THE ROLE OF TYROSINE-283 OF BCR IN HEMATOPOIETIC LINEAGES DURING BCR/ABL LEUKEMOGENESIS

BY AYBIKE ONUR

SENIOR HONORS THESIS
DR. RUIBAO REN

DEPARTMENT OF BIOLOGY
BRANDEIS UNIVERSITY
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ABSTRACT

BCR/ABL induced chronic myelogenous leukemia (CML) is a biphasic disease characterized by the expansion of myeloid lineage granulocytic cells during its chronic phase and the expansion of immature cells during the later blast crisis. In humans, progression into blast crisis is often observed with proliferation of immature myeloid cells, however in one third of the cases proliferation of immature lymphoid cells occur. The mechanism behind different lineage expansion is unknown. Mouse models are efficient in mimicking the chronic phases of the BCR/ABL induced CML and are used to investigate the factors altering the disease phenotype in order to provide the patients with more effective treatment strategies in future. Previous studies showed that different levels of BCR/ABL expression or certain mutations within BCR/ABL can alter the disease phenotype in mouse models.

In the mouse model, BCR/ABL P185 causes a CML-like myeloproliferative disease. BCR/ABL P185 with the point mutation Y283F in its BCR region, however, causes a mixed disease with characteristics of CML and acute lymphoblastic leukemia. The mechanism behind this phenotypic difference has not been well defined. I studied the differences in transformation potentials of the BCR/ABL oncogenes with or without the tyrosine 283. I showed that this tyrosine residue may be important in myeloid lineage transformation and may not be playing a role in lymphoid lineage transformation. Moreover, I revealed a decrease in activation of two signaling proteins, Stat5 and rpS6, in BCR/ABL Y283F compared to P185.
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Results

BCR/ABL P185 and 1-296 transform bone marrow cells at different rates than Y283F and 1-269 \textit{in-vitro} under myeloid conditions.

BCR/ABL P185, Y283F, 1-296 and 1-269 transform bone marrow cells at similar rates \textit{in-vitro} under lymphoid conditions.

GFP sorted 32D cell lines expressing BCR/ABL P185, y283, 1-296 and 1-269 proliferate at similar rates.

BCR/ABL P185 and 1-296 activates Stat5 and rpS6 more than Y283F and 1-269 in 32D cell lines.

Discussion

Tyrosine 283 contributes to potential of BCR/ABL to induce myeloid lineage transformation, but not lymphoid lineage transformation.

Tyrosine 283 contributes to signaling events activating Stat5 and rpS6, which may be important for myeloid disease development.

Acknowledgments

References
INTRODUCTION

Hematopoiesis and Leukemia

Each type of blood cell is generated from pluripotent hematopoietic stem cells (HSC) through a process called hematopoiesis. HSC are found in bone marrow and maintain the blood and blood forming tissues throughout life. Long term HSC can divide infinitely to renew itself or give rise to a short term HSC. Short term HSC can self renew a limited number of times and differentiate into a multipotent progenitor cells. Afterward, a multipotent progenitor cell differentiates either into a common lymphoid or into a myeloid progenitor.

These progenitor cells give rise to all types of blood cells through further differentiation, as depicted in Figure 1. Myeloid lineage cells contain red blood cells, megakaryocytes, macrophages, dendritic cells and granulocytes. These cells have important functions in adaptive and innate immunity, blood clotting and oxygen transportation. Lymphoid lineage cells include B cells, T cells, natural killer cells and dendritic cells, which are the key players of adaptive immunity. This dynamic differentiation process is under the tight regulation of signaling pathways and serves to regenerate the required and diverse population of blood cells.

A deviation from normal hematopoiesis may result in leukemia and other hematological disorders. Leukemia is a cancer of blood or bone marrow and is characterized by the expansion of undifferentiated cells or expansion of just one cell type. Deregulation of the complex signaling pathways controlling hematopoiesis may lead to errors in differentiation and abnormal growth of blood cells. Hematopoietic stem cells are
especially prone to neoplastic transformation that might lead to leukemia because of their unlimited renewal capacity.6

Figure 1. A diagram of hematopoiesis: Hematopoietic stem cells can self-renew or generate committed progenitor cells that eventually produce all blood cells. Red blood cells, megakaryocytes, macrophages, eosinophils, neutrophils, basophils, dendritic cells, T cells, B cells, and natural killer cells are formed through hematopoiesis. The diagram with modifications is adapted from Ren, 2005.3

1 This HSC represents the long term HSC, short term HSC and multipotent progenitor

Chronic Myelogenous Leukemia and the Philadelphia Chromosome

Chronic myelogenous leukemia (CML) represents 15-20% of all leukemia cases in U.S.3 CML was the first type of leukemia to be described8 and the first case when leukemogenesis was shown to be linked to a genetic anomaly9. Over the last decades,
CML's clinical, biological, biochemical, and molecular features have been studied extensively. These studies put CML among the first cancers for which a drug that targets only the malignant cells has been available. There is, however, still more to discover about the disease and its progression to improve the treatment options.

Chronic myelogenous leukemia is characterized by the clonal expansion of a transformed pluripotent hematopoietic stem cell. CML's characteristic genetic anomaly is the Philadelphia chromosome caused by the translocation mutation t(9;22)(q34;q11) (Figure 2). It was first identified as a small unusual chromosome and named Philadelphia chromosome after the location of its discovery. Later on, it was determined that the reciprocal translocation generates the BCR/ABL fusion oncogene by linking the ABL kinase gene to the beginning sequence of BCR. The product, the BCR/ABL fusion protein, has an increased kinase activity essential for neoplastic transformation and several binding domains for intracellular signaling proteins. BCR/ABL oncogene is detected in all blood cells of patients; therefore it is believed that the translocation mutation happens in a long term hematopoietic stem cell.

