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Functional Genomic and Proteomic Analysis of Highly Drug Resistant Chronic Myeloid Leukemia

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Dedication Page

I dedicate this thesis to three important people in my life. The first two being both my parents David and Yvette Oaxaca, for their endless love and support. They have provided me lifelong lessons of how we must work hard for everything we want in life. I also dedicate this thesis to my best friend, and girlfriend, Sarah Chenausky who always displayed patience, enduring love, and belief in me like no other.

FUNCTIONAL GENOMIC AND PROTEOMIC ANALYSIS OF HIGHLY DRUG RESISTANT CHRONIC MYELOID LEUKEMIA

By

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Table of Contents

List of Figures

List of Tables

Abstract

Chronic Myeloid Leukemia (CML) has served as a model for how cancer can be selectively targeted with advanced forms of chemotherapies known as kinase inhibitors. Such new therapies have significantly altered the field of oncology and have led to monumental increases in both survival rates and quality of life for cancer patients. However, approximately one-third of CML patients will go on to develop drug resistance against major kinases inhibitors. Along with this, other cancers are showing increased incidences of drug resistance. Understanding how these cancers, such as CML, overcome drug sensitivity is of major importance within the clinic. To predict potential cell signaling pathways utilized in drug resistant CML, we generated a highly drug resistant T315I BCR-Abl mutant CML cell line from the already established KCL-22 cell line. With our established cell line we analyzed the activity of several cell signaling pathways that serve roles in controlling cellular proliferation, cellular differentiation, and cell death. In contrast to the wild type KCL-22 cell line, we were able to identify MEK1/2 and mTOR dependency within the drug resistant mutant. These findings allowed us conclude that FDA approved kinase inhibitors Trametinib (a MEK1/2 inhibitor) and Temsirolimus (an mTOR inhibitor) may hold therapeutic value in selectively targeting T315I BCR-Abl CML tumor cells. Lastly, using a series of bioinformatics tools such as PROVEAN and SIFT, we analyzed the genomic landscape of both the wild type and artificially established drug resistant CML mutant in order to identify genetic alterations that may be associated with drug insensitivity. From this genomic analysis we were able to identify several novel mutations within the drug resistant mutant that occur within key regulatory sites of various cell signaling molecules. In conclusion, CML drug insensitivity can be overcome by targeting downstream

targets of BCR-Abl such as MEK1/2 or mTOR, and various mutations besides the T315I BCR-Abl variant may contribute to drug insensitivity.

Chapter I: General Introduction

1.1 Cancer and Society

Cancer is a major cause of death worldwide (U.S. Cancer Statistics Working Group, 2013), and is the leading cause of death for Hispanics (Kochanek, 2009). Sophisticated statistical studies supported by the World Health Organization predict cancer to continue to be amongst the top leading causes of global mortality from present day to the year 2030 (Mathers, 2006).

Although trends in cancer rates are predicted to increase, deaths due to cancer are decreasing in developed countries. This decrease is largely due to medical advances, such as the use of tyrosine kinase inhibitors (TKIs) in the treatment of cancer. However, leukemia has been identified as a leading cause of childhood deaths in both developed and underdeveloped countries (Chatenoud, 2010).

Within the United States, cancer rates continue to rise. The National Institutes of Health (NIH) has estimated that $86.6 billion dollars is spent annually on cancer treatment in the United States. Of the $86.6 billion dollars, cancer treatment expenditures in Texas alone account for nearly $7.7 billion dollars, with the Upper Rio Grande and West Texas regions accumulating $417 million dollars in cancer treatment per year. Limiting unnecessary costs on failed treatments, while producing more effective treatments for patients, has become a central issue for both state and national authorities. In El Paso County alone, there were an estimated 3,071 new cancer cases, and 1,063 cancer deaths for 2013. Leukemia and Lymphomas are amongst the leading childhood and adolescent cancers in El Paso (Texas Department of State Health Services, 2013).

These findings have emphasized the importance for further investigation into the exact mechanisms that cause and drive cancer, and more importantly, the underlying causes of drug resistant cancers. With the discovery of Imatinib, chronic myeloid leukemia has served as a model for understanding how a cancer can be selectively targeted. Along with being a pioneer of

personalized cancer treatment, chronic myeloid leukemia has also served as a model for how cancer can evolve (Melo, 2007). Since the newer generation of TKIs are showing promise for cancer treatment, it is also important to attempt to identify as many pathways these small molecule inhibitors act on. Along with this statement, **it is also important to identify any genetic aberrations that may lead to the production of neoplasms. Developing new ways of screening cancer cells against various drugs, and finding ways to navigate the cancer genome,** are of utter importance for the progression of successful new cancer treatment.

1.2 Cancer and Cell Signaling

Within the healthy individual, various cell types are constantly utilizing a vast array of signaling networks in order to maintain homeostasis. Proper functioning of these networks relies on the activity of key proteins. Aberrant changes within the amino acid sequence can lead to alterations in structure and dysfunction of the protein. Such changes can lead to abnormal signaling networks within the cell.

Cancer cells must develop the ability to escape immune surveillance as well as sustain rapid replication. Neoplasms are capable of performing such tasks through the utilization of intracellular pathways such as the JAK/STAT, MAPK/ERK, PI3K/Akt, and TGF-β pathways. Each pathway has it's own function in disease progression, and in some cases is utilized more frequently throughout different cancers (Dreesen, 2007).

Much remains to be discovered about these mechanisms, however the past ten years has seen significant advancements in cancer research. Such research has established a centralized rationale of what characteristics abnormal cells must possess to be classified as cancer. These characteristics, or "Hallmarks of Cancer", are traits that allow the cancer cell to flourish **(Figure 1.1)**. The first of the six accepted cancer traits is the ability to resist cell death. This can result from

various alterations of the PI3K/AKT pathway which drive increased proliferation and pro-survival signals. Cancers that rely heavily on aberrant PI3K/AKT pathway signaling include: lymphoma, thyroid cancer, ovarian cancer, and renal-cell carcinoma (Vivanco, 2002). Additionally, the cancer cell must establish proliferative signaling while at the same time achieve replicative immortality and evade growth suppression or death by the immune system. These three hallmarks are seen in leukemia/lymphoma phenotypes due to the alteration of transmembrane proteins such as NOTCH receptors, and others alike (Aster, 2011). Furthermore, the cancer cell must activate a system that will allow itself to spread (metastasize) to other parts of the body, and establish a source of nutrients by inducing angiogenesis. This aspect of cancer is least understood, but new research continues to highlight the use of cytokines in metastatic progression. (Hanahan, 2011).

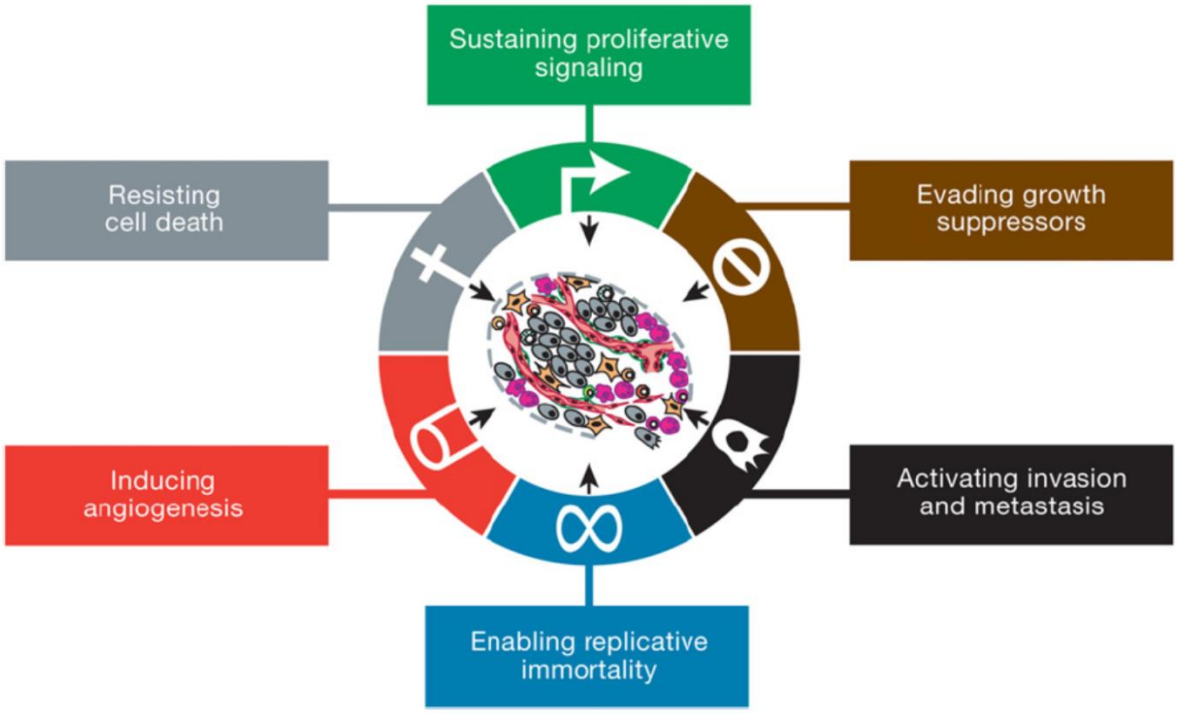


Figure 1.1 Six Hallmark Characteristics of a Cancer Cell. Requirements for a cell to be considered cancerous six hallmarks must be accomplished. (Hanahan, 2011).

1.2.1 Kinase inhibitors and Cancer

The disease specific kinase inhibitors have to offer in treating cancer highlights the importance to continue screening novel compounds for cancer therapy use. A major advancement for cancer treatment was the development of chemotherapy. However, many of the drugs used for conventional chemotherapy have limited success and are not selective in treating cancer. A major flaw in this form of chemotherapy is its inability to discriminate between normal and abnormal cells. This limitation associated with chemotherapy treatment results in the patient experiencing negative side effects. Realizing the limitations of chemotherapy, cancer scientists and oncologists have developed ways to selectively target aberrant cell signaling networks present in cells. Such devices include the use of antibodies, antisense, inhibitors of growth factor receptors and tyrosine kinases. Tyrosine kinases serve major roles in normal and cancerous cell growth. Many pathways utilize tyrosine kinase signaling in order to carry out specific functions. Kinases are enzymes that can range from peptide to lipid molecules that are capable of transferring a phosphate group from adenosine triphosphate (ATP) or guanosine triphosphate (GTP) to the hydroxyl side chain of serine, threonine, or tyrosine residues on target proteins. Transfer of phosphate groups provides the energy needed to drive the signaling process. Since tyrosine kinases serve such important roles in these cancers, scientists have developed therapies that are moderately selective in inhibiting aberrant tyrosine kinase function. One success of these new chemotherapies developed includes that of a BCR-ABL tyrosine kinase inhibitor, imatinib mesylate (Gleevec, Novartis). Gleevec was the pioneer of targeted cancer treatment, and has had major success in treating BCR-ABL positive cancer patients (Aora, 2005). Sorafenib (Nexavar; Bayer Pharmaceuticals) is another landmark for cancer treatment utilizing kinase inhibitors. Sorafenib was developed in 1994 after a large drug screen and was shown to inhibit the proteins Raf-1 and B-Raf. Unlike imatinib, sorafenib has been

shown to block the autophosphorylation of several receptor tyrosine kinases (VEGFR1, 2, and 3, PDGFRβ, c-Kit, and RET), and is used mainly for the treatment of advanced renal cell carcinoma (Wilhelm, 2007).

Since the installation of the tyrosine kinase inhibitor imatinib, many other kinase inhibiting molecules, including sorafenib, have been developed. Since 2001, more than 10,000 kinase inhibitor patents have been filed. Problems that persist in using kinase inhibitors for cancer treatment are that many cancers utilize more than one kinase to survive. Additionally, the networks used by these cancers are also used by normal cell types, which can lead to drug toxicity side effects when targeted for cancer treatment by kinase inhibitors. To avoid drug toxicity, kinase inhibitors must offer high selectivity toward their target. This highlights the importance of screening novel compounds for cancer therapy use. Along with this, screening pharmaceutically established compounds for "off label" use against certain cancers is an area that is beginning to experience growing interest. Developing methods for targeting multiple signaling networks without interfering with heavily trafficked networks is now a major focus of cancer research (Knight, 2010).

1.3 Hematologic Malignancies

Hematologic malignancies are cancers of the blood. The three major types of hematologic malignancies include leukemia, lymphoma, and plasma cell neoplasms, the largest being leukemia. Leukemia can be divided into two categories: lymphocytic or myeloid, depending on which blood cells are impacted. The major disease categories for these diseases include B-, T- acute or chronic lymphocytic leukemia (B-ALL and T-ALL), and acute or chronic myeloid leukemia (AML and CML). According to the National Cancer Institute, an estimated 48,610 leukemia cases will be diagnosed this year in the United States. These new cases will be an addition to the 287,963 people

already living with leukemia (Howlader, 2013). Such high numbers of leukemia cases give credence to the importance of conducting cancer research aimed at improving treatment. Importantly, developing cancer treatments that are selective in their targeting of cancer phenotypes is greatly needed.

These cancers can arise from the bone marrow or from the lymphatic system in response to internal triggers or environmental factors. While the innate immune system is designed to detect and destroy abnormal cells, escape from natural killer (NK) cell surveillance is a main method of hematologic malignancy. NK cells are part of the innate immune system and have anticancer properties. Usually upon recognition of an abnormal cell, the NK cell has the ability to lyse the abnormal cell, and prevent it from further differentiating into other cancerous cells. Avoidance of NK cell surveillance is possible through rapid expansion of cancer cells, which is largely seen in hematologic malignancies. NK cells are heavily researched today due to their potential roles as a cancer therapy (Farnault, 2012).

The internal drivers of hematologic malignancies are the subject of innumerable investigations. For example, the JAK family has been found to play central roles in the development of certain types of leukemia (Scott, 2012). Mutations in key proteins, such as JAK2, are seen in chronic myeloid leukemia and are involved in the mechanism by which the cells sustain uncontrolled cellular proliferation. In addition to leukemia, JAK proteins have been found to serve major roles in other hematological malignancies (Chen, 2012). Another cell survival pathway frequently used by hematological malignancies includes mTOR, an intracellular serine/threonine kinase that functions downstream of many pathways. One of the pathways that activate mTOR includes the PI3K pathway, which leads to the modulation of the apoptosis pathway (Younes, 2011; Green, 2011).

1.4 The Immune System

The ratio of microbes to human beings is overwhelmingly large. Every day the human body fights off potentially dangerous microbes. Although this task seems cumbersome, it is accomplished almost effortlessly by a sophisticated system. The human immune system is an integrated network comprised of cells called leukocytes, collectively known as white blood cells (WBCs). Two major divisions of WBCs exist and belong to either the lymphoid or myeloid lineages. These lineages work together in order to maintain an effective immune response against disease. Any imbalance in this system can lead to the human body being susceptible to pathogenic threats. All WBCs are derived from pluripotent hematopoietic stem cells present in the bone marrow. Through various stimuli, these pluripotent stem cells give rise to other types of stem cells that are limited in their differentiating ability. These derivations of the pluripotent stem cells are the lymphoid and myeloid progenitors. Unlike lymphoid progenitors, myeloid progenitors produce cells that serve roles in other functions in addition to immune processes. Briefly, myeloid progenitors give rise to megakaryocytes, erythroblasts, and granulocytes. These three divisions of myeloid progenitors are involved in platelet and red blood cell formation, and immune responses, respectively. The myeloid lineage gives emergence to many white blood cells that are vital for innate immunity (Murphy, 2008).

1.4.1 The Myeloid Lineage

Currently the accepted model for hematopoiesis suggests that all blood cells are derived from a single hematopoietic stem cell (HSC). Cytokines and growth factors present in the bone marrow (BM) stimulate these HSCs allowing them to differentiate into two distinct lymphoid or myeloid branches. Although the exact mechanisms of this process are debatable, new evidence

highlights interconnecting signaling networks shared between HSC progenitors and mature leukocytes. (Dorshkind, 2010),

On a daily basis, nearly 1-trillion blood cells arise in the BM. These cells are further divided into their specialized roles, depending on the physiological requirements of the individual. The specialized roles can range from immunity to oxygen transportation. Assigning such roles depends on the chemical environment present within the BM and the hematopoietic stem cell's surface expression of certain receptors. The presence of such receptors dictates which lineage (lymphoid or myeloid) the progenitor cells differentiate into. HSCs lose their differentiating ability during their first division, giving rise to multipotent progenitors (MPPs). These MPPs can then be stimulated to form either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). CLPs give rise to T, B, and natural killer (NK) cells, which serve roles in memory (T and B lymphocytes), and innate immune functions (NK cells). The CMP route is more complex, and includes short lived cell types such as granulocytes, erythrocytes, and megakaryocytes (Doulatov, 2012; Iwasaki, 2007).

In the myeloid lineage cell surface markers such as lin-IL7Rα-Kit+Sca-1-CD34+FcγRlo, dictate which MPPs will differentiate into CMPs. These progenitors can in turn give rise to granulocyte-monocyte progenitors (GMPs), which are the primary producers of the myeloid lineage (Friedman, 2007). As hematopoiesis is the term for the development of all blood cell types, myelopoiesis is the term used to describe the formation of all myeloid lineage leukocytes. An immense amount of signaling networks are involved throughout myelopoiesis, and the same amount of signaling pathways are also present during the natural and abnormal functioning of the myeloid leukocyte (Huber, 2012). Understanding the normal process of development that occurs throughout hematopoiesis is crucial to investigating how cancers affecting the blood can arise.

1.5 Chronic Myeloid Leukemia

Myeloid malignancies are clonal diseases of hematopoietic stem cells or their progenitors. These malignancies have genetic alterations that allow for rapid self-renewal, proliferation, and differentiation (Murati, 2012). Chronic myeloid leukemia is a myeloproliferative disease characterized by clonal expansion of transformed, primitive hematopoietic progenitor cells (Faderl, 1999). Chronic myeloid leukemia (CML) has three different phases, which are the accelerated (AP), chronic (CP), and blast phases (BP) (Scholar, 2007). Of these three phases, the blast phase is least understood, and the prognosis in this phase is life expecting. A diagnosis of CML is made when the Philadelphia chromosome is identified along with excessive immature granulocytes present in the patients collected peripheral blood. The large majority of CML patients are diagnosed during the chronic phase of the disease (Melo, 2007). Disease progression into advanced stage CML (blast phase) is poorly understood and is frequently seen in CML patients who develop resistance to cancer treatment. CML patients that enter blast crisis during the course of the disease have a 20% survival rate (Radich, 2007).

Although there has been an extensive amount of research conducted on CML, more research is needed to identify the underlying mechanisms of disease progression and to develop better treatments for the advanced stages of CML.

1.5.1 BCR-ABL Signaling

In 1960, (Nowell et al.) a study consisting of seven chronic granulocytic leukemia (CGL) patients provided results of a recurring minute chromosome, later named the Philadelphia (Ph) chromosome. This study provided the first results of a consistent chromosomal abnormality

associated with a specific type of leukemia, and was a major advancement for cancer research (Deinger, 2000).

Thirteen years after identifying the Ph+ chromosome, a study involving nine CML patients identified a translocation between the long arms of chromosome 9 and chromosome 22. This translocation was concluded to be the primary cause of the Ph+ chromosome (Rowley, 1973). The human cellular homologue (c-abl) of the Abelson (ABL) murine leukemia virus was found to be the gene that was translocated in the Ph+ chromosome (de Klein, 1982). Studies involving CML patients determined that the c-abl gene was specifically involved in Ph+ chromosome CML patients (Bartram, 1983). At approximately the same time, another study by Groffen et al., identified a breakpoint domain present on chromosome 22 in 19 CML patients. This breakpoint domain was then termed "breakpoint cluster region" (BCR). Half a decade later, further research concluded that increased tyrosine kinase activity involving the Ph+ chromosome product protein, BCR-ABL, was present in virtually every chronic myeloid leukemia patient (Daley, 1990; Lugo, 1990).

C-ABL **(Figure 1.2a)** has been found to be involved in cellular functions that govern growth and differentiation. The protein c-ABL is ubiquitously expressed, and is normally found migrating between the nucleus and cytosol. However, c-ABL remains confined to the cytosol when it is fused with the BCR protein **(Figure 1.2b).** The exact mechanisms of the BCR protein by itself are not yet fully understood, but like c-ABL, BCR is also ubiquitously expressed. ABL kinases have been known to serve roles in other cancers, however, only in leukemia cancers is c-ABL fused with BCR to form the constitutively active BCR-ABL protein **(Figure 1.2c)** (Greuber, 2013).

