencing of *ABL1* promoter followed by the tested sequential treatment of TKI and DPH may enable us to examine the potency of the combination therapy on cells obtained from the more fatal advanced phase-CML patients.

Our findings demonstrate the necessity of monitoring normal *ABL1* expression which when lost can accelerate disease progression and if present, can function as a therapeutic target. In conclusion, we have established ABL1 as a tumor suppressor in BCR-ABL1 mediated leukemia and a therapeutic target in Philadelphia chromosome positive- as well as other oncogenic FTK-mediated leukemias.

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