![Diagram of the Philadelphia chromosome translocation](image)

**Figure 2. The Philadelphia chromosome:** The translocation mutation t(9;22)(q34;q11) gives rise to BCR-ABL oncogene.
CML is a biphasic disease with an initial chronic phase and a final blast crisis. The chronic phase resembles a myeloproliferative disease and is characterized by the expansion of granulocytes over a many months or couple of years\textsuperscript{3,10}. Although the exact mechanism is unknown, the disease is thought to progress into the blast crisis upon accumulation of other mutations that cause a premature block in differentiation\textsuperscript{3}. As a result, immature myeloid or lymphoid cells proliferate rapidly and compose over 30\% of the peripheral blood cells\textsuperscript{12}. In majority of cases the patients display signs of myeloblastic leukemia resembling acute myeloid leukemia (AML). In about a third of the cases, however, they experience a blast crisis resembling acute lymphoblastic leukemia (ALL)\textsuperscript{13}. Figure 3 illustrates the cell expansions observed in BCR/ABL induced leukemia.

Hematopoiesis is impaired in BCR/ABL expressing blood cells. Differentiation in myeloid lineage is blocked as one cell type (granulocytes or immature cells) expands greatly. It is suggested that differentiation of lymphocytes is compromised by BCR-ABL expression as well\textsuperscript{3}. Lineage commitment studies of BCR-ABL expressing hematopoietic stem cells show that lymphocyte populations, especially T cells, have decreased amount of BCR-ABL\textsuperscript{+} cells when compared to other cell types of the same origin\textsuperscript{14}. Studying how BCR-ABL influences differentiation during hematopoiesis is important to understand the mechanism of leukemogenesis and may also give clues about normal hematopoiesis.
Figure 3. Abnormal hematopoiesis during chronic and blast crisis phases of CML: Normal differentiation and development of blood cells are prevented by BCR/ABL. Blue line illustrates the origin of granulocytic expansion during the chronic phase of the disease. Red lines depict the proliferation of immature myeloid or immature lymphoid cells during blast crisis.

BCR-ABL as an Oncogene

Fusion of the non-receptor tyrosine kinase, ABL, and the signaling molecule, BCR, forms the oncogene BCR/ABL. In-frame fusion of these genes creates an oncoprotein with a constitutively active kinase that localizes in the cytoplasm and influences several cellular pathways leading to neoplasm. Downstream targets of
BCR/ABL include several signaling proteins involved in pathways leading to increased proliferation and transformation, altered adhesion properties and inhibited apoptosis (Figure 4)\textsuperscript{15}.

**Figure 4. Downstream targets of BCR/ABL:** This figure from Acquaviva (2007)\textsuperscript{1} illustrates the signaling pathways downstream of BCR/ABL. P210 and its significant domains for protein interactions.

Certain domains of BCR-ABL have been identified crucial for its oncogenic activity. A functional ABL kinase activity is essential for activating signaling pathways leading to transformation\textsuperscript{3}. Moreover, several regions of both ABL and BCR are
important for recruiting the downstream targets. BCR's important domains are Y177 (GRB2 adapter protein binding site), phospho-serine-threonine-rich sequence (SH2 binding domains), coiled coil motif (for oligomerization), and guanine exchange factor (GEF)\(^9\). ABL contains SH2, SH3, actin binding and DNA binding domains\(^9\). All these domains do not contribute equally to leukemogenesis of BCR/ABL. While some mutations in these regions decrease the transformation potential of the oncogene both in vivo and in vitro, some do not have any effect in vivo and in vitro or only influence in vivo transformation\(^1\). For instance, a BCR/ABL Y177F mutant can transform certain cell types in vitro, however in vivo this mutant fails to induce a CML-like disease observed with the wild-type BCR/ABL and induces an ALL\(^16\). It is important to study the role of the different domains in BCR/ABL in order to understand the mechanism of the disease.

BCR-ABL activates RAS, JAK-STAT, PI3K pathways and alters focal adhesion kinase (FAK) activity (Figure 4 above). A significant increase in STAT5 activation was shown in myeloproliferative diseases\(^17\). Likewise, increased activity of JAK-STAT pathway intensifies the STAT5 activity in BCR/ABL\(^+\) cells, which show myeloid lineage expansion.\(^3\). Increased STAT5 activity contributes to leukemogenesis by promoting proliferation and inhibiting apoptotic pathways\(^18\). Activated RAS initiates the cascade of signaling events activating RAF, MEK and ERK\(^2\), which turn on the gene expression and alter the cell cycle progression\(^9\). RAS activation induce growth factor-independent cell proliferation\(^19\) and further activates PI3K\(^3\). PI3K pathway promotes survival by induction of anti-apoptotic genes such as Bcl-2\(^20\). Another downstream protein, FAK, alters cell adhesion properties and thus contribute to leukemogenesis\(^10\).
Depending on the breakpoints of ABL and BCR genes, a variety of BCR-ABL isoforms can form. These isoforms include P185, P210 and P230 (Figure 5). Among the oncogenic forms of BCR/ABL, BCR-ABL p210 is the most common one detected in patients with CML. Rarer forms, such as P185 and P230, are associated with acute lymhoblastic leukemia (and rarely with CML) and chronic neutrophilic leukemia, respectively\(^9\). Different than the most common isoform P210, P185 isoform lacks the C terminal of BCR which contains the guanine exchange factor (GEF) domain. Different phenotype observed in humans might be due to lack of interactions with certain proteins (that bind to this region) or an increased expression of a smaller gene. P185 still contains the serine-threonine kinase, oligomerization domain, and GRB2 binding site that contribute greatly to its oncogenic potential.

**Figure 5. Isoforms of BCR/ABL**: Oligomerization domain (OD) and the serine threonine kinase domain are present in all BCR/ABL isoforms. Presence of other domains in the BCR region causes different diseases in humans. The guanine exchange factor domain, calcium phospholipid binding domain (CalB) and Gap-Rac domain account for the different disease progression. P185 causes acute lymhoblastic leukemia (and rarely CML), p210 causes CML, and p210 causes chronic neutrophilic leukemia. This figure with modifications is from Advani, et al.\(^5\)
There is a potent and highly specific kinase inhibitor targeting the ABL kinase in BCR/ABL \(^8\). This inhibitor is known as imatinib mesylate or Sti-571 and is marketed to patients as Gleevac by Novartis. Sti-571 suppresses the oncogenic activity of BCR/ABL both \textit{in vivo} and \textit{in vitro} by inhibiting phosphorylation of target proteins; however it does not eliminate the BCR/ABL\(^+\) cells in a cell culture or stem cell population of an organism \(^{21,22}\).