A result of the BCR to ABL fusion is the constitutive activation of the ABL kinase protein. Three different BCR-ABL proteins have been identified and include P210, P185, and P230. The three proteins differ in the numbers of BCR coding sequences present in the fusion protein. P210 has been identified as the hallmark BCR-ABL protein form active in CML. BCR-ABL remains active because BCR fusion prevents ABL kinase auto-inhibition capability (Greuber, 2013). This constant activated state of BCR-ABL present in the cytosol leads to activation of other proteins and ultimately to cytokine independence. The proteins that are frequently activated by BCR-ABL also serve as important intermediates in pathways such as the Ras/Raf/Mek/Erk, PI3K/Akt, SFK, SAPK, and JAK/STAT pathways **(Figure 1.3).** Adapter molecules such as Grb-2 can lead to recruitment of Sos proteins, which are capable of activating Ras via utilization of GTP (Pendergast AM, 1993). Other known substrates of BCR-ABL known to activate the Ras pathway are Shc and CrkL. CrkL is a tyrosine kinase with SH domains that are capable of binding active and inactive ABL proteins (Oda, 1994). JNK/SAPK signaling is also utilized by BCR-ABL signaling along with the combinational use of the p38 protein. These pathways have been shown to serve important roles in BCR-ABL's transforming capabilities (Deininger, 2000). The exact mechanisms of how BCR-ABL utilizes the Ras/Raf/Mek/Erk pathway are not fully understood.

The JAK/STAT pathway has also been found to be effected by BCR-ABL. BCR-ABL-transformed cell lines such as the K562 leukemia cells show constitutive activation of STAT5a/b and STAT3 (de Groot, 1999). Other studies involving multiple cell lines have shown that STAT proteins do not rely on JAK activation, but may be directly regulated by BCR-ABL. Along with these findings studies have also shown that the activated JAK/STAT pathway in BCR-ABL positive cells does not require IL-3 or GM-CSF for activation (Chai, 1997). Kabarowski et al. showed that this growth factor independence may be due to BCR-ABL activation of Ras.

Establishing multiple ways of sustaining cytokine independent cell growth is necessary for any cancer, and is seen clearly in BCR-ABL signaling.

The PI3K pathway is a pathway that regulates apoptosis. In BCR-ABL positive cell lines, increased BCR-ABL activity leads to elevated PI3K activity. This effect is mediated through Cbl proteins along with the previously mentioned CrkL adapter proteins. A downstream target of the PI3K pathway is the protein Bad, which is responsible for apoptosis initiation. In BCR-ABL mutated cell lines, Bad has been shown to be inactive. Such results prompted Keeshan et al. to investigate the effects of BCR-ABL on Bad functions. Their results showed that cells expressing high levels of BCR-ABL were able to mimic IL-3 stimulation, and prevent Bad from performing it's apoptotic function (Deininger, 2000; Keeshan, 2002).

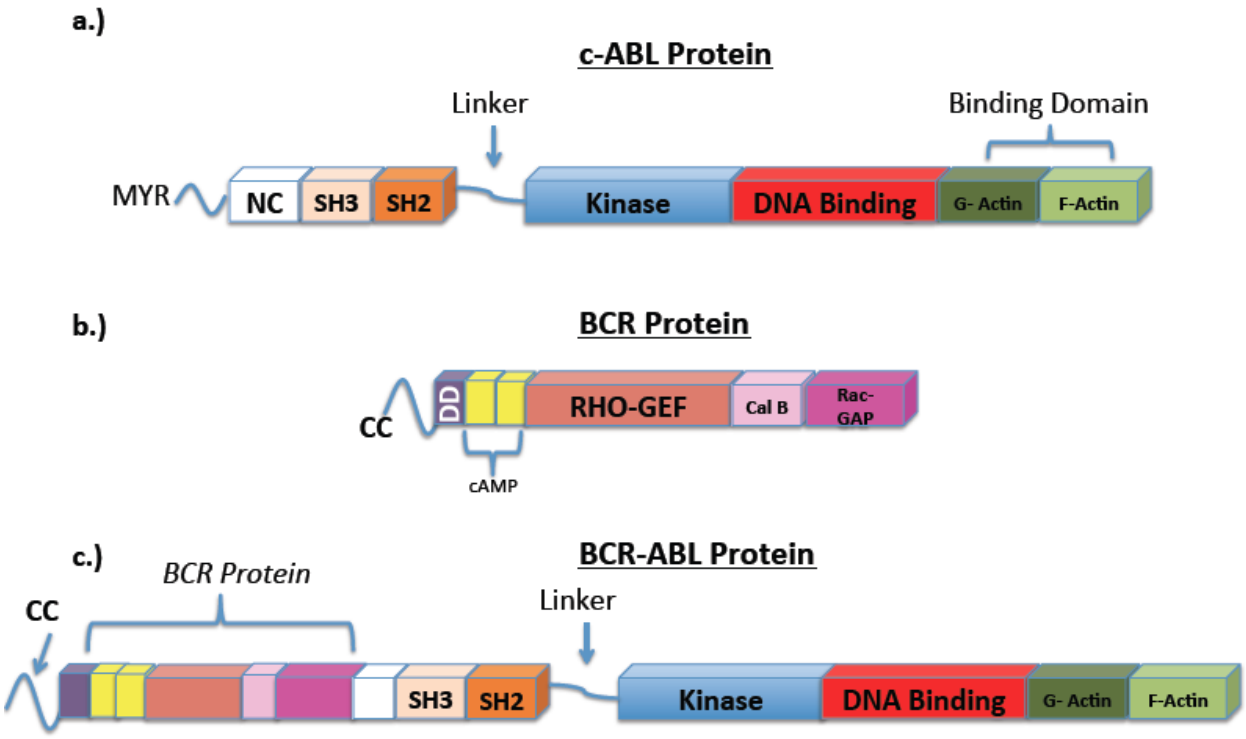


Figure 1.2 Structure of BCR-ABL Protein. a.) ABL Protein - SRC homology domains SH3 & SH2 are located near the NH_2 terminus (Orange & Peach respectively). These domains allow for interactions between other proteins. Linker region connects kinase domain (blue) with SH2/SH3, and serves a role in regulating autophosphorylation. N-terminal cap NC (white) with myristoylation (MYR) serves dual roles in membrane localization and kinase activity. Near the 3' end, nuclear localization signals, DNA-binding, and actin-binding motifs are found (red and green respectively). **b.)** BCR Protein – A coiled-coiled domain is found at the N-terminal end, which serves a role in dimer formation. Dimerization domain (DD, purple) along with two cyclic adenosine monophosphate (cAMP) kinase homologous domains (yellow) are utilized in Grb-2 binding, which plays a role in activating the Ras pathway. The RHO-GEF center of the molecule is involved in Rho guanidine exchange factors, and acts in activating transcription factors such as NF-κB. The c-terminus has a Rac molecule, which also serves a purpose in activating the Ras pathway (Deininger, 2000). **c.)** BCR-ABL Protein – Myr on ABL is replaced with BCR, causing fusion between BCR and ABL. The fusion of the proteins causes a large portion of the N-cap region to be removed keeping regions of the BCR and ABL proteins intact (Panjarlan, 2013).

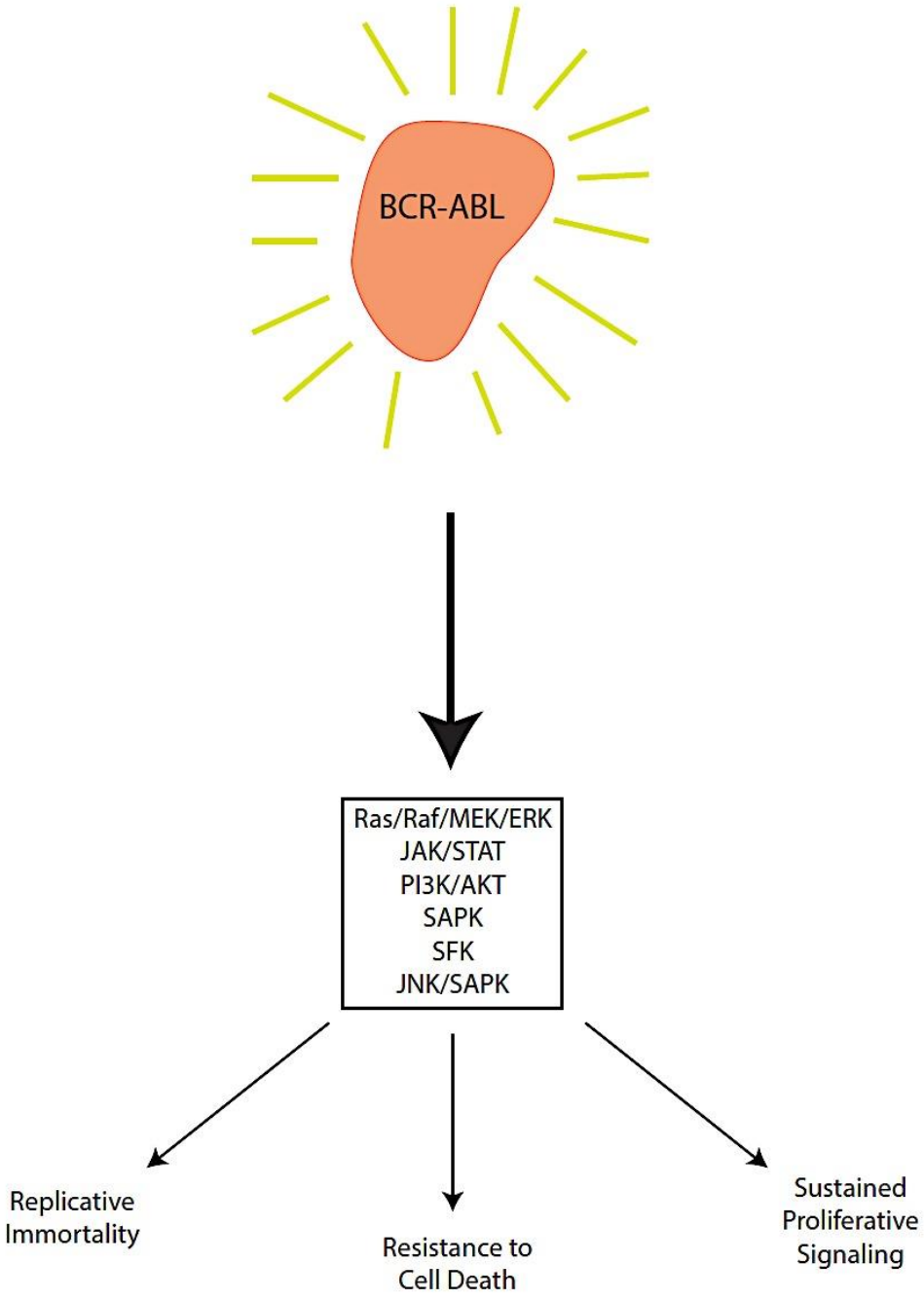


Figure 1.3 Constitutive activation of BCR-Abl influences activity of multiple pathways. BCR-Abl is a constitutively activated tyrosine kinase with dual kinase activity that allows it to modulate various signaling cascades within the cell.

1.5.2 BCR-ABL Independent Pathways

Besides BCR-ABL, other mutations can lead to a CML phenotype. One particular oncogenic mutation occurs in the fms-related tyrosine kinase 3 (FLT3) receptor. During blast crisis in CML patients, FLT3 has been shown to be overexpressed. This mutation has been found to lead to increased cellular proliferation by activating downstream signaling cascades such as the ones already mentioned (Small, 2006; Weisberg, 2010) FLT3 mutations are heavily investigated for their role in drug resistant CML and AML. Particularly in AML, FLT3 inhibition has shown to be an effective way of stopping disease progression (Leung, 2013). The JAK2 V617F mutation is another BCR-ABL independent pathway seen frequently in CML patients. This mutation results in a loss of autoinhibitory function of JAK2 leading to increased activation. In patients with this mutation, downstream targets such as STAT5 and STAT3 are found to be constitutively activated (Staerk, 2012; Scott 2012). Another BCR-ABL independent pathway found in CML patients involves the Tel-Platelet-derived Growth Factor Receptor β (Tel-PdgfR β). This pathway is rarely seen in CML patients but deserves mentioning because of its ability to achieve constitutive tyrosine phosphorylation. Knowledge of this pathway's exact mechanisms in sustaining CML disease is unknown, however, studies have shown Tel-PdgfR β to have influence on PI3K signaling, and regulation of transcription (Huang, 2012).

1.5.3 Current Therapies in the Treatment of CML

Due to the discovery of Imatinib's capability to inhibit BCR-ABL tyrosine kinase activity through binding of the ATP pocket domain, CML treatment has forever changed. Prior to the tyrosine kinase inhibitor era, the main treatment for CML was interferon therapy, and prior to that was standard chemotherapy (busulfan and hydroxyurea). Interferon therapy works by targeting

CML stem cells, but is mainly beneficial for patients in the early stages of the disease (Talpaz, 2013). Although tyrosine kinase inhibitors have shown promise in improving the prognosis of CML patients, approximately one-third of CML patients will develop resistance to Imatinib (Marin, 2013). Such incidences of drug resistance has prompted researchers to find new TKIs capable of inhibiting these types of cancers.

Second generation TKIs such as Dasatinib (Sprycel, Bristol-Myers) and Nilotinib (Tasigna, Novartis) were developed soon after the discovery of drug resistant CML patients. Drugs such as Dasatinib have the ability to target multiple kinases at low concentrations and thus are considered pan-kinase inhibitors. However, the full spectrum of TKI activity remains to be discovered, and is being studied using methods that measure the activity of many different cellular pathways simultaneously (Karaman, 2008; Rix, 2007).

Since 2001, more than 15 kinase inhibitors have been approved by the FDA for treatment of various cancers. One persistent problem is that many cancer patients do not respond to second generation TKIs. Patients, who have exhausted all options of cancer treatment, usually end up receiving hematopoietic stem cell transplantation. Although transplantation is a cure for CML, the problem of finding matching donors for CML patients is a significant limiting factor (Pavlu, 2011).

In addition to treating CML with these small molecule inhibitors, they also show promise in selectively treating other cancers. **The importance of identifying enzymes, such as kinases, that serve active roles within various drug resistant cancer signaling networks, along with identifying genetic aberrations that drive the malfunctioning of such proteins, makes this research a priority.**

Chapter II: Signaling Networks within Myeloid Cells

In order to understand the mechanism by which BCR-Abl is capable of sustaining cytokine independent growth, a brief overview of the common signaling pathways within myeloid cells is necessary.

2.1 JAK-STAT Pathway

During a normal physiological state, myeloid and monocyte differentiation is governed by hematopoietic cytokines. Janus tyrosine kinases (JAKs) and signal transducers and activators of transcription (STATs) are proteins intricately involved in cytokine stimulated intracellular signaling (Liu, 1998).

To date, seven STAT proteins have been identified, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Along with the STAT proteins, four members of the JAK family (JAK1, JAK2, JAK3, and Tyk2) have also been discovered (Leonard, 1998).

JAKs have seven JAK homology (JH) domains (JH1-JH7). Each domain has its own unique role in the catalytic activity of the protein. STATs also have special domains present within their protein structure that dictate their interactions with other intracellular signaling networks. For example, each STAT molecule contains six domains that are required for the transactivation and DNA binding property of the protein. (Ross, 2007). Stability of such domains is required for the proper functioning of the cell. For example, mutations within the transactivation domain of STAT5a can lead to increased activation. Gu et al demonstrated that the amino acid variant phenylalanine replacing serine at amino acid position 710 promotes migration and invasion of prostate cancer cells, which is a prerequisite for metastases.

Cytokine initiated JAK/STAT signaling pathways are orchestrated through various cytokine receptors. Each receptor activates a specific set of JAKs and STATs, and does so through the previously described domains that are present in these proteins (JH domains of JAKs; SH2 and

other domains of STATS). Receptors associated with activating JAK proteins are the interferon (IFN) and interleukin (IL) receptors. The cytokine activated cascade in the JAK/STAT pathway begins with the docking of a cytokine (interferon or interleukin) to its respective receptor **(Figure 2.1).** Docking of the cytokine then leads to dimerization of the two receptors while simultaneously recruiting JAK proteins to the cytoplasmic domains of the receptors (Kirken, 1994; Ihle 1997).

Instantaneously, when the two receptors dimerize, the receptor bound JAK proteins autophosphorylate each other. Once phosphorylated, STAT docking sites are formed on the surface of the interleukin receptors. In close proximity, STAT proteins interact with the phosphorylated JAK proteins via their SH2 domain, and then become phosphorylated. After the STAT proteins are phosphorylated they form dimers with other phosphorylated STAT proteins present in the cytoplasm. The activated STATs translocate to the nucleus of the cell where they interact with specific binding sites present on DNA. The nuclear activity of dimerized STAT proteins subsequently leads to gene transcription within the cell (Levy, 2002; Schindler, 2007).

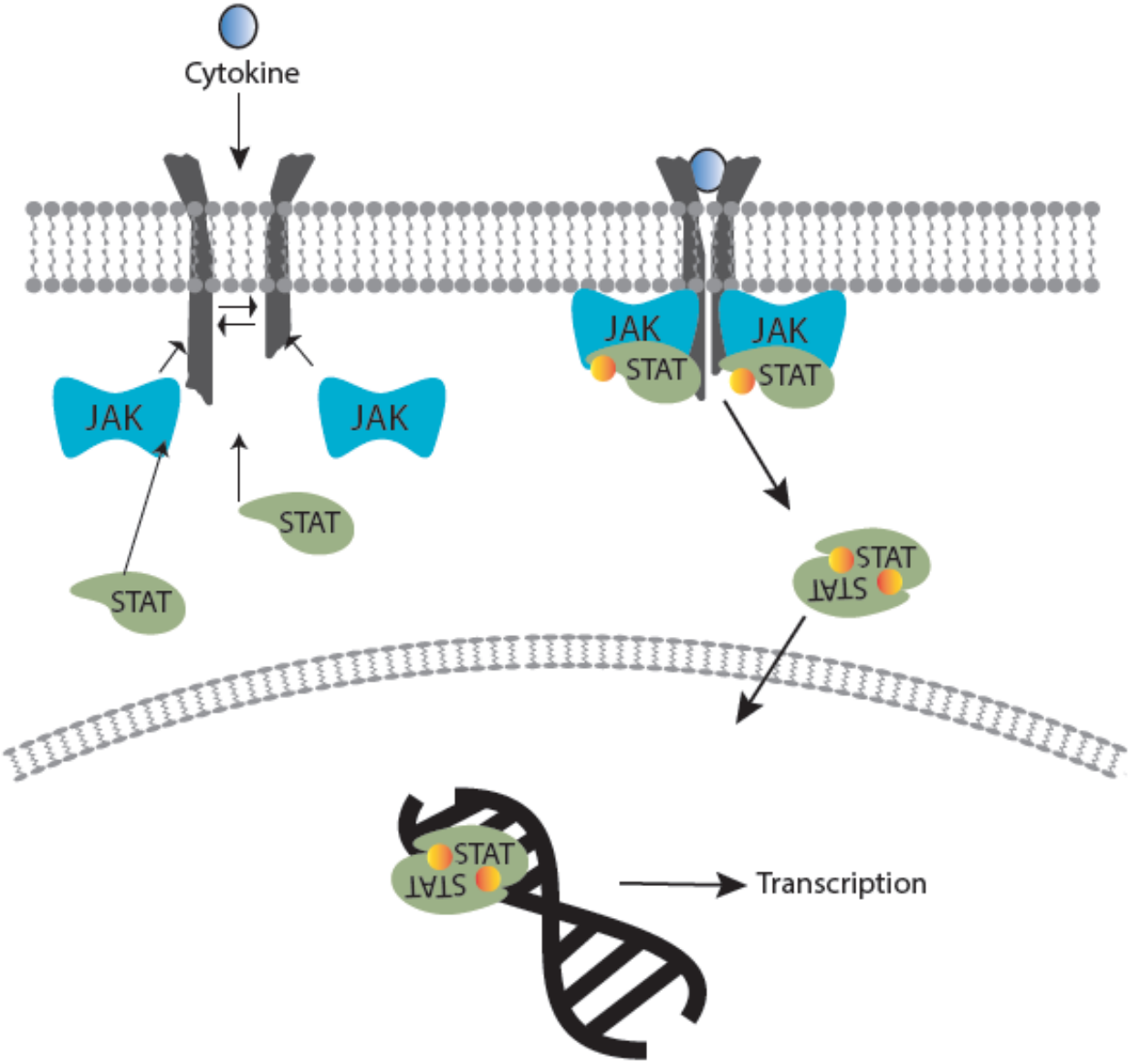


Figure 2.1 Schematic representation of the JAK/STAT pathway. Cytokine/growth factor docking to it's respective receptor is essential for the activation of the pathway. Different cytokines/growth factors (IL-2, IL-3, and G-CSF) recruit JAK proteins, and lead to the activation of Stat proteins. Activated Stat proteins then translocate to the nucleus and bind DNA sites to activate transcription.