\textbf{Murine Model for CML}

Efficient \textit{in-vivo} models are necessary to study the roles of BCR/ABL domains during disease development. Several models were created to study the leukemogenesis of BCR/ABL. A xenografting model was not successful due to the inefficient interactions of BCR/ABL\(^+\) cells from patients and mouse hematopoietic niches \(^{23}\). BCR-ABL knock-in mice were fetally lethal, and other knock-in methods that dealt successfully with fetal-lethality could not mimic the clonal disease because of wide expression of the oncogene \(^{23}\).

Infecting donor hematopoietic stem cells with BCR-ABL using murine stem cell virus and transplanting these cells into lethally irradiated mice was shown to be the most effective way (100% efficiency) to mimic CML in the mouse \(^{24}\). Irradiation empties the hematopoietic niche for better reconstitution of donor cells \(^{25}\). The disease in mouse resembles the chronic phase of CML in humans with increased number of white blood cells in peripheral blood due to expansion of granulocytes and enlarged spleen. The disease never develops into blast crisis in mouse because infiltration of granulocytes
results in pulmonary hemorrhages that are the main cause of death in mice. The pulmonary hemorrhages rarely occur in human. Despite the phenotypic differences in humans, in this in-vivo murine model, the three isoforms of BCR/ABL (P185, P210 and P230) induce CML-like myeloproliferative diseases with similar characteristics but varying latencies.²³

The role of Tyrosine 283 in BCR-ABL Leukemogenesis

Even though BCR/ABL P185 has the potential to transform lymphoid lineage blood cells, myeloproliferative disorder is observed in the mouse model because the myeloid diseases develop faster. Retroviral transduction and bone marrow transplantation model for CML is used to conduct structure-function analysis of BCR/ABL domains in order to identify the roles of these domains in lineage transformation. In these studies, a BCR/ABL mutant with only the first 222 amino acid residues of its BCR region was shown to induce a mixed disease with the simultaneous expansion of mature granulocytes and immature B cells without a delay in disease latency.²⁶ To identify the smallest mutation that can cause this mixed disease, smaller deletion mutations and point mutations were tested with our mouse model (Figure 6).

Dr. Catherine Spicer, a previous graduate student in Ren Lab, narrowed down the region responsible for the phenotype change to amino acid residues between 269 and 296. In addition, she identified a tyrosine between 269th and 296th amino acid residues, which is important to induce only an MPD. A point mutation at this tyrosine with is sufficient to induce the change in disease phenotype (Figure 6).
Figure 6. BCR/ABL and disease phenotype: Results from mouse experiments with BCR/ABL P185 and several deletion or point mutations. B-ALL (B cell acute lymphoblastic leukemia) is characterized by expansion of immature B cells. The figure is adapted with modifications from Spicer, 2003\textsuperscript{2}

\textsuperscript{1} Mixed disease was developed with 90\% efficiency, while other constructs had 100\% efficiency.

This point mutation replaces the tyrosine at 283\textsuperscript{rd} position with a phenylalanine, thus inhibiting the phosphorylation of this residue. This tyrosine is a site of autophosphorylation and probably a binding site. Phosphorylation of this residue is important for protein interactions and signal transduction of BCR/ABL\textsuperscript{2}. The role of tyrosine 283 in BCR/ABL leukemogenesis is not yet known. Phosphorylation at this tyrosine may be inducing events promoting BCR/ABL induced myeloid lineage expansion or inhibiting lymphoid lineage expansion. Y283F mutation may be inhibiting or weakening the interactions with some target proteins of BCR/ABL\textsuperscript{2}. 
My research project investigated how the Y283F mutation causes the change in disease phenotype from an MPD to a mixed disease with MPD and B-ALL. I compared signal transduction in cell lines, which are expressing BCR/ABL P185, Y283F, 1-296 and 1-269, looking for a clue about which signaling pathways are affected. I also compared the myeloid lineage and lymphoid lineage transformation potentials of these BCR/ABL constructs using primary cells.
MATERIALS AND METHODS

A) Cell lines and cultures

32DC13 (32D):

32D is a myeloid progenitor cell line from C3H/HeJ mouse. The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS), 100 μg/ml penicillin, 100 μg/ml streptomycin (1X P/S) and 10% WEHI3B-conditioned media, a source of interleukin 3 (IL-3), unless noted differently. They were split 1:10 as needed to avoid cells from becoming over-confluent in the flask.

Bosc23:

Bosc23 cells are the murine stem cell retrovirus packaging cell line. The cells were cultured in DMEM with 10% FBS and 1X P/S. The cells were washed with phosphate buffer saline (PBS) and incubated with trypsin-EDTA to disengage from the plate. They were split 1:5 when the plate became confluent. They were not split to a lower concentration to avoid clumping of cells.

NIH3T3:

NIH3T3 cells are fibroblast cells used in DNA transfection assays from NIH Swiss mouse embryo\textsuperscript{27}. The cells were cultured in DMEM with 10% calf serum (DCS) and 1X P/S. The cells were washed with phosphate buffer saline (PBS) and incubated with trypsin-EDTA to disengage from the plate. They were split 1:5 when the plate became confluent.
B) Retroviruses

Retrovirus was used to integrate desired genes into the genome of cell lines and bone marrow cells. Retrovirus was prepared by the packaging cell line, Bosc23, by transfecting it with murine stem cell vectors (MSCV). The cell lines I used required the plasmid vector, pClEco, which contains cDNA for the viral proteins, for optimum transfection. Bosc23 cells were thawed at least four days before transfection and split twice to ensure cells’ viability.

1.8*10^6 Bosc23 cells were plated on each 60mm plate with 4 ml of media 16 hr before transfection. For each plate, 5μg of DNA construct (MSVC) was dissolved in 438 ml ddH₂O. 62 ml CaCl₂, 500 ml 2x HEPES-Buffered Saline (HBS), and 1μg pClEco were bubbled into the solution in given order. 1ml solution was added to each plate and incubated for 9-12 hours. After the incubation period, the media was replaced with 3 ml of fresh media.

In 24 hours, the transfection was confirmed by detection of green fluorescence in cells under UV-light microscope. The retrovirus containing the media was collected at 48th hour. The cell debris was removed by spinning the media at 1200 rpm for 5 min and transferring the supernatant into a clean conical tube. The retrovirus was kept on ice and in dark until used.

C) Infections

NIH3T3 cell infections were used to match the retroviral titers. The cells were infected using the following procedure: 1*10^5 cells were plated on each 60 mm plate 12-16 hours before infections. The cells are incubated in 1 ml media, 1 ml retrovirus, and
8μg/ml polybrene for 4-6 hours. After the incubation period, the media is replaced with fresh media. At 48th hour, GFP expression of infected cells was measured by flow cytometry analysis.

32D Cl3 cells were infected by the spin-inoculation method. 1*10^6 cells, which were suspended in 1.5 ml media in a well of 6-well plate, were mixed with 1.5 ml retrovirus and 8μg/ml polybrene. The cells were centrifuged for 90 minutes at 1000rcf at 25°C, incubated for 4 hours and then the media was replaced with fresh media. At 48th hour, GFP expression was measured by flow cytometry.

D) DNA constructs

I used the MSCV constructs prepared by Catherine Spicer. MSCV construct contains retrovirus packaging genes, promoter regions, genes for bacterial transformation and cloning sites. A diagram of the P185 BCR/ABL MSCV construct is seen in Figure 7.

![Diagram of MSCV construct]

**Figure 7. A murine stem cell virus construct:** IRES allows co-expression of GFP and BCR/ABL by providing a second ribosome binding site for translation of the mRNA

Different BCR-ABL mutants have been inserted into MSCV vectors using the restriction enzyme sites (not shown in the figure). Internal ribosome entry site (IRES) allows translation of the second protein coding gene downstream from the one on a single mRNA by mimicking the 5' cap^28 for ribosome binding thus enabling co-expression of
GFP with BCR-ABL protein. Green fluorescent protein (GFP) is used to detect BCR-ABL+ cells. The constructs I used and their BCR/ABL region are shown in Table 1.

**Table 1: MSCV constructs**

<table>
<thead>
<tr>
<th>Name</th>
<th>Construct (5' → 3')</th>
<th>BCR/ABL region</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>MSCV-IRES-GFP</td>
<td></td>
</tr>
<tr>
<td>P185</td>
<td>MSCV-p185-IRES-GFP</td>
<td>BCR ABL</td>
</tr>
<tr>
<td>Y283F</td>
<td>MSCV-p185Y283F-IRES-GFP</td>
<td>BCR ABL</td>
</tr>
<tr>
<td>1-269</td>
<td>MSCV-p185Δ269-IRES-GFP</td>
<td>BCR</td>
</tr>
<tr>
<td>1-296</td>
<td>MSCV-p185Δ296-IRES-GFP</td>
<td>BCR</td>
</tr>
</tbody>
</table>

GFP served as a control in the experiments. In mouse experiments, P185 and 1-296 developed an MPD, while Y283F and 1-269 developed a mixed disease. These constructs were compared with each other to investigate why the disease phenotype differs.

Competent *Escherichia coli* was transformed to prepare more DNA constructs needed for experiments. 10µg of DNA was added to 50µl competent cells under sterile conditions. The cells were incubated on ice for 30 minutes and then in 42°C water bath for 2 minutes (heat shock). After adding 1 ml LB broth (Luria-Bertani media), the cells were incubated in 37°C water bath for 40 minutes. Cells were spun down at 5000 rpm for 2 minutes to remove excess LB broth and then plated on LB/Ampicillin plates.

The plates were incubated upside down at 37°C for 12 hours. A colony was then grown in 250 ml LB broth with 50 µg/ml ampicillin. High purity plasmid maxiprep
system (Marligen Biosciences, Inc.) was used to purify the vectors as described in the insert until the plasmid DNA precipitation step. At this point 10.5 ml isopropanol was added to the eluate. The solution was centrifuged at 15000g at 4°C for 30 minutes. After discarding the supernatant, the pellet was resuspended in 500 µl ddH₂O and transferred to an eppitube. 50µl sodium acetate and 1 ml 95% cold ethanol were added to the tube and the solution was centrifuged at 14000 rpm for 20 minutes. The supernatant was removed and DNA pellet was washed with 70% ethanol twice. The pellet was air-dried in a tissue culture hood until it became transparent and resuspended in sterile ddH₂O.

DNA concentration and purity (DNA/protein ratio) was measured using UV spectrophotometer. DNA is diluted 1:200 for OD260 and OD 280 readings. The concentration of DNA in µg/µl (mg/ml) was calculated by multiplying the OD260 reading by 10. OD260/OD280 ratio needed to be between 1.6 and 2.0, because below 1.6 there are not enough proteins to support the DNA and above 2.0 there is protein contamination. All DNA constructs I used had an OD260/OD280 in this range.

Purified DNA vectors were tested for the existence of certain restriction sites and certain length DNA pieces to confirm that the desired vector was purified. 1 µg of DNA, 1µg of restriction enzyme (Table 2), loading buffer and BSA (if needed) were added to

<table>
<thead>
<tr>
<th>Construct name:</th>
<th>Restriction enzyme:</th>
<th>Buffer:</th>
<th>Bands:</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>Eco/Not I</td>
<td>B3, BSA</td>
<td>1.3, 5.2 Kb</td>
</tr>
<tr>
<td>P185 and Y283F</td>
<td>Aat II/ Cla</td>
<td>B4, BSA</td>
<td>1.5, 2.7, 3.3, 4.2 Kb</td>
</tr>
<tr>
<td>1-296 and 1-269</td>
<td>Aat II</td>
<td>B4</td>
<td>1.4, 2.7, 7.0 Kb</td>
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</table>
30 μl water and put in a 37°C bath for 45-60 min. 5 μl loading buffer was added to samples prior to loading into 1% agarose gel with 5μl ethidium bromide. The voltage was set to 130V. Bands became visible under UV light as they separated from each other.

E) Flow cytometry

Flow cytometry measurements were done on a FACSCalibur machine to determine the percentage of BCR/ABL expressing cells in a culture. The cells were washed twice with PBS and suspended in 250 μl PBS containing 2.5 μg/ml propidium iodide (PI). PI labels dead cells and forward scatter is used to eliminate the debris29. FlowJo v.4.6 software was used to analyze the FACS data. PI vs. forward scatter analysis graph was used to choose live cell population, and GFP histogram was used to determine the percentage of GFP⁺ (and also BCR/ABL⁺) cells among the live population.