Within neutrophilic and granulocytic precursor cells, the JAK/STAT pathway is utilized in cell development. Granulocyte colony-stimulating factor (G-CSF) regulates the proliferation and differentiation of neutrophilic-granulocyte precursor cells present in the bone marrow. G-CSF receptors share similarities with interleukin recepters. *Tian et al*. showed that STAT3 and STAT5 are utilized in G-CSF initiated signaling. Other studies have shown STAT3 and STAT5 have roles in controlling myeloid differentiation through other receptor systems such as signal transducing subunit gp130 and erythropoietin receptor signaling. The latter system is primarily involved in erythroid cell differentiation. In addition to cell development, STAT5 also serves an important role in myeloid cell survival (Smithgall, 2000).

2.2 Src Family Kinases (SFKs)

Src proteins were the first type of protein kinases to be discovered that showed phosphorylation at tyrosine residues, giving rise to a new division of protein kinases. Src proteins contain regions of homology that have been coined Src homology (SH) domains. Along with these SH domains, Src proteins also contain a catalytic domain where the activating tyrosine residue resides (Martin, 2001).

SFKs include the Src, Lyn, Fyn, Yes, Fgr, Hck, Blk, Zap-707, and Yrk non-receptor protein tyrosine kinases that respond to various stimuli including growth factors, cytokines, and steroids. SFKs remain in close proximity or bound to the plasma membrane of the cell. Fgr and Hck kinases are the major SFKs expressed within myeloid cells. Hck inhibits expansion of the myeloid progenitor pool, and works with Fgr to promote survival and function of differentiated myeloid cells. SFK may control functions that govern cytoskeleton rearrangement, cell motility, cell adhesion, translation, metabolism, proliferation, and cell survival within myeloid cells (Huber, 2012).

Activation of SFKs is carried out by receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs). Once SFKs are activated they can initiate various signaling cascades **(Figure 2.2).** In myeloid cells, SFK can initiate phosphorylation of STAT proteins, specifically STAT3. Along with direct activation of certain proteins, SFKs initiate activation of Ras/Raf/MEK/ERK, PI3K/AKT, and PKC signaling cascades (Miranda, 2007). SFKs are involved in many processes, and are ubiquitously expressed in many cell types. As you will see later, SFKs also play vital roles in cancer progression.

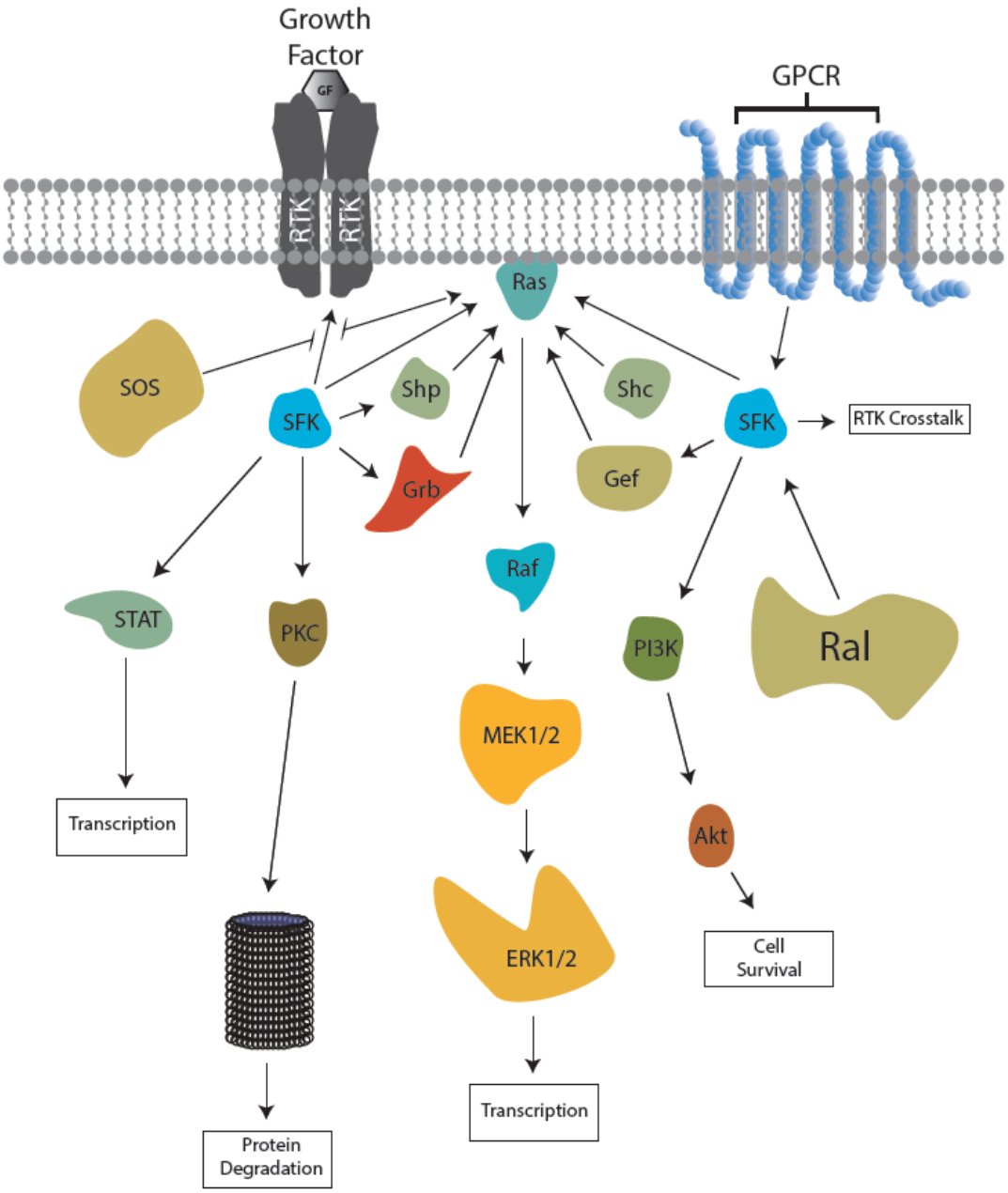


Figure 2.2 Src Family Kinases (SFKs) Signaling Networks. SFK regulate multiple pathways. SFKs (blue colored proteins) activate pathways that lead to cell survival, gene transcription, and protein degradation. In this figure SFKs interact with PKC signaling to initiate protein degradation via proteasomes. SFKs are also depicted interacting with STAT signaling and the Raf/MEK/ERK pathway to activate transcription. SFKs also interact with PI3K networks to regulate apoptosis.

2.3 Ras Signaling

The Rat sarcoma protein (Ras) family consists of a large number of similar structured GTPase proteins that are bound to the plasma membrane of the cell. The primary Ras GTPase proteins are the H-, K-, N-, E-, R-, and M-Ras proteins and are activated by mitogens, cytokines, and growth factors. Ras proteins function in cellular proliferation and cell cycle progression through transcription, translation, and degradation of key cell-cycle components **(Figure 2.3).** In human hematopoietic cells, progenitor cells of the myeloid lineage rely largely on the activity of H-Ras (Huber, 2013).

The mammalian cell cycle consists of four phases: the first gap phase (G1 phase), the DNA synthesis phase (S phase), the second gap phase (G2 phase), and mitosis (M phase). Transition between phases is policed in part by cyclin-dependent kinases (CDKs) along with their regulatory subunits, cyclins throughout Ras signaling. Two major cyclin subunits are involved in the Ras signaling network, D- and E-type cyclins. Each cyclin has it's own unique feature on it's respective CDK. These features can range from activation to inhibition of other proteins. A major downstream target of Ras GTPases is the MAPK signaling cascade, which includes RAF, MEK, MAPK, and ERK proteins. Through the latter pathway, GTP bound Ras is able to regulate G1-through S-phase cell cycle progression. One major function for Ras proteins is the inhibition of the retinoblastoma protein (RB) through the activation of the RAF-MEK-ERK/MAPK pathway. RB is an important growth suppressing protein, and inhibiting it's activity can lead to cellular proliferation and increase the likelihood of cancer. Other targets of Ras proteins include phosphatidylinositol 3-kinases in which cellular apoptosis can be regulated. Finally, Ras proteins can also target a family of GTPases that contribute largely to cell-cycle progression, RAL proteins. (Coleman, 2004).

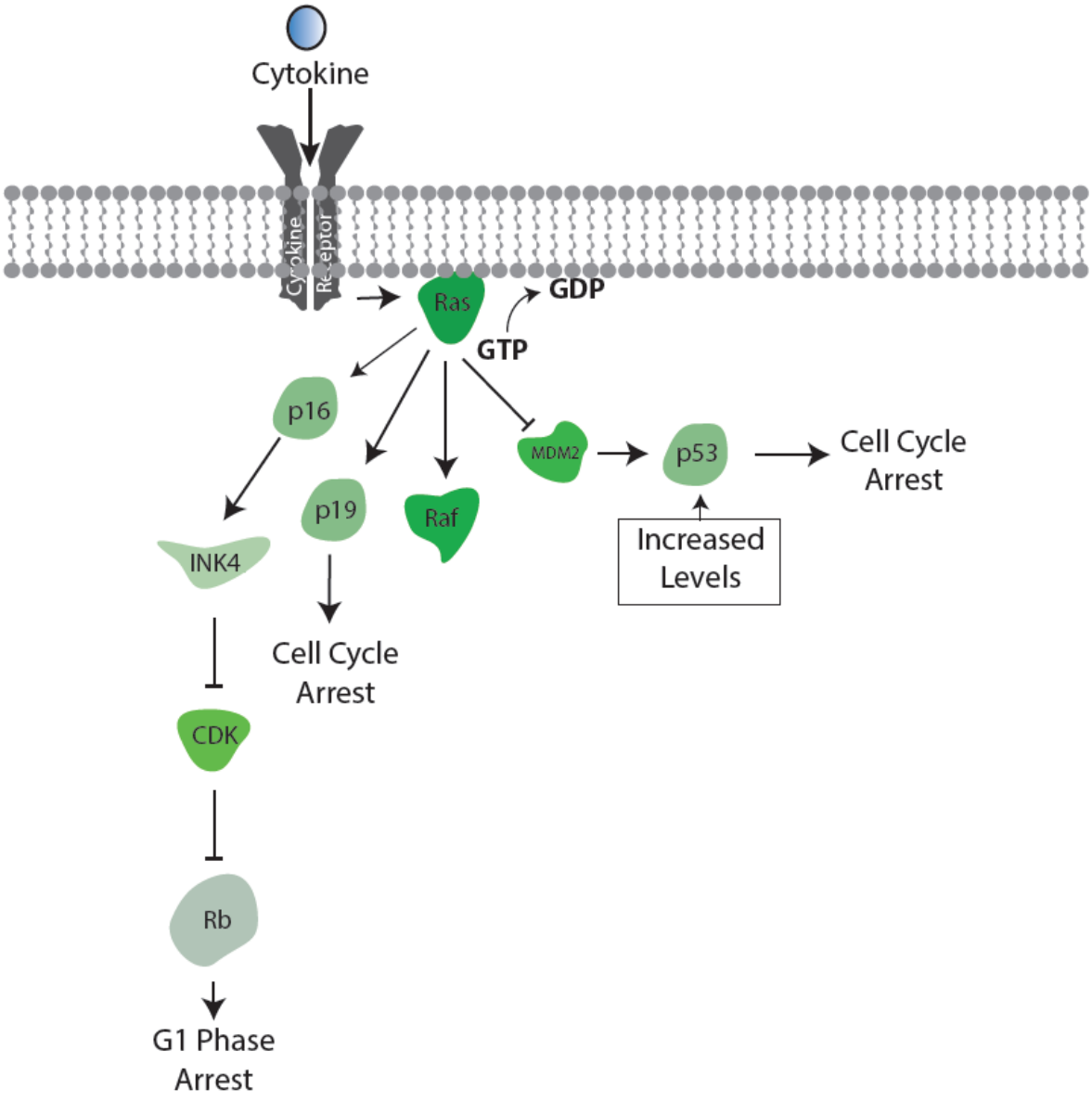


Figure 2.3 Ras Signaling Pathway. Ras carries out its GTP initiated activity at the cell membrane. Four different paths can lead to cell cycle arrest at different phases of the cell cycle. For example, Ras can activate p16 which can then activate a downstream target INK4 which prevents INK4 from activating CDK and RB, ultimately leading to G1 phase arrest. Another route includes inhibiting MDM2 which leads to the accumulation of p53 within the cell, and eventually to cell cycle arrest.

2.4 Raf/MEK/ERK Pathway

As mentioned previously, the Raf/MEK/ERK pathway is an important downstream signaling cascade that is targeted by RAS GTPase proteins. The Raf family is composed of three members: A-Raf, B-Raf, and C-Raf. Each isoform contains three conserved regions: CR1, CR2, and CR3. These three regions serve roles in Ras-binding, apoptosis regulation, and kinase activity respectively **(Figure 2.4)**. Mutations within Raf proteins, such as the V600E B-Raf mutant, have contributed to the prevalence of many cancers, the most common being melanoma (Sun, 2014). Although the Raf proteins are major targets for the Ras GTPase protein, they do not require Ras to be activated. However, Ras proteins do recruit Raf proteins towards the cellular membrane, and allow Raf to interact with other proteins along the way. Mitogen-activated protein kinase/ERK kinase proteins (MEK) are the downstream targets for Raf proteins. MEK proteins are part of the larger family of mitogen-activated protein kinases (MAPK) that are major players in pathways that regulate embryogenesis, cell differentiation, proliferation, and death. MEK proteins are also capable of phosphorylating serine/threonine and tyrosine residues, making them dual specificity kinases (Pearson, 2001).

The MEK family consists of five different proteins: MEK1, MEK2, MEK3, MEK4, and MEK5. In myelopoiesis, MEK1/2 are the primary acting MEK proteins. Currently the only known substrates for MEK are the four members of the extracellular signal-related kinase (ERK) family: ERK 1/2, JNK 1/2/3, p38 MAPK, and ERK5. MEK1/2 proteins primarily interact and activate ERK1/2 proteins, which in turn transition into the nucleus to initiate proliferative signals. Interleukin 3 (IL-3), G-CSF, and granulocyte macrophage colony-stimulating factor (GM-CSF) are all initiators of the RAF/MEK/ERK pathway in myeloid lineage cells (Chung, 2011).

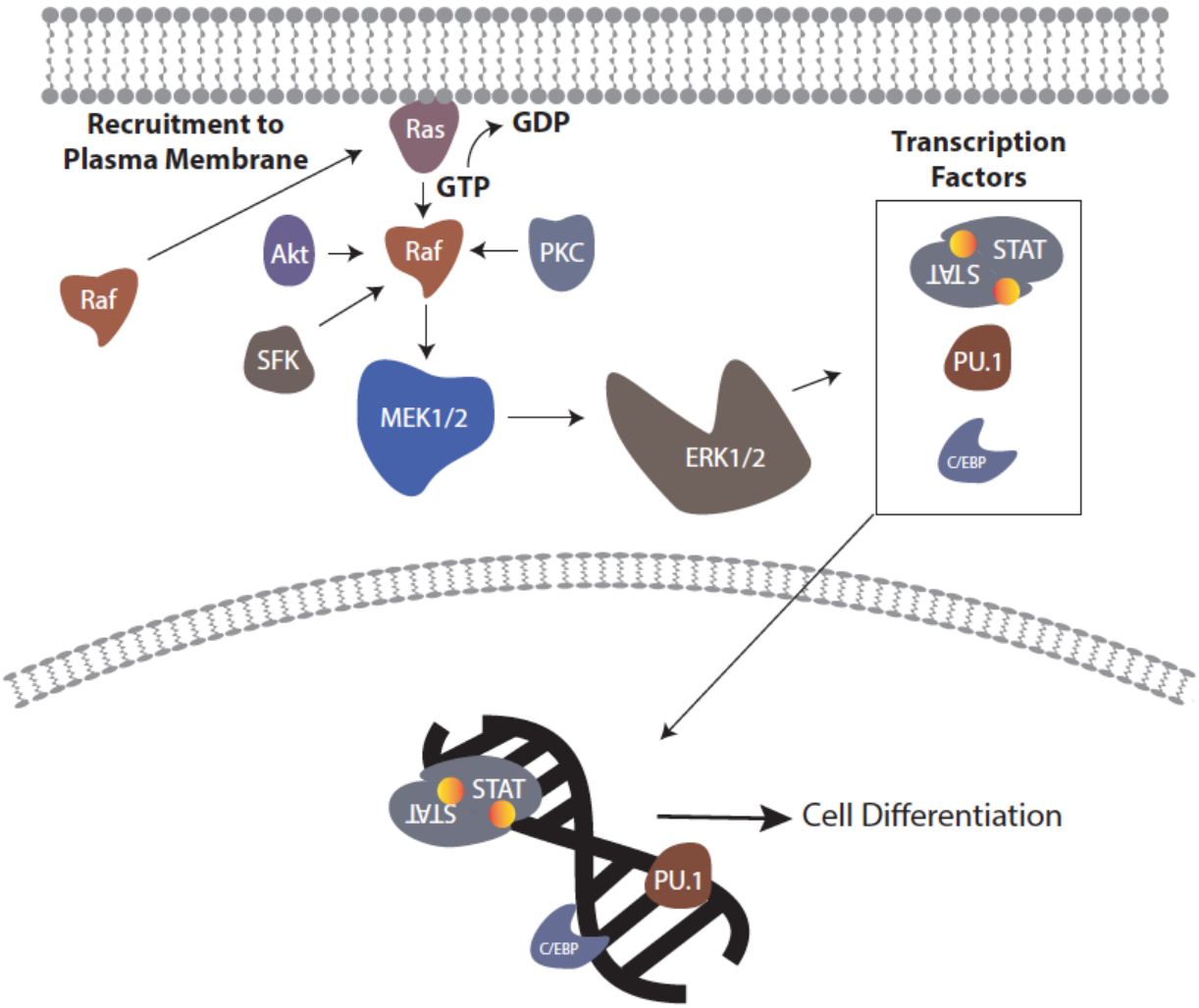


Figure 2.4 Raf/MEK/ERK Pathway. This pathway is activated by proteins from other pathways such as Akt (PI3K Signaling), PKCs , and SFKs. This pathway also utilizes other pathway substrates, such as STAT proteins to regulate cell differentiation.

2.5 P38 MAPK and JNK/SAPK

Four genes encode p38 MAPKs: MAPK11, MAPK12, MAPK13, and MAPK14. Jun N-terminal kinase (JNK) proteins are encoded by three genes: MAPK8, MAPK9, and MAPK10, which are translated to JNK1, JNK2, and JNK3 respectively. JNK proteins and p38 MAPKs function in response to environmental and genotoxic stresses **(Figure 2.5)**. Along with these functions, the two interact at with one another to control cell proliferation, differentiation, and cell survival (Wagner, 2009).

In myeloid cells, TGFβ and interferon binding to their respective receptors leads to activation of the MAPK pathway. These activated receptors carry out their functions internally through utilization of proteins such as G-proteins, myosin light chain kinases (MLKs), and the p21 protein. This pathway has positive and negative regulatory effects. Positive regulation includes p38 MAPK activation of C/EBPε, which then leads to C/EBPε binding to DNA and sequentially initiates cellular differentiation. In myeloid cells, this process is responsible for early multi-lineage differentiation of myeloid cells. Interestingly, this same pathway can also lead to cellular growth suppression, depending on the growth factor/cytokine that initiated the signaling cascade. JNK to SAPK signaling acts as a negative regulatory mechanism of this pathway. In this pathway, JNK and SAPK are activated by phosphorylated MKK 4/7 or activated cytokine receptors. IL-3 and G-CSF are the known growth factors involved in JNK to SAPK signaling. After JNK/SAPK has been activated, the signaling cascade triggers the release of cytochrome c from the mitochondria leading to apoptosis (Miranda, 2007).

Other studies have shown JNK to be activated by Hematopoietic Progenitor Kinase 1 (HPK1) protein. Such activation has been demonstrated to lead to cell survival and differentiation. To date, limited information is available to what other factors stimulate JNK (Huber, 2012).

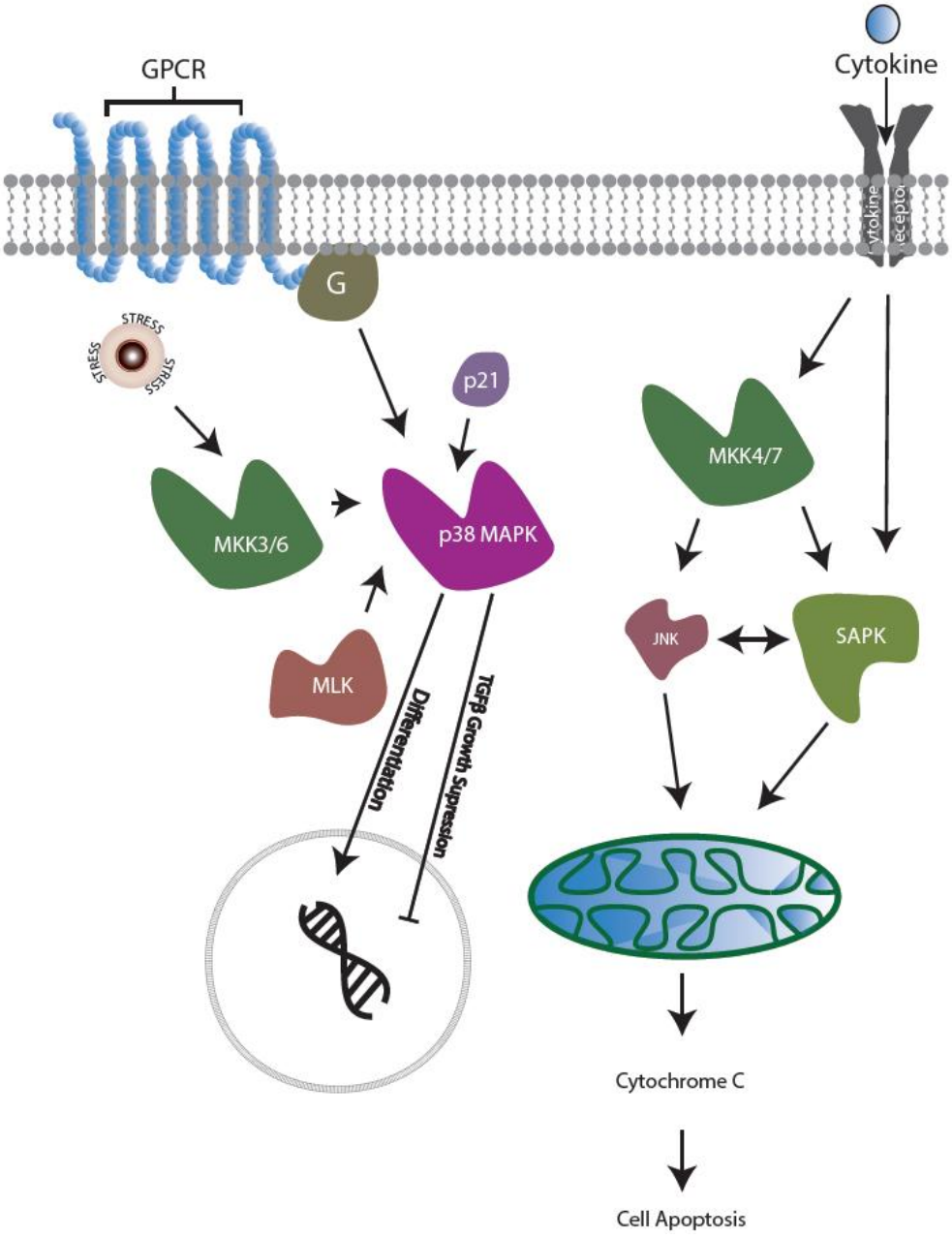


Figure 2.5 P38 MAPK and JNK/SAPK Pathway. This pathway is unique in that there are two different results of it's functioning, differentiation or growth suppression. Through p38 MAPK, the pathway can result in growth suppression or cellular differentiation. JNK/SAPK signaling can also lead to cell apoptosis through the release of cytochrome C from the mitochondria.

2.6 PI3K Signaling

PI3K is a lipid kinase protein that is involved in a multitude of intracellular signaling pathways **(Figure 2.6)**. The PI3K family consists of three PI3K subclasses, and class I PI3K proteins have been shown to play major roles in cellular differentiation, glucose uptake, vesicular trafficking, and apoptosis within myeloid cells (Yao, 1995).

PI3K class I proteins have three different catalytic classes p110α, p110β, and p110δ. These proteins are activated by specific kinases and form heterodimers with adapter molecules such as p85α, p85β, p50α, p55α, and p55γ (Polak, 2012). For simplicity, instead of listing each class and subclass present within a signaling cascade, we will refer to the term PI3K to collectively represent these classes. PI3K is also known to regulate cellular differentiation, glucose uptake, vesicular trafficking, and apoptosis through signaling cascades initiated by tyrosine residue phosphorylation on selected proteins (Kapeller, 1994).

As mentioned earlier, G-CSF is involved in granulocyte differentiation and proliferation. Studies have shown G-CSFR recruits PI3K to the specific domains on the receptor. Such recruitment of PI3K has been proven to lead to inhibited apoptosis, and enhanced proliferation (Hunter, 1998). Normal physiology of monocyte and granulocytes rely heavily on PI3K signaling.

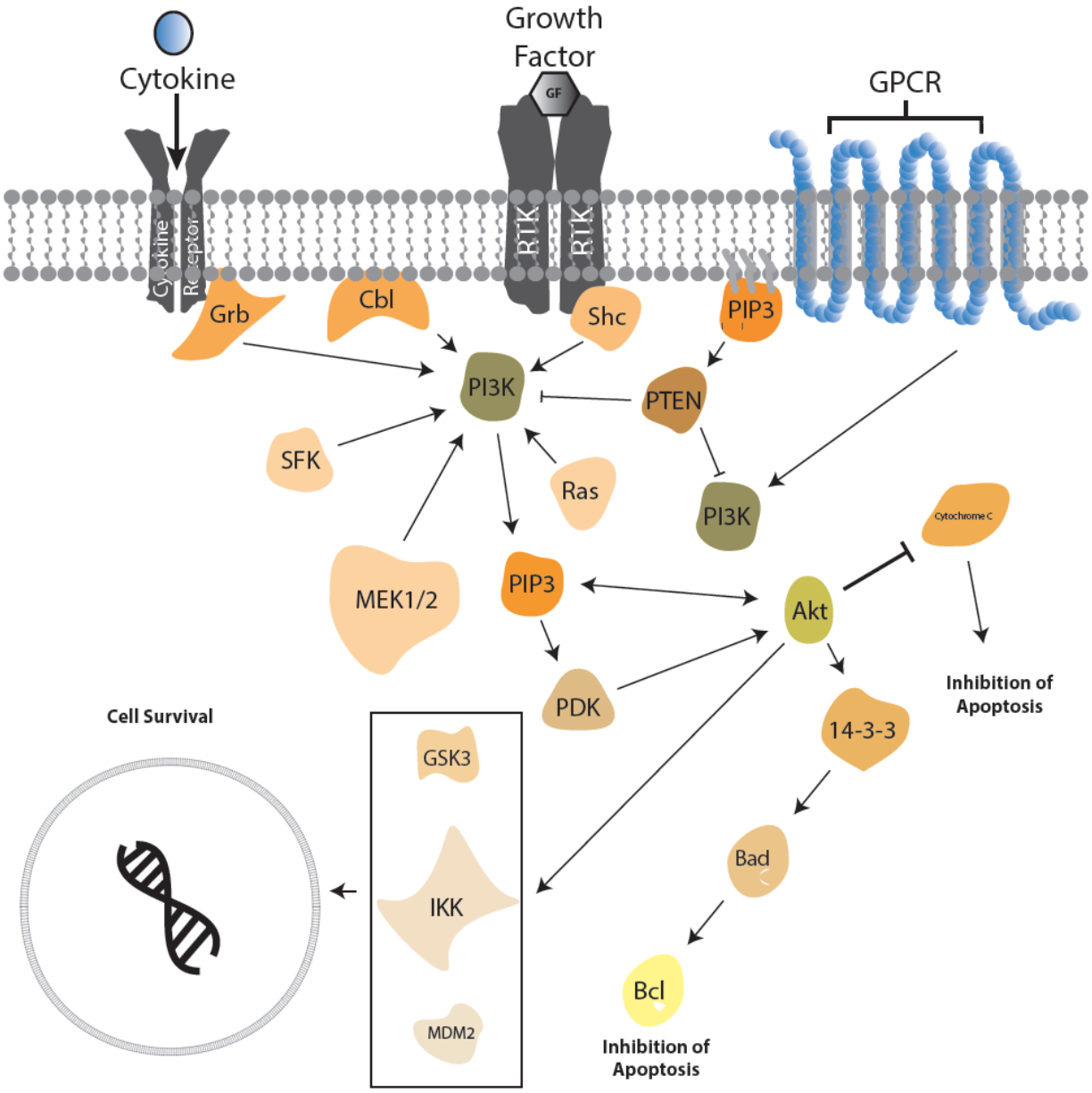


Figure 2.6 PI3K Signaling Pathway. PI3K signaling is a well-integrated pathway with connections to almost every other signaling network. Cells of the myeloid lineage utilize this pathway mainly for functions involving apoptosis and cell survival. SFKs, MEK1/2, Grb, Cbl, and PIP3 are all capable of activating PI3K (Note in this figure that PIP3 is a membrane bound protein. One of the two PIP3 proteins shown, is not attached to the cell membrane for clarity of highlighting the flow of the pathway). In most cases, PI3K activates PIP3, which then leads to activation of Akt. After Akt is activated, multiple downstream apoptosis proteins such as the caspases are deactivated.

2.7 TGFβ/SMAD Pathway

Transforming growth factor beta (TGFβ) and small body size mothers against decapentaplegic (SMAD) proteins are regulators of cellular transcription through their respective serine/threonine receptor kinases (TGFβR) **(Figure 2.7).** The binding of TGFβ to it's tyrosine receptor kinase can lead to the internal activation of SMAD proteins through serine/threonine activity. Depending on the receptor activated, different SMAD proteins will respond. SMAD protein homologs SMAD-1, -2, -3, -5, and -8 have been shown to target and activate downstream SMAD-4 which ultimately leads to gene transcription, and cellular differentiation. On the other end, SMAD-6 and -7 have been shown to activate p21 protein which then leads to growth suppression of the cell. Besides having several roles in myeloid differentiation, this pathway is also actively involved in various blood cell lines (Miranda, 2007).

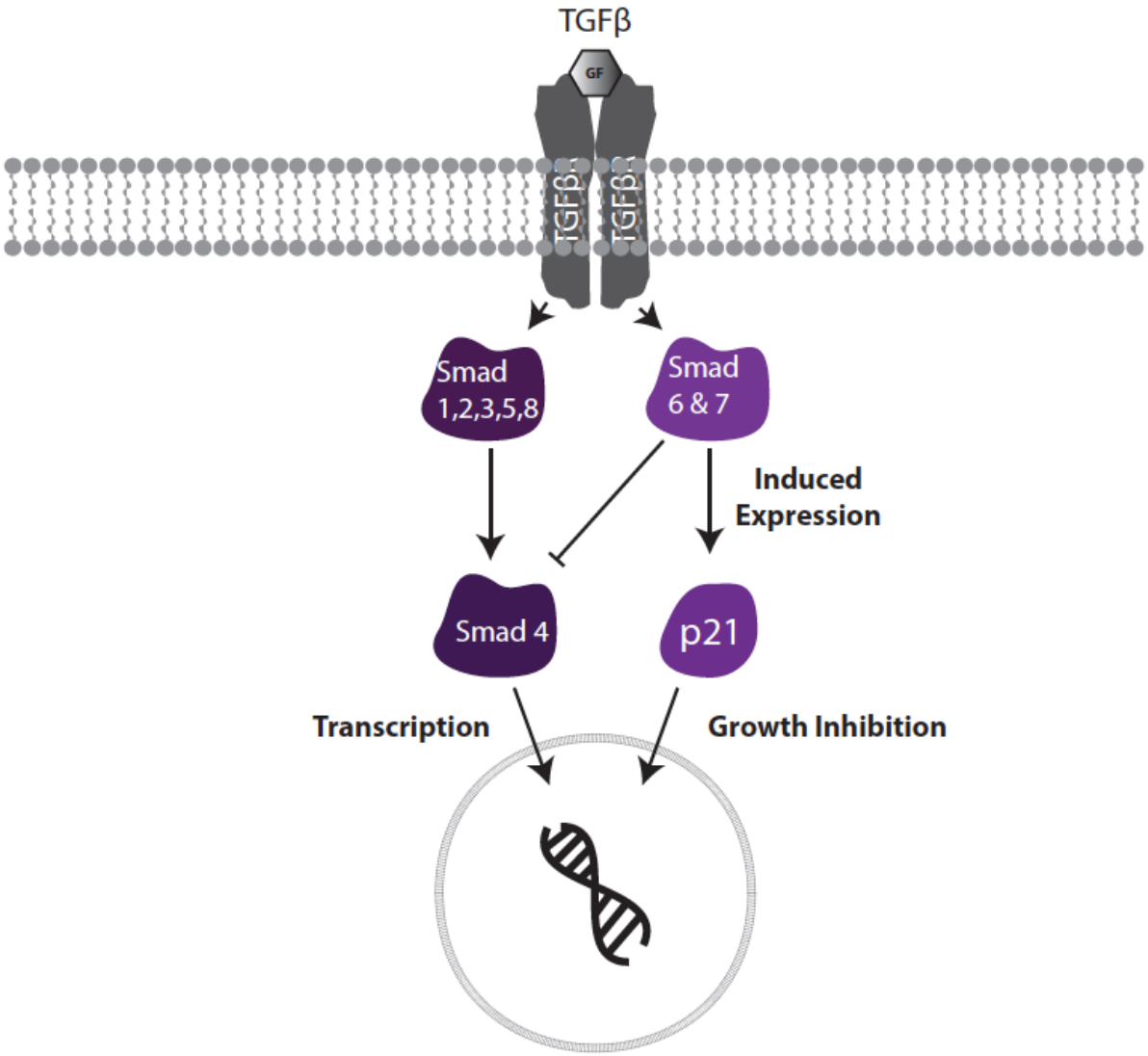


Figure 2.7 TGFβ/SMAD Pathway. This pathway utilizes receptor tyrosine kinases to initiate the signaling cascade. The effects of the signaling cascade can be either negative or positive depending on which Smad proteins are activated during the process.

2.8 Protein Kinase C (PKC)

The protein kinase C (PKC) family consists of 12 serine/threonine kinases that serve roles in cell proliferation, differentiation, apoptosis, and angiogenesis. PKCs are calcium dependent and are activated by both phophotidylserine (PS) and diacylglycerol (DAG). Recent studies have shown that PI3K pathways, and phospholipase C (PLC) can also indirectly activate PKCs **(Figure 2.8).** Little is known about PKC signaling, but to date, the MEK-ERK pathway is known to serve an important role in PKC signaling (Mackay, 2007).

In primary granulo-monocytic progenitors, PKCs serve major roles in lineage commitment. Along with this mechanism, some PKCs, such as PKCδ are phosphorylated by SFKs. Signaling networks involving PKCs are diverse, and information regarding the exact mechanisms of such pathways is limited. However, studies continue to elucidate the potential roles PKCs serve as therapeutic targets in various malignancies based on their interactions with other signaling networks (Miranda, 2007).

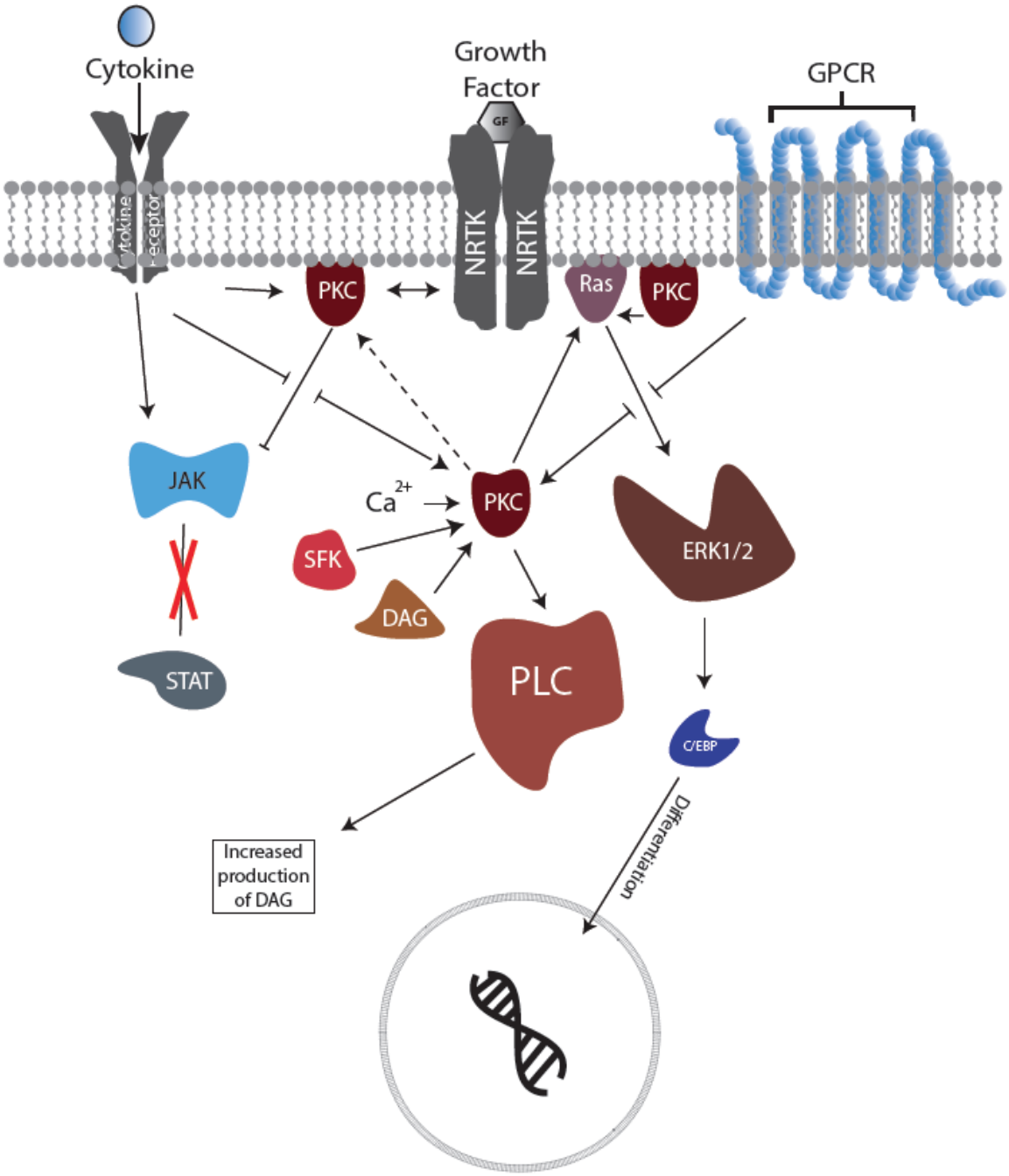


Figure 2.8 Protein Kinase C (PKC) Signaling Pathway. PKC is capable of being activated by either GPCRs, NRTKs, and RTKs. Myeloid cells ability to differentiate relies heavily on PKC functioning. PKCs carry out their activity at the cytosolic side of the plasma membrane, where they interact with GPCRs and other receptors. PKCs can also initiate a looping network by activating Phospholipase C (PLC), which leads to increased production of DAG, and ultimately increased activation of PKC by DAG.