F) BCR/ABL infected 32D cell lines

Upon confirming GFP expression in infected 32D cells, the cells were sorted for GFP in Flow and Affymetrix Core Facility at Brandeis University. 800-1700 cells were sorted per construct and cells were grown in cultures containing 2μM Sti-571 kinase inhibitor (Gleevec by Novartis) which specifically inhibits the kinase activity of BCR/ABL. GFP expression of the grown cultures was confirmed to be higher than 95% by flow cytometry analysis.

G) 32D Growth curve

2*10⁴ cells/ml were seeded in 5 ml DMEM with 10% FBS (no Wehi3B or Sti-571) in triplicates. The cells were counted every day to observe the rate of growth.
**H) Immunoblots and cell lysates**

Sorted 32D cell lines were grown in media with Sti-571 to minimize the effect of BCR/ABL protein on cell signaling while establishing a cell culture. To examine each construct’s influence on cell signaling, whole cell lysates of the 32D cells were prepared 12-13 hours after removal of Sti-571 and Wehi3B from the media. This time span allowed BCR/ABL to change the signaling pathways at detectable levels.

The media was removed from cells and the cells were resuspended in 1x protein loading buffer with 100mM DTT to a concentration of $10^6$ cells/ml. The mixture was heated on a 100°C heat block for 5 min and centrifuged at 14000 rpm for 20 min. Lysates were aliquoted and used or stored in the -70°C fridge.

6-18% gradient polyacrylamide gels were used to separate the proteins. The voltage was set to 100V through stacking gel and to 150-165V through the gradient gel. The proteins were transferred to nitrocellulose paper either at 100V for 1.5 hours or 30V overnight in the cold room. After the transfer, Ponceau dye was added to the nitrocellulose paper to visualize the proteins and cut the blot at required molecular weight line; then the dye was washed away using 1x TBST.

The blots were blocked with 5% dry milk in TBST for 30 min at room temperature to prevent unspecific binding of antibodies. Blots were washed with the primary antibody added to the 5% milk overnight in the cold room (see Table 3 for antibody details). The next day, the blots were washed with TBST for 20 minutes three times to clear the primary antibody. Horseradish peroxidase (HRP) labeled secondary antibody in 5% milk was added to blot for a 45-60 min wash at room temperature, and
then the blots were washed again with TBST for 20 minutes three times. SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) was poured on each blot for 5 minutes so they would gain chemiluminescense for developing. Several exposures of the blots were taken. In most cases, total proteins were blotted for after stripping and reprobing the blots with non-phospho specific antibodies.

Table 3: Antibodies and their dilutions used for immunobloting

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary antibody and concentration</th>
<th>Secondary antibody and concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCR-ABL</td>
<td>anti- Ab-3: 1/500</td>
<td>Mouse(^1): 1/5000</td>
</tr>
<tr>
<td>Dynamin</td>
<td>anti- Dynamin: 1/1000</td>
<td>Mouse(^1): 1/10000</td>
</tr>
<tr>
<td>Actin</td>
<td>anti- Actin: 1/20000(^3)</td>
<td>Mouse(^1): 1/10000</td>
</tr>
<tr>
<td>Stat5</td>
<td>anti- p- Stat5: 1/2000; anti- Stat5: 1/1000</td>
<td>Mouse(^1): 1/5000</td>
</tr>
<tr>
<td>ERK 44/42 (MAPK)</td>
<td>anti- p- ERK: 1/1000; anti- ERK: 1/1000</td>
<td>Rabbit(^2): 1/5000</td>
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<td>AKT</td>
<td>anti- p- AKT: 1/1000; anti- AKT: 1/1000</td>
<td>Rabbit(^2): 1/5000</td>
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<tr>
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<td>Rabbit(^2): 1/5000</td>
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<td>anti- myc: 1/1000</td>
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<td>anti- p53: 1/25</td>
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<td>anti- GFP: 1/3000</td>
<td>Mouse(^1): 1/5000</td>
</tr>
</tbody>
</table>

\(^1\) Southern Biotech, HRP Goat anti Mouse IgG 1 mg/ml; \(^2\) Southern Biotech, HRP Goat anti Rabbit IgG 1 mg/ml; \(^3\) 1/10000 of the diluted actin antibody
When there was a significant difference in protein activation, the blot was analyzed using NIH image 1.63. The “Analyzing Electrophoretic Gels” technique from NIH Image website was used to quantify the bands of the blots. After doing a linear calibration with lightest and darkest point of the graph, the bands of the blot were analyzed.

1) Soft agar bone marrow colony assay

Transformation potentials of different BCR/ABL constructs were measured with soft agar bone marrow colony assays. The assays were conducted under different conditions to reveal if the oncogene has different effects on myeloid and lymphoid cells. Student’s t test was used to test if the results were statistically significant.

Bone marrow colony assay under myeloid conditions:

Five days before bone marrow isolation, donor mice were injected with 0.2-0.3 ml 5-fluorouracil (5-FU). 5-FU kills dividing cells and thus eliminates the early progenitor cells. As a result, the stem cells start to divide to make up for the lost cells and the stem cell population is enriched. The next day, Bosc23 cells were transfected, and on the third day NIH3T3 cells were infected with retrovirus for determining the viral titer. On the day of bone marrow isolation, infected NIH3T3 cells were analyzed with flow cytometry for the percentage of GFP+ cells to match the viral strengths. Bone marrow was isolated from leg bones of mice under sterile conditions (approximately 2*10^6 cells/mice).

7.3 ml media cocktail (4.5 ml DMEM, 0.117 ml PSA, 0.117 ml L-glut, 1.71 ml FBS, 508 µl Wehi3B, 5.4 µl IL-3, 14.6 µl IL-6, 321 µl stem cell factor (SCF), and 9.4µl polybrene) was added to every 10*10^6 bone marrow cells. The cells were divided equally
for each type of retrovirus and titer-matched retrovirus was added to media to a retrovirus:media ratio of 4.2:7.3. The cells were incubated overnight. NIH3T3 cells were also infected with titer-matched retrovirus to compare the actual viral strengths. Following day the media containing the retrovirus was removed; fresh media and retrovirus were added in the same amounts for another overnight incubation. On the last day, the media was removed, the cells were resuspended in DMEM with 20% FBS and 2x P/S and counted for soft agar plating (see below for procedure). For each construct, 2*10^5 cells/well were plated in triplicates.