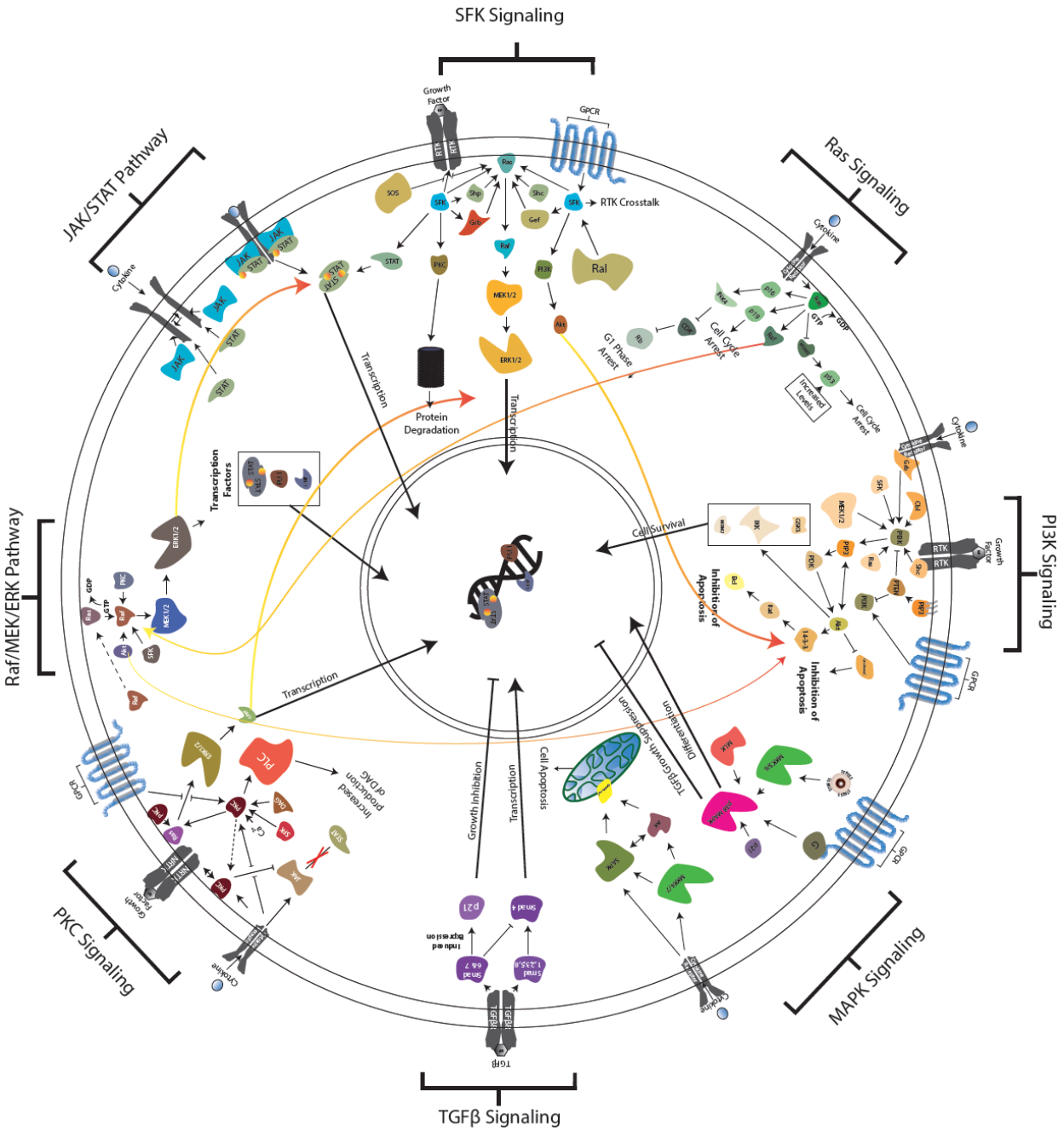


Figure 2.9 Crosstalk between pathways within myeloid cells. All pathways are connected between one another through one or more intermediates. This feature is important in understanding cell signaling.

2.9 Hypothesis and Significance

All the signaling cascades described above **(Figure 2.9)** allow for cells within the myeloid lineage to perform their everyday functions within the innate immune system. Mutations can arise within the genome of these cells resulting in severe changes with protein function and normal signal transduction. Such alterations can lead to aberrant activation or function of the aforementioned pathways ultimately resulting in malignancy or immune dysfunction.

Modern cancer drugs are designed with the intent to selectively inhibit specific pathways. Although cancer deaths have dramatically decreased within the developed world, the number of drug resistant forms of cancer continues to rise. Additionally, many underrepresented minorities, and citizens of developing countries suffer from high cancer mortality rates. Discovering methods that will allow for clinical detection of a patient's likelihood of developing drug resistance is of extreme importance. Also, identifying compounds that are capable of reversing drug resistance are of great benefit for the cancer patient.

Chronic myeloid leukemia has served as a model for how cancer can be selectively targeted by special small molecule inhibitors, and has brought an entire new meaning to personalized medicine. Since 2001, the survival rates of chronic myeloid leukemia have decreased significantly, but mortality rates have continued to remain a problem within the El Paso County area **(Figure 2.10).** Besides serving as a model for personalized medicine, CML has also served as a model for drug resistance. Since the installation of imatinib in treating CML many cases of drug resistant CML have arose. Such cases have illuminated how cancers can acquire drug resistance over time. It is therefore important that we continue to utilize CML as a model to further explore the genomic and proteomic landscape of drug resistance within cancer.

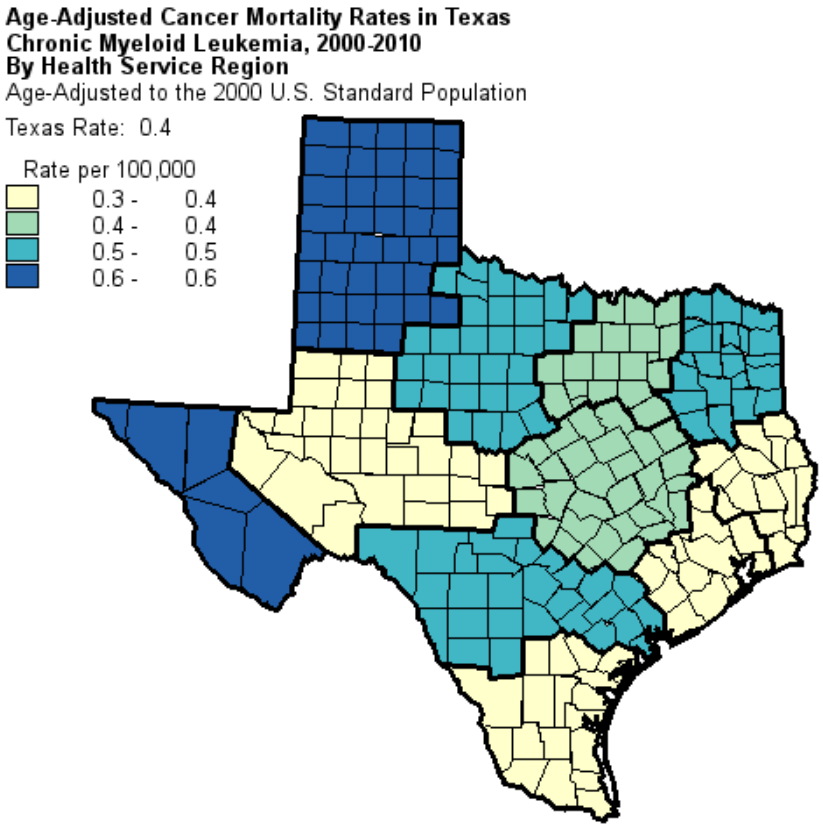


Figure 2.10 CML Mortality Rates in Texas. Between 2000 and 2010, El Paso had one of the highest mortality rates due to CML. (Texas Department of State Health Services, 2014).

The main objective of this research was to test the hypothesis that **artificially generated imatinib resistant tumor cell lines can reveal intra and inter oncogenic drivers as novel therapeutic targets in the treatment of CML. With this hypothesis our main research goal was to identify multiple ways that BCR-ABL signaling in drug resistant CML can be exploited using small molecule inhibitors.**

Chapter III: Activated Pathways that are Secondary to Treatment by Small Molecule Inhibitors

3.1 Introduction

There exist a multitude of mutations within the BCR-Abl protein that gives rise to drug resistant CML. Of these, the T315I mutation is the most common, and one of the most drug resistant mutants **(Figure 3.1).**

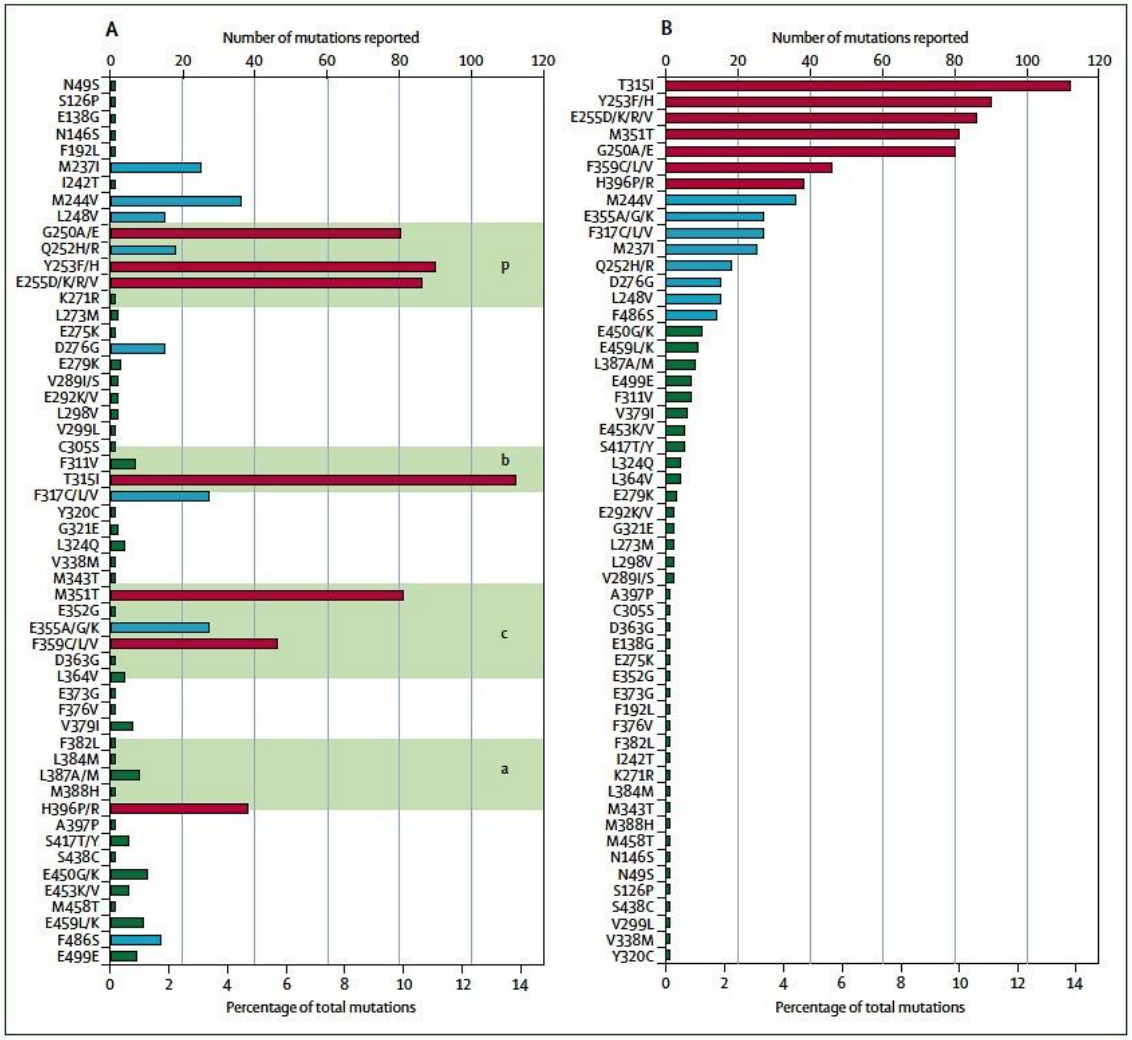


Figure 3.1 Incidence of mutations in clinical practice. All BCR-Abl mutations within CML that have been reported. (p: P-loop/ATP binding site; b: imatinib binding site; c: catalytic site; a: activation site), (Apperley, 2007).

Mutations differ from one another depending on which residue within the protein they impact. For example, the four most common BCR-Abl mutations occur within the ATP-binding,

imatinib binding, catalytic, or activating domains of the protein (highlighted in green in Figure 14.) The T315I mutation occurs within the Imatinib binding pocket **(Figure 3.2)**, and results in a threonine to isoleucine variant. The threonine amino acid contains a hydroxyl group that is necessary for imatinib attachment. Without threonine present, imatinib along with other kinase inhibitors are unable to bind properly and perform their inhibitory actions on the T315I mutant. Many CML studies have been aimed at designing small molecules that selectively inhibit BCR-Abl mutants. Second generation TKIs such as Nilotinib and Dasatinib have shown high selectivity for various SFK proteins, and have shown even greater response towards the majority of BCR-Abl drug resistant mutants.

However, the T315I mutant is resistant to second generation TKIs. Third generation TKIs such as Ponatinib have emerged on to the scene in treating T315I mutant CML cases, and have had success. As of 2013, Ponatinib was recalled by the FDA due to its adverse side effects largely seen within patients administered the medication. Overcoming such negative side effects in drug development remains an obstacle for advancing targeted cancer therapy. One fact to keep in mind is that TKIs have only been in clinical use for less than two decades. Hence, this field of cancer therapy development is still in its early years.

To investigate our hypothesis, we developed an artificially generated imatinib resistant cell line from an established cell line, KCL-22. The KCL-22 cell line is originally a human chronic myeloid leukemia in blast crisis. The cell line was established from the pleural effusion of a 32-year old woman with Philadelphia chromosome-positive CML in blast crisis; described to contain the t(9;22) b2-a2 fusion gene and a p53 mutation (Kuonishi, 1983;Drexler, 1999).

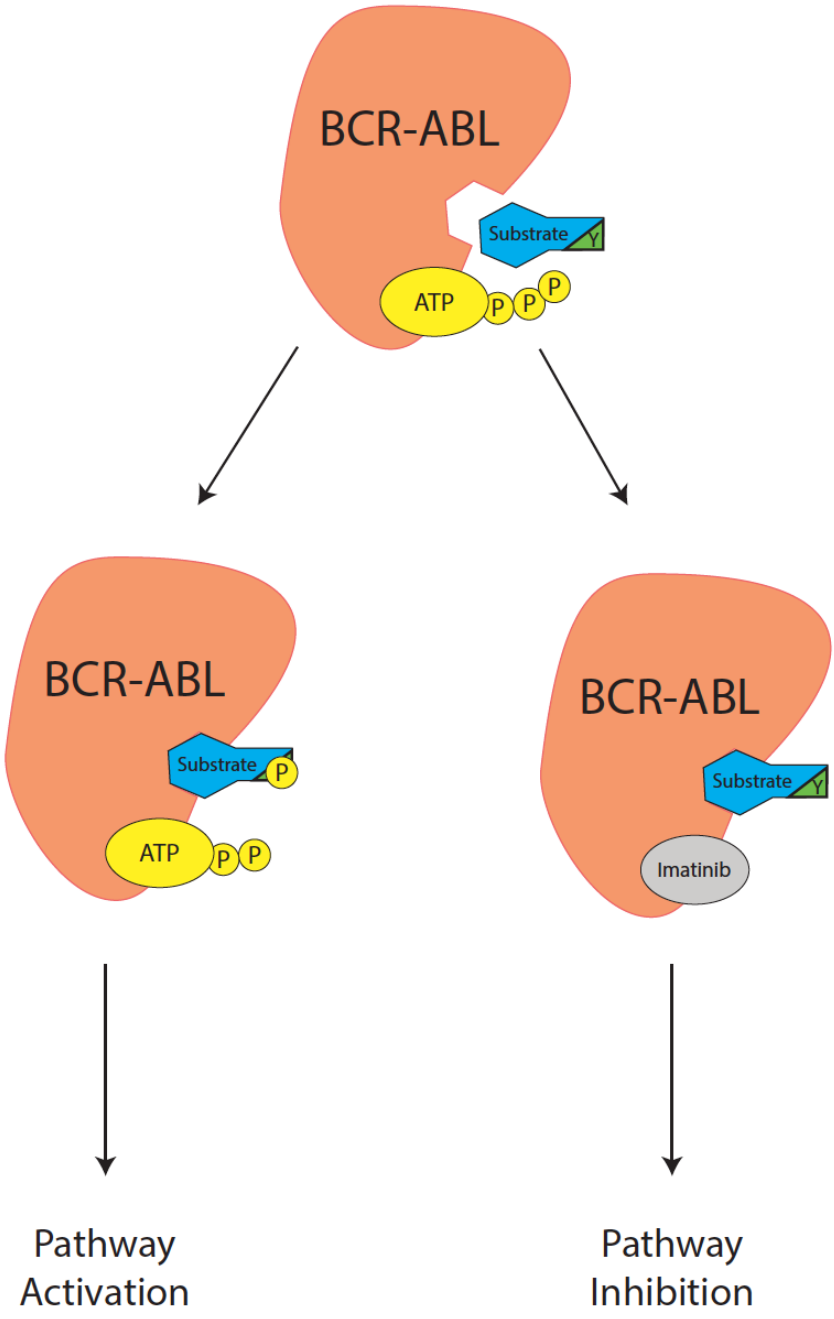


Figure 3.2 Imatinib Mechanism of BCR-Abl Inhibition. Imatinib, like other tyrosine kinase inhibitors is an ATP competitive inhibitor. Imatinib binds to the ATP pocket and prevents phosphorylation of the enzymes substrate.

3.2 Materials and Methods

3.2.1 Cell Culture: The CML blast crisis cell line KCL-22, K562, and YT cell lines were maintained using RPMI 1640 1x media (HyClone, UT) containing 1% Penicillin/Streptomycin (Corning, VA), 1% L-Glutamine (Corning, VA), and 10% heat inactivated fetal calf serum (Atlanta Biologicals, GA) as reported previously (Nagy, 2009). Incubation conditions of cells were at 37 °C in humidified 5% carbon dioxide (CO2) atmosphere, in a regular water jacketed incubator.

3.2.2 Establish imatinib resistant cell model: Cells were kept in culture with imatinib concentrations of 500 nM, 750 nM, and 1 μM during weeks 1-2, weeks 3-4, and weeks 4-6 respectively. During these six weeks, cells were passaged at 40% of the standing population every three days. Cell population density was consistently maintained between 5-10 million cells in a T75 flask.

3.2.3 Drug Concentrations: Imatinib and other kinase inhibitor concentration dose curves were obtained from literature regarding phase I evaluations of each drug. Literature provided peak concentrations (Cmax) values for each drug. Peak Concentrations for FDA approved drugs used in primary dose curves were as follows: Imatinib (4,478.0 ng/ml) (Peng et al., 2004), Gefitinib (443.88 ng/ml) (Milton et al., 2007), Dasatinib (46 ng/ml) (Demetri et al., 2009), Sunitinib (70.9 ng/ul) (Faiyre et al., 2006), Erlotinib (1,737 ng/ul) (Hidalgo et al., 2001), Lapatinib (2470 ng/ul) (Chu et al., 2008), Nilotinib (3.6 uM) (DeRemer et al., 2008), Sorafenib (2,248 ng/ul) (Strumberg et al., 2005), Trametinib (33.4ng/mL) (Infante et al., 2012), and Vemurafenib (61μg/mL) (Grippo et al., 2013).

3.2.4 Luminex Analysis: Phosphorylation activity and protein expression were analyzed utilizing Luminex 100/200 System, Austin, TX. Milliplex panels were obtained from Millipore, Billerica, MA, USA. Panels used included: SFK Panel (Blk:Tyr389, Fgr:Tyr412, Fyn:Tyr420, Hck:Tyr411, Lck:Tyr394, Src:Tyr419, Yes:Tyr412), RTK Panel (Pan Tyr), 9-Plex MultiPathway Panel, Apoptosis (Caspase3:Active, GAPDH:Total, PARP:Cleaved), STAT Panel(STAT1:Tyr701, STAT2:Tyr690, STAT3:Tyr705, STAT5A/B:Tyr694/699, STAT6 (Tyr641), BCR-ABL Panel (BioRad Laboratores), and Early Apoptosis Panels Panel (Akt:Ser473, BAD:Ser112, Bcl-2:Ser70, Active Caspase 8:Total, Active Caspase 9:Total, JNK:Thr183/185, p53:Ser46) were used. Manufacture protocol was followed for each panel.

3.2.5 Proliferation Assays: Cells were cultured in complete media (previously described, Ross 2007) with Cmax concentration of respective drug. Cells were seeded at a concentration of 1.0 x 10^4 cells per well in a 96-well plate. Plate was kept in incubation for 72-hours. After 72-hours, CellTiter96 MTS Reagent Promega was used, and manufacture protocol was followed. Plates were read using a plate reader set to wavelength of 490 nm.

3.2.6 Cell Cloning: KCL-22 cells were cloned using serial dilutions in a 96-well plate. Cells were cultured in imatinib (Cmax concentration) containing media. After 1.0 x 10^7 cells arose from one clone, a sub-cloning process was performed. Cells were placed into expanded culture after population grew to 1.0 x 10^7 cells a second time.

3.2.7 PBMC Stimulation: Normal PBMCs were stimulated using phytohaemagglutinin (PHA). Cells were kept in culture with PHA for 72-hours in complete media.

3.2.8 Statistical Analysis of Protein Activation: Statistics employed were performed utilizing Minitab Statistical Software and SAS Statistical software. Statistics consultation was obtained with Dr. Julia Bader, Research Specialist UTEP Mathematical Sciences.

3.2.9 Cell Staining: Phosphorylated JAK3 Tyr980 (sc-16567), JAK3 B-12 (sc-6932), and STAT5 C-17 sc-835 antibodies were obtained from Santa Cruz Biotechnology, and were used at a dilution 1:250 in Tween-PBS. Phosphorylated ERK1/2 Thr202/Tyr204 (9101), Phosphorylated C-Raf Ser338 (9427) and Phosphorylated STAT5 Tyr694 (9427) antibodies were obtained from Santa Cruz Biotechnology, and were used at a dilution 1:250 in Tween-PBS. Cells were left in primary antibody over night, washed with Tween-PBS, and were stained with respective Cy2 or Cy3 secondary (dilution 1:400) and DAPI (1:800) for one hour.

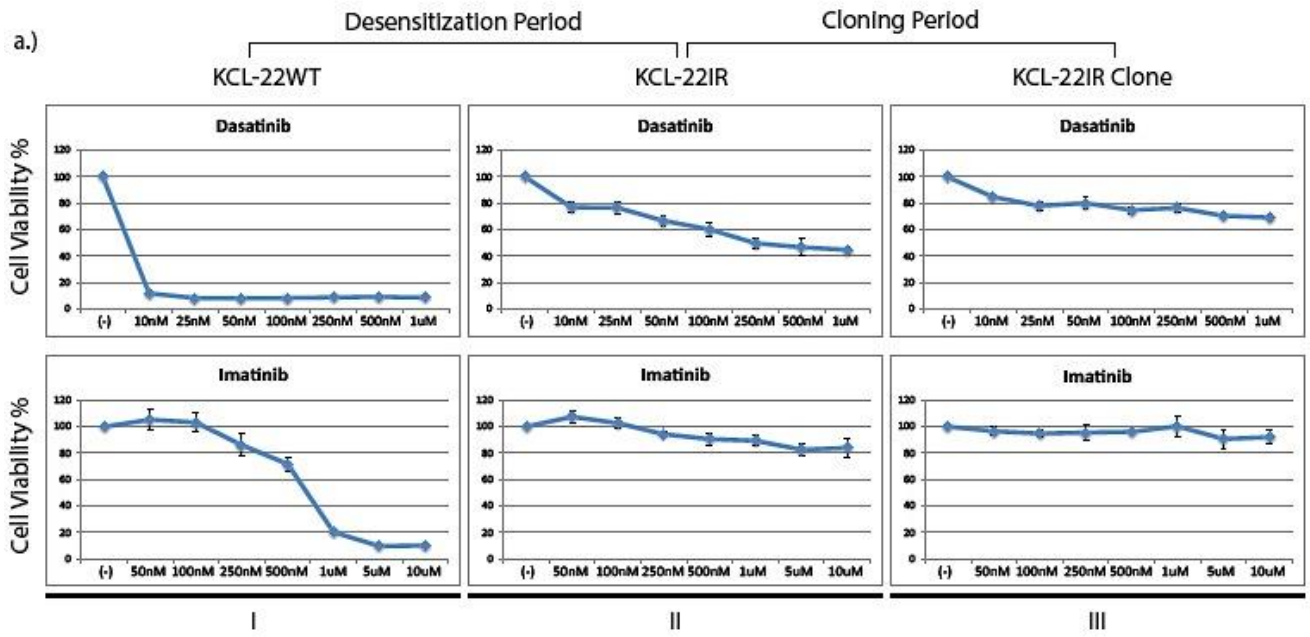
3.2.10 Immunofluorescent Confocal Microscopy: Cells were adherent, and were cultured on sterile cover slips inside a six-well culture plate, containing complete RPMI media. The cells were visualized using a Zeiss LSM 700 confocal microscope using a 40× oil immersion objective (Carl Zeiss) in the multitrack scanning mode with excitation wavelengths set at 488 (argon laser), 543, and 633 nm (He-Ne lasers); emission wavelengths were 505–530 nm and >560 nm for detection of the Cy2 and Cy3 coupled secondary antibodies, respectively. Images were captured using the ZEN 2009 (version 5.5) software and exported in a 12-bit TIFF RGB format to Adobe Photoshop and Illustrator CS4 for processing.

3.3 Results

3.3.1 Generation of Imatinib Resistant KCL-22 Cell Line

KCL-22WT cells were placed in imatinib containing culture, and viable cells were selected for. After six weeks of increasing concentrations of imatinib, KCL-22 cells displayed insensitivity towards Cmax concentrations of imatinib in comparison to KCL-22WT cells. Initially, KCL-22 cells showed an IC50 with imatinib less than 1 μM, and an IC50 with dasatinib less than 10 nM. After KCL-22 cells were desensitized to imatinib over six weeks, KCL-22 cells displayed IC50 values to imatinib and dasatinib at >10μM. KCL-22IR cells were then placed into imatinib free culture for four weeks in order to eliminate the possibility of imatinib insensitivity being caused by increased cellular metabolism of drug. Imatinib insensitivity remained after imatinib withdrawal period of four weeks. After demonstrating that KCL-22 cells were now insensitive to imatinib, cloning of KCL-22IR cells was performed as described in methods. This was done in order to acquire a homogenous population of KCL-22 resistant cells. At the end of the cloning process, approximately eight weeks, KCL-22IR cells no longer displayed an IC50 value with Cmax concentrations of Dasatinib, and cell % viability did not fall below 90% with Cmax concentrations of imatinib **(Figure 3.3a).**

a.)



b.)

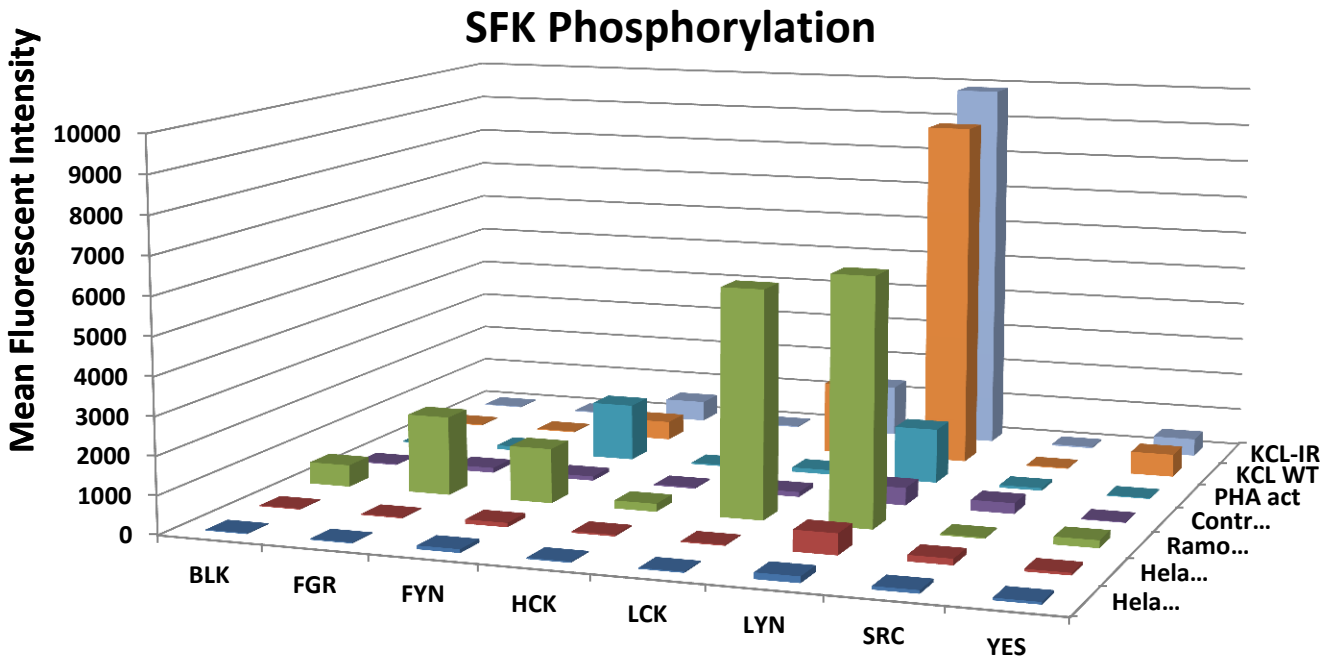
Cellular Pathways Analyzed	Targets
Apoptosis	GAPDH, Active Caspase 3, Cleaved PARP
Early Apoptosis	JNK, Bad, Bcl-2, Akt, Caspase 9, p53, Caspase 8
BCR-Abl	Phospho and Total BCR-Abl
Receptor Tyrosine Kinases	VEGFR1, VEGFR2, c-Kit, VEGFR3, MCSFR, TIE2, TIE1, PDGFRb, FLT3, PDGFRa
Src Family Kinases	Src, Fyn, Yes, Lck, Lyn, Fgr, Blk, Hck
MAPK/transcription- Pathways	CREB, NFKb, p38, ERK1/2, p70S6k,
JAK3/Raf/Shc/PI3K/Receptor Protein- Pathways	Shc, JAK3, Raf-1, PLCy, Tec, Itk, PI3k, LAT, Zap70
STAT Pathway	STAT2, STAT1, STAT3, STAT5, STAT6

Figure 3.3 KCL-22 Cells Become Insensitive to Imatinib. **aI.**) WT KCL-22 Cell line was treated with two TKIs (Dasatinib & Imatinib). The WT KCL-22 cell line is sensitive to Dasatinib and Imatinib with IC50 concentrations of ~<10 nM and ~<1 μM, respectively. **aII.)** After culturing KCL-22 cells in imatinib, KCL-22 cells became insensitive at significant clinical concentrations and showed a significant increase in insensitivity towards Dasatinib. Imatinib did not reveal any IC50 concentration, and only presented an IC20 concentration at 10 μM. Dasatinib displayed an IC50 concentration of 1 μM. **aIII.).** After cells were cloned and grown in 10 μM of Imatinib, cells showed almost 100% insensitivity to Imatinib, and no longer displayed an IC50 with Dasatinib. An IC30 concentration is seen with Dasatinib at 1 μM. These results compliment Imatinib and Dasatinib's inefficacy at inhibiting ATP activity, seen in highly drug resistant CML patients. N=3. **b.)** List of pathways analyzed utilizing Luminex. Ras/Raf/MEK/ERK, JAK/STAT, Akt/PI3K, Apoptosis, RTK Signaling, SFK, Protein Kinase signaling pathways are represented by one or several targets analyzed.

3.3.2 Identification of Activated Pathways in Response to Imatinib Resistance

To identify potential compensatory pathways that are activated secondary to small molecule inhibitors KCL-22IR cells were analyzed for changes in protein activation. Forty-eight proteins were analyzed utilizing Luminex technology **(Figure 3.3b).** SFK phosphorylation levels between KCL-22WT and KCL-22IR were markedly similar; however there were gradually increased phosphorylation activity seen with LYN and FYN proteins in KCL-22IR cells. Mildly decreased activity in LCK was also noted in KCL-22IR cells **(Figure 3.4a).**

a.)



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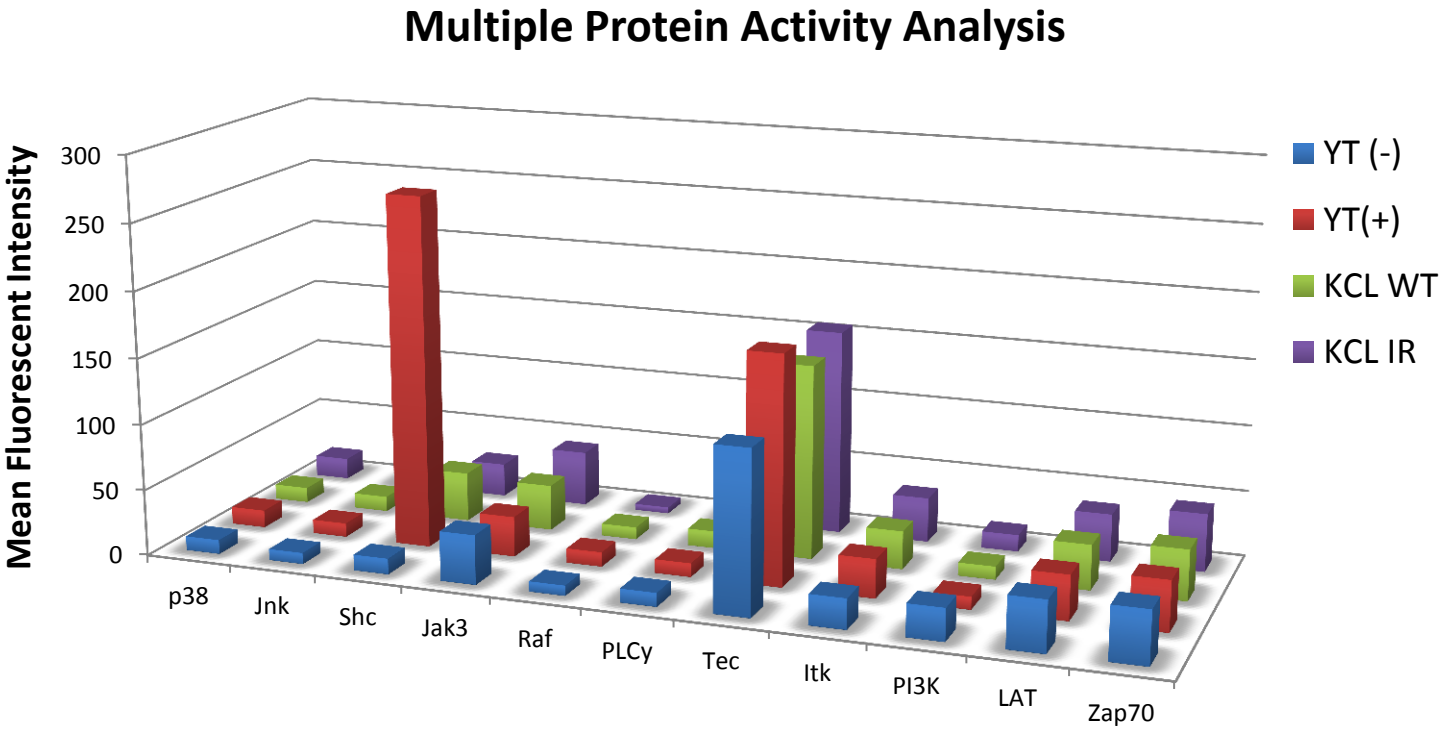

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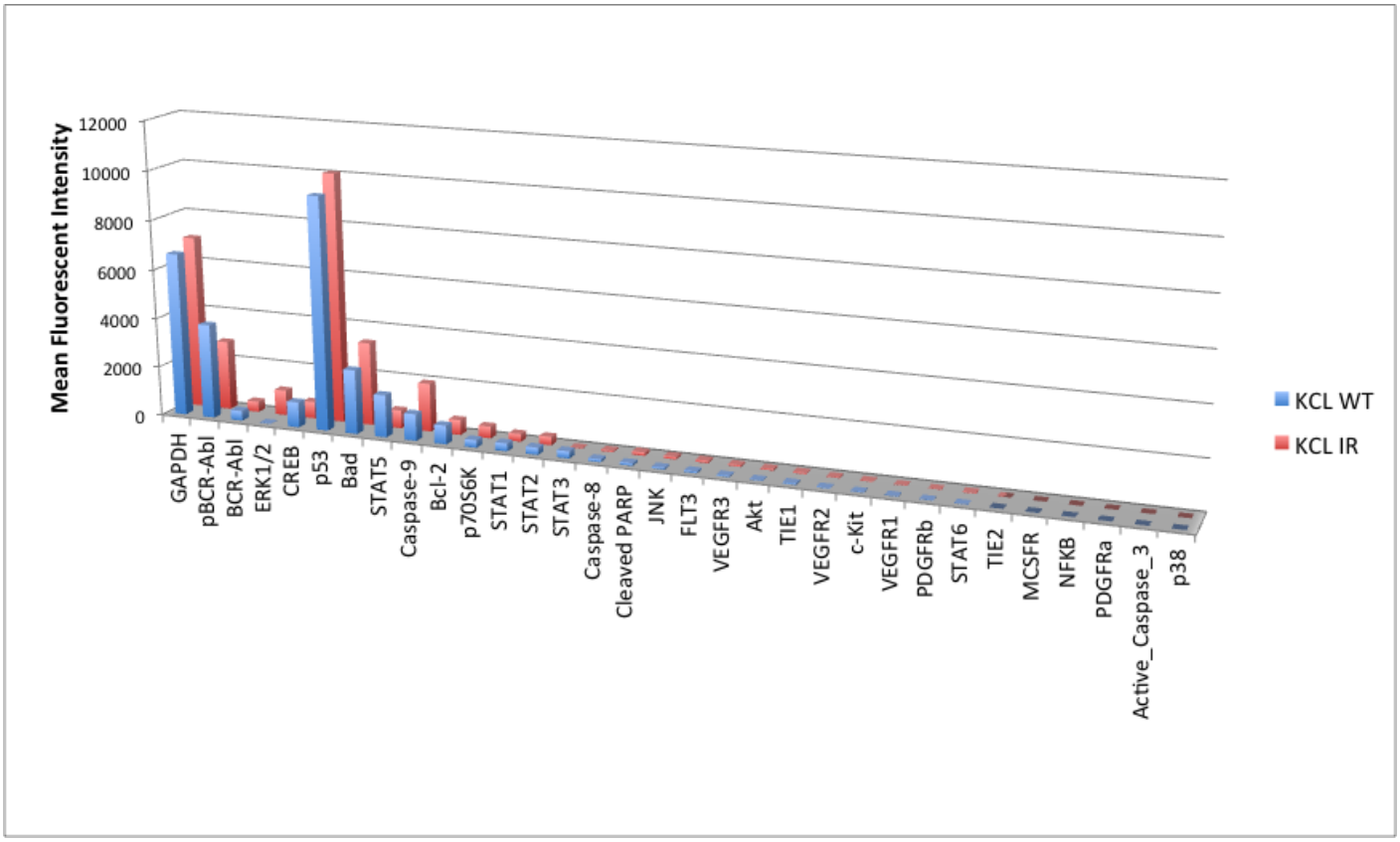


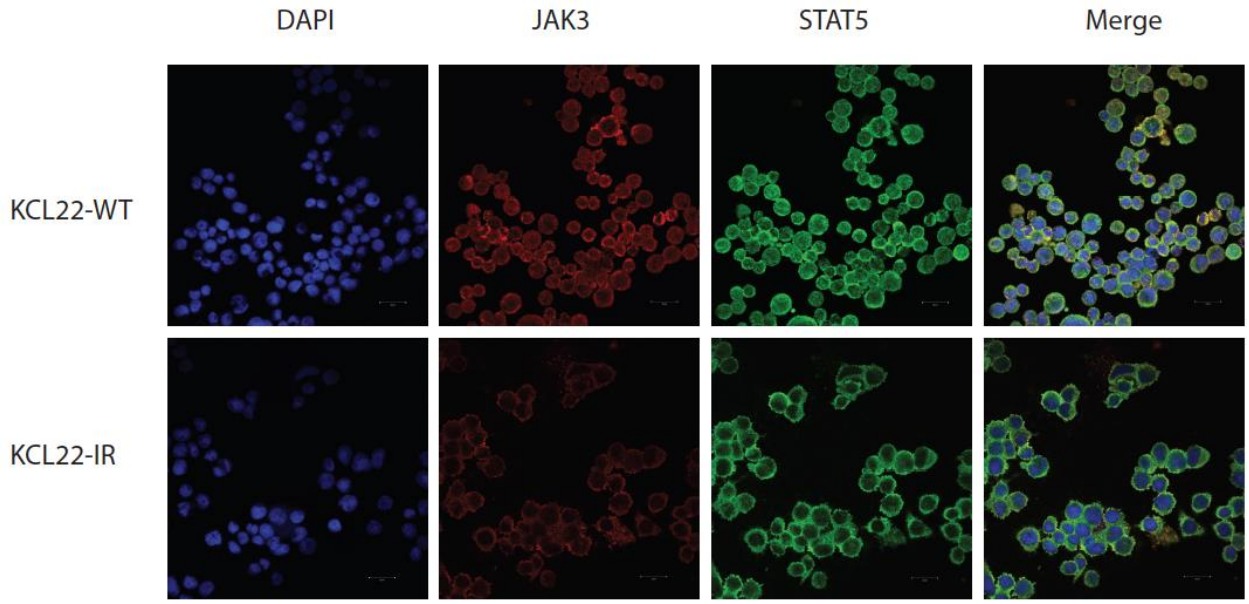
Figure 3.4 KCL-22IR vs WT KCL-22 Pathway Activation Profile. a.) KCL-WT and KCL-IR SFK phosphorylation levels were measured using Luminex technology. Graph shows Mean Fluorescent Intensity which indicates significance of phosphorylated state. Cells were compared against five controls. Three controls were provided by the SFK Panel Manufacturer (HeLa Unstimulated, HeLa Pervanadate, and Ramos Pervanadate). Two other controls used were normal PBMCs and PHA activated PBMCs. Both KCL-22 cell types showed increased LYN activity in comparison to controls. N=2. **b.)** Various pathways are represented in comparison to YT negative and YT IL-2 stimulated cells. **c.)** Various pathways (MAPK, STAT, RTK, RPK, Apoptosis) are represented including BCR-Abl activity. N=2.