**Bone marrow colony assay for lymphoid conditions:**

Retrovirus was prepared and the titers were matched as described above. Bone marrow cells were isolated from non-5-FU treated mice (approximately 50*10^6 cells/mice). 1 ml of RPMI with 20% Wehi3B, 20% FBS, 100 µl IL-7, 30 µl polybrene and 2x P/S was added to every 10*10^6 cells. Cells were divided equally between the constructs and retrovirus was added in 1:1 ratio. Cells were centrifuged at 1000rcf (1500 rpm) at 25°C for 90 min, incubated for 90 min and then counted for soft agar plating (see below for procedure). For each construct, 1*10^6 cells/well and 5*10^5 cells/well were plated in triplicates.

**Soft agar plating:**

1.2% bactoagar was prepared (100 ml ddH₂O and 1.2g bactoagar) and autoclaved the day before plating. It was melted in microwave and placed in 42°C water bath for an hour. At this temperature 1.2% bactoagar stays liquid and is cold enough not to damage the media or the cells. 2x media was prepared and warmed in the same bath. For myeloid conditions 2x media contained 2x DMEM, 40% FBS, 4x P/S, and 5mM β-
mercaptoethanol, and for lymphoid conditions it contained 2x RPMI, 40% FBS and 5mM β-mercaptoethanol. Then 1x media with 0.6% bactoagar was prepared by mixing the 2x media and 1.2% bactoagar with a 1:1 ratio, and this media was kept in 42°C bath when not used.

Infected bone marrow cells were suspended in 4.5 ml of 1x media at desired concentrations. Each well in a 6-well plate was first covered with 1x media with 0.6% bactoagar. Then 4.5 ml of 1x media with 0.6% bactoagar was added to the cells, mixed thoroughly and divided equally between 3 wells. After an hour of solidification, the plates were put in the incubator and the colonies were counted in 10-12 days.
RESULTS

BCR/ABL P185 and 1-296 transform bone marrow cells at different rates than Y283F and 1-269 in-vitro under myeloid conditions

Biological studies with mouse models showed that tyrosine 283 influences the disease phenotype. Absence of the tyrosine 283 changes the disease from a CML-like myeloproliferative disorder to a mixed disease with MPD and B-cell-ALL. I compared the myeloid transformation abilities of the wild-type and mutant BCR/ABL genes in primary cells from mouse bone marrow to reason the difference in disease phenotype. Bone marrow was collected from 5-FU treated donor mice. 5-FU treatment kills the dividing cells and promotes the stem cells to divide thus enriching the HSC population in the bone marrow. The collected cells were infected under conditions that supported myeloid differentiation and growth with the presence of cytokines IL-3, IL-6 and stem cell factor (SCF).

Primary cells have been infected with titer matched retrovirus as described in materials and methods. Titers were matched using pre-titer FACS data and virus concentrations that lead to approximately 25-40% GFP+ cells in NIH3T3 cells. NIH3T3 infections for actual titers matched closely as well.

After the infections, 2*10^5 cells per well were plated in soft agar in the absence of cytokines. 10 days later transformation rates were compared by the numbers of colonies formed. Bone marrow cells infected with the GFP construct did not form any colonies. Under conditions that supported myeloid lineage growth, BCR/ABL P185 and 1-296 transformed the cells at a significantly higher rate than Y283F and 1-269 (Figure 8).
Table 4 includes the number of colonies counted in each well, their average, the standard deviations and errors. Student's t test results support that the difference between the number of colonies between P185 and Y283F wells and between 1-296 and 1-269 wells were statistically significantly (both P values are less than 0.0001). These results suggest that P185 and 1-296 have a greater potential to transform cells into myeloid lineages than Y283F and 1-269. Same pattern was observed in previous experiments (data not included) that gave out fewer colonies.

<table>
<thead>
<tr>
<th></th>
<th>GFP</th>
<th>P185</th>
<th>Y283F</th>
<th>1-296</th>
<th>1-269</th>
</tr>
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<tbody>
<tr>
<td>Well #1</td>
<td>0</td>
<td>493</td>
<td>338</td>
<td>564</td>
<td>366</td>
</tr>
<tr>
<td>Well #2</td>
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<td>485</td>
<td>346</td>
<td>586</td>
<td>379</td>
</tr>
<tr>
<td>Well #3</td>
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<td>330</td>
<td>546</td>
<td>372</td>
</tr>
<tr>
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<td>495</td>
<td>338</td>
<td>565</td>
<td>372</td>
</tr>
<tr>
<td>Standard deviation</td>
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</tr>
<tr>
<td>Standard error</td>
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<td>6</td>
<td>5</td>
<td>12</td>
<td>4</td>
</tr>
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</table>

Student's t test's P value: 1.69*10^{-5} 4.61*10^{-5}
Figure 8. Results from soft agar colony assay under myeloid conditions: (a) Under myeloid conditions, $2 \times 10^5$ infected bone marrow cells from 5-FU treated mice formed different number of colonies in each construct. There is a significant difference in transformation rates by P185 and Y283F and by 1-296 and 1-269. GFP is not sufficient to induce colony formation. (b) Pictures of a sample well from the colony assay.

**BCR/ABL P185, Y283F, 1-296 and 1-269 transform bone marrow cells at similar rates in-vitro under lymphoid conditions**

Primary cells from the bone marrow of non-5-FU treated mice were used to study the lymphoid lineage transformation potentials of different constructs. These primary
cells consist of HSC, common myeloid and lymphoid progenitors and other more
differentiated cells. These cells were infected and incubated in media containing the
cytokines IL-3 and IL-7, which promotes proliferation of lymphoid cells. These two
cytokines are not sufficient to promote the myeloid lineages; therefore the results of this
experiment reflected the transformation rates of lymphoid cells.

Primary cells from bone marrow were infected with titer matched virus (same
virus as for the myeloid condition). 5*10^5 cells/well were plated in soft agar in the
absence of cytokines. Colonies were counted on day 10 (Table 5). Bone marrow cells
infected with the GFP construct did not form any colonies. Transformation rates were
almost the same for each construct (Figure 9). According to Student’s t test, the wells do
not show a statistically significant difference in number of colonies formed by each
construct. On day 10 some wells had several large colonies, while others had fewer large
colonies and more small ones (data not shown).

Table 5: Number of colonies formed in the bone marrow colony assay under lymphoid
conditions

<table>
<thead>
<tr>
<th></th>
<th>066</th>
<th>P185</th>
<th>Y283F</th>
<th>I-296</th>
<th>I-269</th>
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<tbody>
<tr>
<td>#1</td>
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<tr>
<td>#2</td>
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<td>34</td>
<td>37</td>
</tr>
<tr>
<td>#3</td>
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<td>25</td>
<td>24</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td>Average</td>
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<tr>
<td>Standard deviation</td>
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<tr>
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<td>5</td>
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</tr>
</tbody>
</table>

Student’s t test’s P value: 0.35 0.40
Figure 9. Results from soft agar colony assay under lymphoid conditions: (a) $5 \times 10^5$ infected bone marrow cells were plated in soft agar under lymphoid conditions. There is no significant difference between transformation rates of P185 and Y283F and of 1-296 and 1-269 under lymphoid conditions. GFP is not sufficient to induce colony formation. (b) Not all the colonies are visible in these pictures as the colony sizes varied from small to large.
GFP sorted 32D cell lines expressing BCR/ABL P185, Y283F, 1-296 and 1-269 
proliferate at similar rates

Having studied a feature of leukemogenesis biology, I decided to study the 
biochemical role of tyrosine 283 as well. For these experiments, I chose to use the 32D 
cells, which form a homogenous myeloid progenitor cell culture. 32D cells are dependent 
on interleukin-3 (IL-3) for survival and growth. BCR/ABL expression overcomes the 
dependence on IL-3.

I prepared 32D cell lines expressing GFP, P185, Y283F, 1-296 and 1-269 
constructs at similar levels in order to compare their growth rates and affects on signaling 
pathways. To create the cell lines, wild-type 32D cells were infected with MSCV virus 
constructs and grown for a week with Wehi3B and Sti-571. Sti-571 is a kinase inhibitor 
that suppresses the oncogenic activity of BCR/ABL by blocking the ABL kinase. 
Wehi3B is a source for IL-3 and is necessary for supporting the cell growth and survival.

One week after the infections, the GFP+ cells were sorted out. Figure 10a shows 
the pre-sorting GFP histogram of infected cells and the GFP expression level chosen to 
sort the cells (red lines). Figure 10b shows the post-sorting GFP histogram from cell 
cultures after a week. These cells were grown with Sti-571 to prevent cells with oncogene 
from proliferating rapidly and accumulating additional mutations and with WEHI3B. The 
data shows that the sorting was successful; the culture had 99% BCR/ABL+ cells and 
GFP expression levels of the cells were similar.
Figure 10. FACS analysis of retrovirus infected 32D cell lines: FACS analysis of 32D cells before (a) and after (b) sorting. The sorting was successful and created cell lines expressing GFP at similar levels.

Sorted 32D populations were seeded in equal numbers into DMEM with FBS but no kinase inhibitor (Sti-571) or Wehi3B to observe the rate of cell proliferation. The control cells with GFP died out quickly because they needed the IL-3 stimulation (WEHI3B). BCR/ABL containing cells were not dependent on IL-3 and proliferated at very similar rates (Figure 11). I stopped counting on the third day when they became over-confluent.

Similar rates of proliferation of 32D cells expressing BCR/ABL P185, 1-296, Y283F or 1-269 suggest that these forms of BCR/ABL do not have any proliferative advantage over each other, at least under in vitro conditions. Therefore tyrosine 283 may not be playing a significant role in promoting cell proliferation in vitro.
Figure 11. Growth curve of 32D cell lines: The results show that cells infected with BCR/ABL P185, Y283F, 1-296 and 1-269 proliferate at the same rate in-vitro.

BCR/ABL P185 and 1-296 activates Stat5 and rpS6 more than Y283F and 1-269 in 32D cell lines.

32D cells were used to study the protein activation, because the homogeneity of the cell culture allowed accurate comparison of different constructs. Primary cells from bone marrow are heterogeneous, and the different signaling patterns occurring in one cell type may not be observable in a diverse population of cells.

Immunoblots were used to investigate the altered signal transduction due absence of tyrosine 283 of BCR/ABL. Whole cell lysates prepared from sorted 32D cell lines were run on a 6-18% polyacrylamide gel. The proteins were then transferred on a nitrocellulose paper and the blot was probed for several antibodies. Figure 12 shows the results of several immunoblots.
Dynamin and actin were used as loading controls. Two sets of 32D lysates were prepared in order to repeat significant results observed with one lysate. *In-vitro* phosphotyrosine profiles of the four forms of BCR/ABL are very similar. The ABL kinase activity is evident as the phosphorylated tyrosine levels are higher in all BCR/ABL expressing cells compared to the GFP control. There are two proteins at around 60kDa and 110 kDa (shown with arrows) that are activated differently P185 and Y283F BCR/ABL compared to 1-269 and 1-296. Previously it has been suggested that the lane around 60 kDa corresponds to phosphorylation of p62<sup>DOK</sup> protein<sup>2</sup>. I have found no information on the lane around 110 kDa. I did not study these differences any further as they did not show the same pattern as disease phenotype changes and are probably due to missing domains after 296<sup>th</sup> residue.

Several signaling proteins were probed for to determine if any of the signaling pathways downstream of BCR/ABL has been activated differently in BCR/ABL genes missing the Y283. Of the proteins studied, there were only two proteins that showed different phosphorylation levels in the same pattern as the disease phenotype. P185 and 1-296 was shown to activate Stat5 and rpS6 more than Y283F and 1-269 do.

There was no significant difference in activation of the other proteins in downstream pathways: AKT, Erk 1/2, Mek 1/2, RAF1, PDK, mTor, JNK, c-myc, p53 and PI3K.
Figure 12. Immunoblots of 32D whole cell lysates: Activation of signal transduction proteins were compared with immunoblots of 32D whole cell lysates. Phosphorylated protein levels are labeled with a prefix “p-” and total protein levels are labeled only with the protein name. Raf1, Erk 1/2, Mek 1/2, PDK, AKT, mTor, JNK, c-myc and p53 showed similar levels of activation in four forms of BCR/ABL. Stat5 and rpS6, however, are upregulated more in BCR/ABL constructs with tyrosine...
DISCUSSION

In my experiments, I used *in-vitro* methods to determine why BCR/ABL Y283F and 1-269 cause a mixed disease (B-ALL and MPD) while BCR/ABL P185 and 1-296 cause an MPD. Transformation potential of an oncogene is a combination of its influence on cell proliferation, differentiation and adhesion properties. According to the previous mouse experiments, tyrosine 283 may be associated with signaling pathways that promote BCR/ABL to transform the pluripotent stem cells into myeloid lineages only\(^2\). It was not clear why there was no lymphoid lineage expansion with P185 BCR/ABL in mouse models.