In comparison to WT KCL-22 cells, KCL-22IR cells demonstrated decreased Shc and Raf activity. However, there was significant phosophorylation increases seen in JAK3, Itk, and Zap70 proteins **(Figure 3.4b).**

Figure 3.4c shows final comparison of phosphorylation levels between KCL22-IR and KCL22-WT cells. Phosphorylated BCR-Abl was shown to be markedly decreased within IR cells, however total protein expression of BCR-Abl was almost identical for both IR and WT cells. Also noted was increased activation of Caspase 9 seen in IR cells. There were extremely significant differences in ERK1/2 activation with IR cells showing nearly a 10-fold increase in activation compared to WT KCL-22 cells. Interestingly there was also decreased activation of CREB and STAT5 seen with IR cells in comparison to WT cells. RTK activity was measured and was demonstrated that neither WT nor IR cells contain significant activation of RTK activity. These findings suggest that cellular proliferation is being driven by an internal mechanism such as BCR-Abl activity **(Figure 3.4c).**

Visualized in cell staining, we were able to observe expression of both JAK3 and STAT5 in both WT and IR KCL-22 cells. STAT5 appears to be more nuclear within the WT cells **(Figure 3.5a),** and more cytoplasmic within the IR cells**.** Phosphorylated states of JAK3 and STAT5 were also confirmed **(Figure 3.5b).** Phosphorylated STAT5 appears to be more nuclear within the WT cells, and more cytoplasmic within the IR cells, as confirmed by total antibody staining shown in Figure 3.5a. Increased phosphorylated ERK1/2 levels were also noted in the IR cells **(Figure3.5c)**, whereas the WT cells demonstrated increased phosphorylated C-Raf **(Figure3.5d)**. These results confirm the findings noted in the multipathway analysis present in Figure 3.4.

a.)



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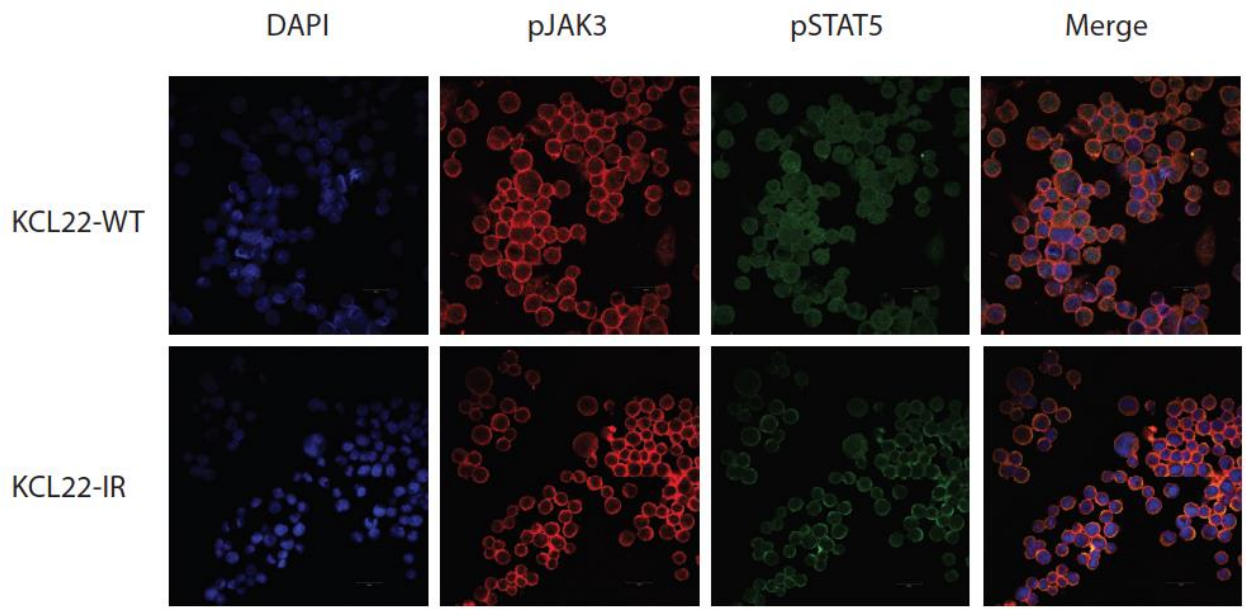

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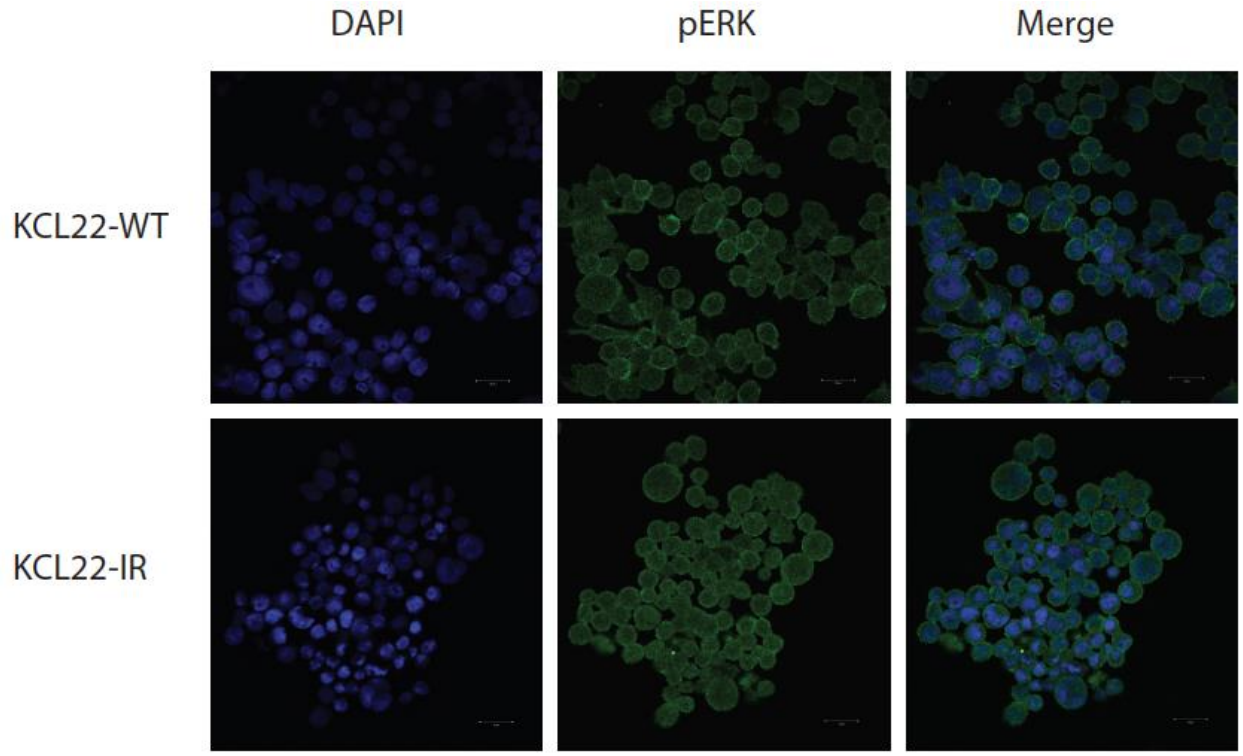


d.)

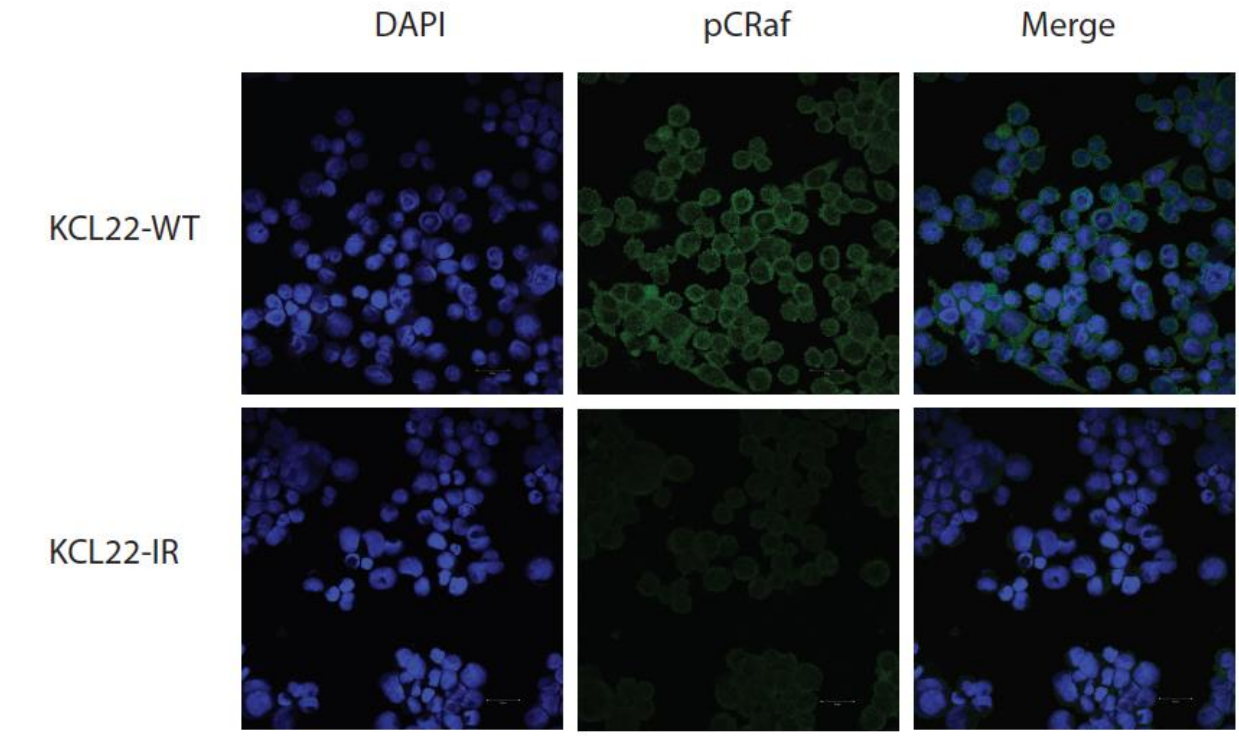


Figure 3.5 Immunofluorescent Staining of KCL-22IR/WT cells. a.) Total JAK3 (red channel) and total STAT5 (green channel) are shown with nuclear staining (blue channel). b.) Phosphorylated- JAK3 (red channel) and STAT5 (green channel) are shown with nuclear staining (blue channel). Overall intensity of activated JAK3 is seen increased in KCL22-IR cells. c.) Phosphorylated ERK1/2 is shown (green channel) with nuclear staining (blue channel), overall intensity of active ERK1/2 is increased in KCL22-IR cells. d.) Phosphorylated C-Raf is shown (green channel) with nuclear staining (blue channel), overall C-Raf intensity is seen increased in KCL-22WT cells.

3.4 Discussion

In order for cells to function properly they must be able to maintain a homeostatic balance when encountered with an environmental stress. Possessing such functions allows for cancer cells to circumvent cell death caused by small molecule inhibitors. As demonstrated in our results, various proteins expressed altered states of activation in response to imatinib insensitivity. Noteworthy is the decreased active state of BCR-Abl within the KCL-22 imatinib resistant daughter cells. Theoretically, a CML possessing such insensitivity as seen within our IR generated cell line would be problematic to treat inside a clinical setting. Rationally, one would think that if KCL-22IR cells were not responsive to BCR-Abl selective drugs, then they would possess higher levels of BCR-Abl activation. This rationalization is contradicted to an extent within our results. Even more interesting is the increased levels of activation seen with JAK3 within the KCL-22IR cells. This finding is noteworthy due to the activation state of the common JAK3 substrate STAT5. In the KCL-22IR cells STAT5 activation levels are nearly 33% the activation levels of STAT5 seen in the WT cell line. These results imply that activated JAK3 is not acting on STAT5.

Danial et al. demonstrated that within murine pre-B lymphocytes transformed with v-abl constitutive activation of JAK-STAT signaling was present. Such findings have led several scientist and clinicians to investigate the role of JAK inhibitors in treating drug resistant CML patients (Warsch, 2013). Also interesting was the presence of decreased Shc activity seen in the

KCL-22IR cells. Shc proteins are involved in regulation of apoptosis and are seen largely in drug resistant cancers. Due to the high tolerance towards strong kinase inhibitors such as imatinib and dasatinib, one would expect Shc activation levels to remain high within KCL-22IR cells. This rationalization is contradicted with findings observed in our studies. Finally, ERK1/2 activity was almost one-hundred times greater in the KCL-22IR cells compared to the KCL WT cells. As described earlier, ERK1/2 serves major roles involved in cell survival and growth. Our results support the findings demonstrated by Fenouille et al. which showed that Fyn/ERK kinase signaling is increased in patient's treated with imatinib. However, in their study, imatinib resistance was due to increased Fyn/ERK activity in response to upregulation of a matricellular protein called SPARC. Such protein activation changes have been shown to contribute to acquired imatinib resistance, however our findings show that Fyn activity is decreased within the KCL-22IR cells. Along with this, our KCL-22IR cells show extreme tolerance towards high levels of dasatinib, which is a SFK (encompassing FYN) inhibitor, and shows inhibition of such proteins at concentrations <10nM. These results suggest that Fyn may not be driving ERK activity. Another important finding to realize is the raf-1 activity levels were significantly lower in KCL-22IR cells. From the previous chapter we know that Raf activity is vital in the activation of it's downstream target ERK1/2. Finally, KCL-22IR cells showed increased levels of phosphorylated Itk, Tec, and Zap70 in comparison to KCL-22WT.

In conclusion, increased ERK1/2, JAK3, and LYN activity demonstrated within our imatinib resistant generated cell line has led us to conclude the possibility that the activation states of these proteins is secondary to imatinib treatment, and possibly confers insensitivity to imatinib and dasatinb. Based on the protein expression profiles demonstrated in figures 3.4 and 3.5. We

have proposed KCL-22IR BCR-Abl signaling in the following flow diagram to illustrate pathways altered in response to drug insensitivity **(Figure 3.6)**.

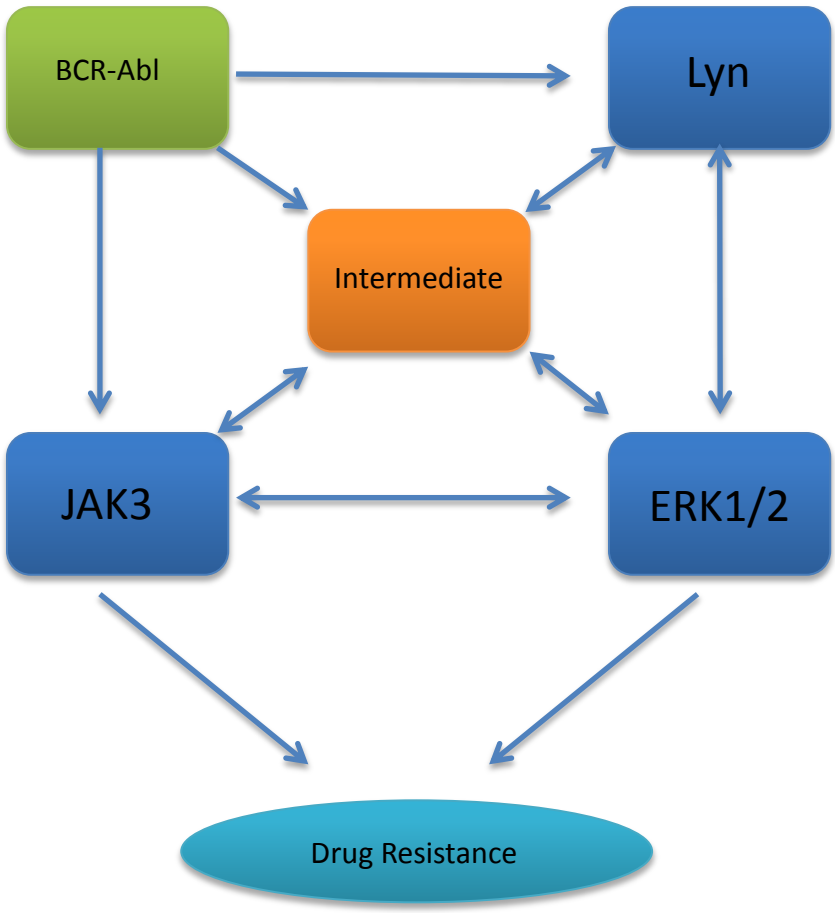


Figure 3.6 KCL22IR Utilizes JAK3 and/or ERK1/2 Signaling in Establishing Cell Survival. Based on the phosphorylation profiles shown earlier, BCR-Abl may be utilizing JAK3 or ERK1/2 signaling through one or more intermediates.

Chapter IV: Identification of Small Molecule Inhibitors that Selectively Induce Cell Death in Imatinib Resistant Chronic Myeloid Leukemia

4.1 Introduction

Recently, third generation kinase inhibitors are in phase III clinical trials for FDA approval. One of these third generation kinases, Ponatinib (Iclusig, ARIAD) was developed with high selectivity for the T315I BCR-ABL mutant. The FDA approved Ponatinib for the treatment of chronic myeloid leukemia in December of 2012. However, in October of 2013, the FDA withdrew it's approval due to increased cardiac complications seen in CML patients treated with Ponatinib. As of November 5, 2013, the FDA asked the manufacturer of Ponatinib to suspend all sales of the drug. Ponatinib was a beacon for the treatment of T315I drug resistance seen in CML patients, and it's implications in clinical use could have been as monumental as Imatinib's activity. Now that Ponatinib has been discontinued for use in the clinic, a new therapy is now needed for the treatment of drug resistant CML patients with the T315I mutation. Approximately fourteen years have passed since the advent of TKIs in clinical usage. Much still remains to be explored about these drugs mechanism of action within cancer. Using our imatinib resistant generated cell line, KCL-22IR, we attempted to identify an already FDA approved small molecule inhibitor capable of inhibiting a highly drug insensitive cancer. This approach allowed us to identify new pathways to uncouple KCL-22IR cell line growth.