One of the several possible explanations is that tyrosine 283 may associate with targets that induce myeloid cell transformation strongly or suppress lymphoid cell transformation. The same effect could be true for lineage commitment. Another possibility is that tyrosine 283 promotes signals that cause a high rate of proliferation in myeloid lineages and a rapid disease development before the lymphoid lineages had a chance to expand. This study suggests that tyrosine 283 does not have an effect on proliferation rates, that it is necessary for activation of signaling pathways associated with Stat5 and rpS6, and that it favors myeloid lineage transformation but does not influence lymphoid lineage transformation.

**Tyrosine 283 contributes to the potential of BCR/ABL to induce myeloid lineage transformation, but not lymphoid lineage transformation**

Bone marrow colony assays compared the transformation potentials of the four BCR/ABL constructs. The results suggest that the tyrosine 283 has an important role in
myeloid lineage transformation. When bone marrow cells from 5-FU injected donor mice were infected with our constructs, P185 and 1-296 transformed more cells into colonies than Y283F and 1-269 under conditions that supported myeloid differentiation and not lymphoid (due to cytokine and media content). Presence of tyrosine 283 is shown to increase the rate of transformation by 1.5 fold when compared to BCR/ABL constructs missing it.

On the contrary, when the bone marrow cells from non-5-FU injected mice were infected with our constructs and cells were infected and plated under conditions that would support lymphoid differentiation and not myeloid, the cells transformed into colonies at rates not significantly different from each other. This result suggests that tyrosine 283 does not suppress the lymphoid differentiation. This data is consistent with the results of previous experiments that showed that BCR/ABL P185 may also cause a mixed disease if the viral titers are very low.26

**Tyrosine 283 contributes to signaling events activating Stat5 and rpS6, which may be important for myeloid disease development**

Tyrosine 283 increases the Stat5 activation in the 32D cell lines. Stat5's role has been previously identified in myeloid cell differentiation32 and establishment of CML by BCR/ABL.33 It is a necessary cytokine for myeloid differentiation 34. Its role in myeloproliferative disease development has also been shown previously in studies comparing BCR/ABL and v-Abl. V-Abl is the oncogenic form of the cellular Abelson kinase and induces pre-B cell leukemia in mice. It was shown that BCR/ABL induced
higher Stat5 activation than v-Abl in NIH3T3 cells\textsuperscript{32}. Stat5 is often associated with other myeloproliferative diseases as well\textsuperscript{17}. The explanation behind the correlation of myeloid expansion and Stat5 activation may be Stat5’s role in induction of anti-apoptotic and proliferative signals\textsuperscript{32}.

Stat5 does not have a known binding site on BCR/ABL, however it is established that BCR/ABL activates Stat5. In future protein binding assays may be used to determine how Stat5 and tyrosine 283 of BCR/ABL are connected and if there are intermediate proteins. These studies will help determine further the role of Stat5 in CML and other myeloproliferative diseases.

Results from the experiments measuring growth rate of 32D cell lines suggest that the tyrosine 283 does not have an influence on proliferation potential \textit{in-vitro} in the 32D cells. Cells expressing P185 and 1-296 grew at similar rates as Y283F and 1-269 \textit{in-vitro} in the absence of cytokine (IL-3) signaling. Therefore the effect of Stat5 may be anti-apoptotic more than proliferative. Though, it is possible that the influence of tyrosine 283 could not be observed because the cell line is already transformed. Further studies are needed to come to a concrete conclusion about the different proliferation potentials of these constructs and Stat5’s role in it.

I observed a significant activation difference of ribosomal protein S6 (rpS6) between P185 and Y283F and between 1-296 and 1-269 as well. RpS6 is phosphorylated and activated by the S6 kinase which is under the regulation of mTor and AKT pathways\textsuperscript{36}. RpS6 influences the cell size, glucose homeostasis and protein synthesis within the cell\textsuperscript{36}. The role of rpS6 in neoplastic growth is not yet fully determined. It is possible that rpS6 increases the cell proliferation rate and anti-apoptotic signals leading to
a faster progression of the disease, thus not permitting the lymphoid disease to rise. As a result, myeloid cells dominate and an MPD is observed.

There are many other proteins under the control of S6 kinase which phosphorylates rpS6. A difference in rpS6 activation is an indication for increased S6 kinase activity. Therefore in the future it is important to investigate if any other downstream targets of S6 kinase are influenced by the absence of Y283. Identification of gene transcription factors or other factors that may contribute to leukemogenesis under S6 kinase control would be important in depicting the role of Y283. Moreover, S6 kinase may link us to other neoplastic myeloid transformation factors, which may be found upstream of S6 kinase and downstream of BCR/ABL.

It is important to note that the cells of different lineages are under the control of different signaling events and that the intracellular wiring of signal transduction pathways may vary in different blood lineages. My experiments suggest that BCR/ABL contributes to rapid transformation of myeloid lineage with activation of Stat5 and rpS6, but these proteins are not as important for lymphoid lineage transformation. When the progression of myeloid disease is slowed down due to dampening signals downstream of tyrosine 283 deficient BCR/ABL, lymphoid cells have a chance to expand and thus a mixed disease with B cell and immature myeloid cell expansions is observed in mouse models.
ACKNOWLEDGEMENTS

I dedicate this work to my parents, Aybek and Turgut, who have been the greatest source of inspiration and cheeriness throughout my life

"Let the beauty of what you love be what you do." Rumi

My countless thanks go to Dr. Ruibao Ren and Dr. Jaime Acquaviva. They have made this very valuable experience possible.

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Finally I want to remember, thank and wish a peaceful journey to all the mice that have been sacrificed to increase our knowledge and understanding of life.
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