4.2 Materials and Methods

4.2.1 Proliferation Assays: Cells were cultured in complete media (previously described) with Cmax concentration of respective drug. Cells were seeded at a concentration of 1.0×10^4 cells per well in a 96-well plate. Plate was kept in incubation for 72-hours. After 72-hours, CellTiter96 Promega MTS Reagent was used, and manufacture protocol was followed. Plates were read using a plate reader set to wavelength of 490nm.

4.2.2 Drug Concentrations: Imatinib and other kinase inhibitor concentration dose curves were obtained from literature regarding phase I evaluations of each drug. Literature provided peak concentrations (Cmax) values for each drug. Peak Concentrations for FDA approved drugs used in primary dose curves were as follows: Imatinib (4,478.0 ng/ml) (Peng et al., 2004), Gefitinib (443.88 ng/ml) (Milton et al., 2007), Dasatinib (46 ng/ml) (Demetri et al., 2009), Sunitinib (70.9 ng/ul) (Faiyre et al., 2006), Erlotinib (1,737 ng/ul) (Hidalgo et al., 2001), Lapatinib (2470 ng/ul) (Chu et al., 2008), Nilotinib (3.6 uM) (DeRemer et al., 2008), Sorafenib (2,248 ng/ul) (Strumberg et al., 2005), Trametinib (33.4ng/mL) (Infante et al., 2012), and Vemurafenib (61μg/mL) (Grippo et al., 2013).

4.2.3 Statistical Analysis of Protein Activation: Statistics employed were performed utilizing Minitab Statistical Software and SAS Statistical software.

4.2.4 Drug Treatments and Lysing of Cells: Cells were seeded at a concentration of 1.0×10^6 per mL, and 5mLs was added to individual wells of a six well plate. Cells were left in culture with designated concentrations of kinase inhibitors. After two hours, cells were harvested and immediately lysed. Cell lysis buffer was supplied by Millipore Luminex Kit, and was supplemented with complete protease inhibitors, n=3.

4.3 Results

4.3.1 KCL22-IR Cells Display Resistance Against Multiple Kinase Inhibitors

Cells were challenged against fifteen FDA approved small molecule inhibitors **(Figure 4.1)**, the majority of which are intended to treat other cancers besides CML. KCL-22R cells were also tested against one experimental small molecule inhibitor, EP009**.** EP009 was developed within our

laboratory for the treatment of T-cell acute lymphoblastic leukemia by selectively targeting JAK3, previously described (Ross, 2013). KCL-22IR cells' IC-50 values were compared to KCL-22WT cells and K562 BCR-Abl (+) cells. The KCL-22 imatinib insensitive clones demonstrated mark resistance towards multiple kinase inhibitors **(Figure 4.2a).**

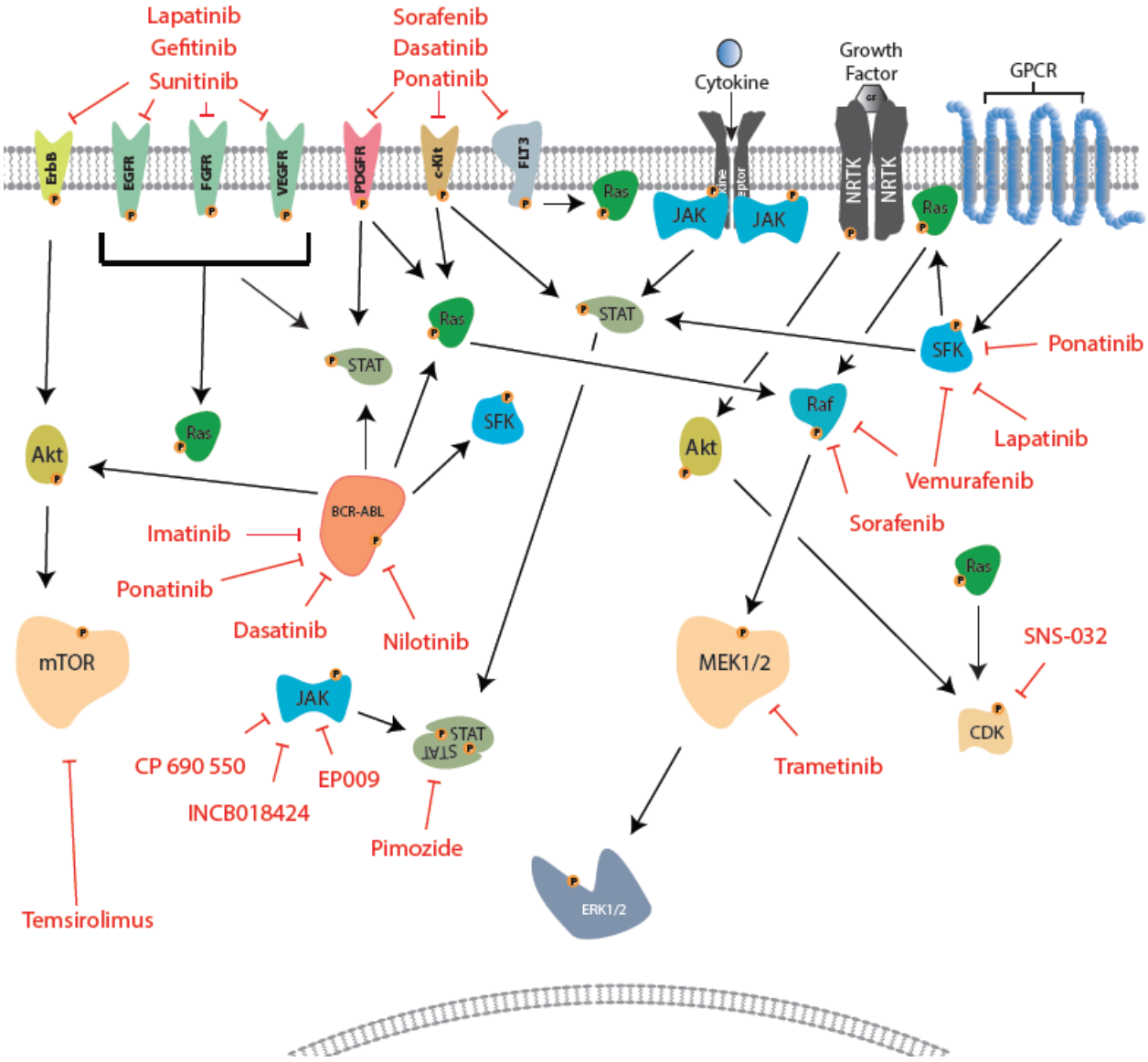


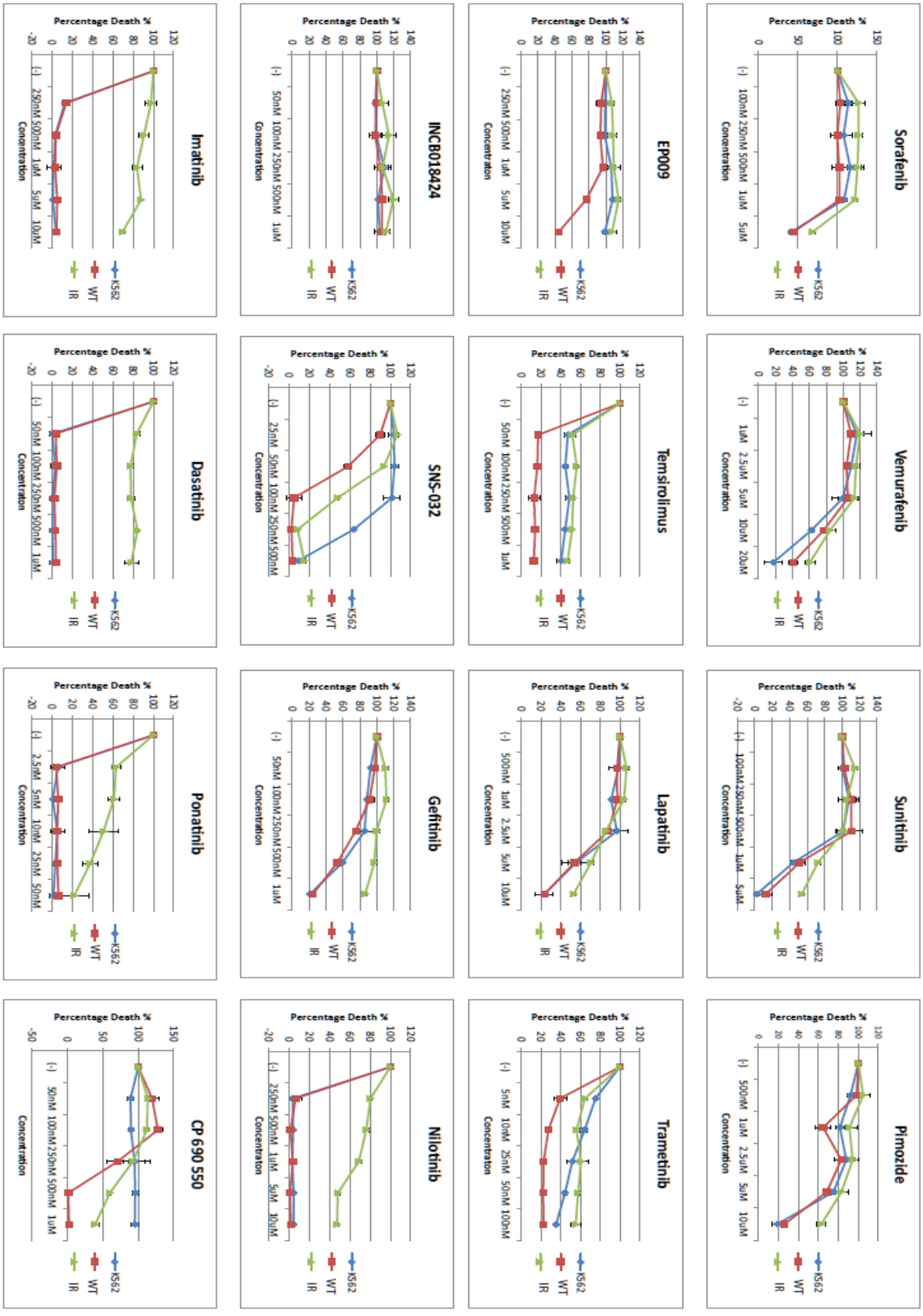
Figure 4.1 Schematic of Cell Signaling Pathways for Select FDA anti-neoplastics. Kinases inhibitors are listed in red, and known target is referenced with red arrow. For full detail on inhibitor, please see table 1 in appendix.

Compared to the WT cells and K562 cells, the KCL-22IR cells demonstrated significant insensitivity towards the majority of FDA approved kinase inhibitors. Inhibitors that showed the most potency in inhibiting proliferation were ponatinib, temsirolimus, and nilotinib. However, the concentrations of temsirolimus and nilotinib are in the upper range of peak clinical concentrations. Interestingly, both WT and IR KCL-22 cells were markedly more sensitive to CP 690 550 than K562 cells. However, IR cells displayed a higher insensitivity towards CP 690 550 than WT cells. Trametinib, a MEK1/2 inhibitor displayed moderate potency towards all three cells, however IR cells remained more insensitive to trametinib than both WT and K562 cells. Overall, cells showed various levels of sensitivity towards different kinase inhibitors. Suggesting that specific proteins are dominating proliferation machinery **(Figure 4.2b).**

4.3.2 KCL22-IR Cells Dependency on ERK1/2 Activity

Due to the results demonstrated in the previous chapter regarding increased ERK1/2 activation within KCL-22IR cells, and the results demonstrated in Figure 4.2a with KCL-22IR cells demonstrating sensitivity towards trametinib (a MEK1/2 inhibitor), inhibitor influence on activated proteins was investigated. Cells were treated with select inhibitors (Trametinib, Dasatinib, Imatinib) with three dose points for approximately two hours. Starting with the first dose point we began at 50% Cmax value and increased in equal increments according to concentrations used in Figure20a. Cells were lysed according to manufacture protocol, and the active states of forty-eight proteins in response to inhibitor treatment were analyzed **(Figure 4.3).**

a.)



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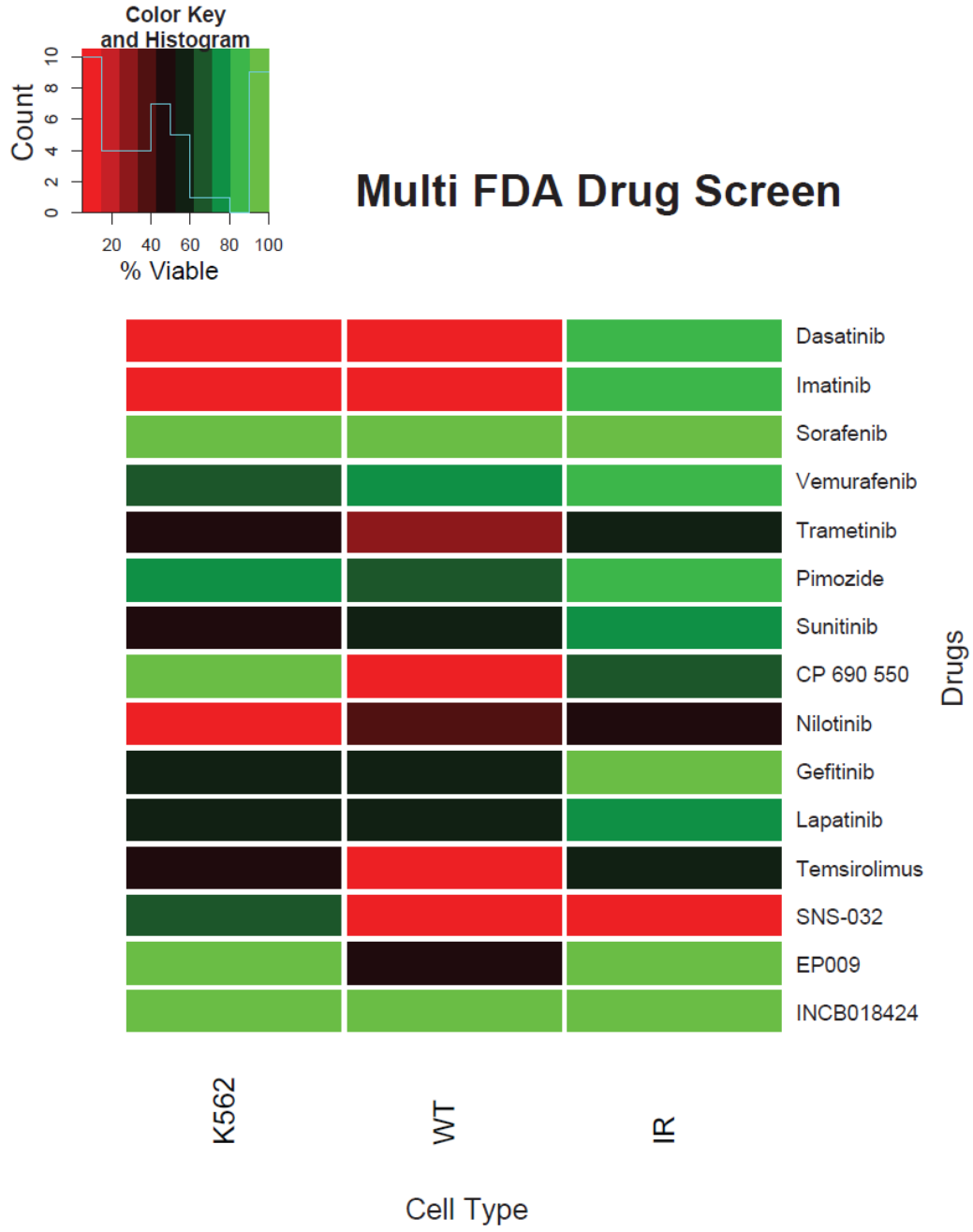
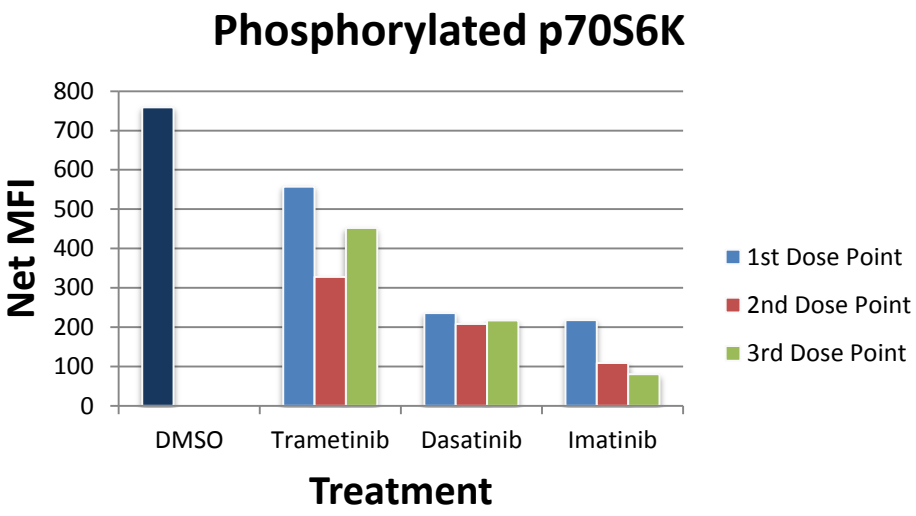
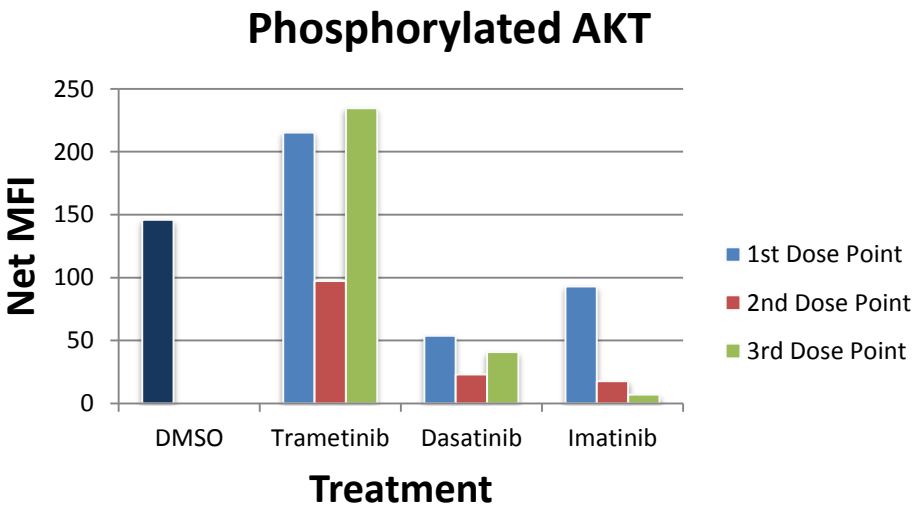
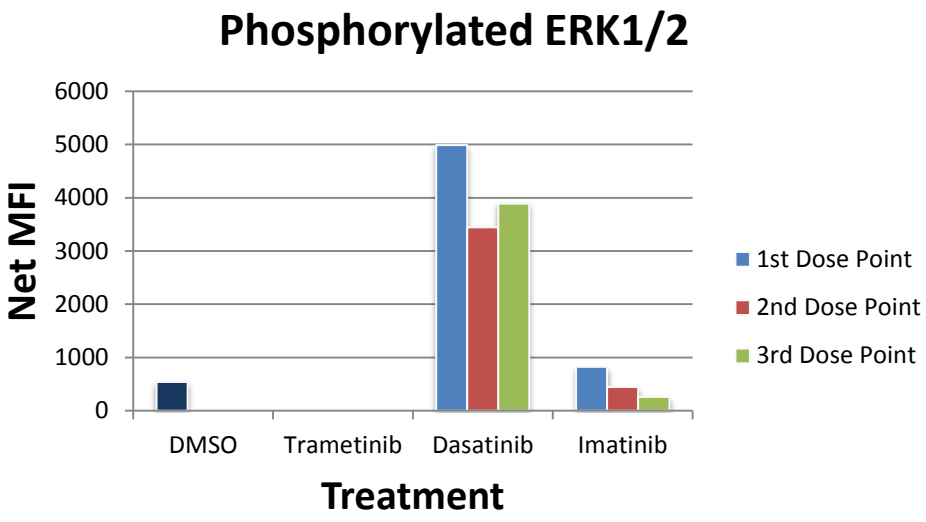
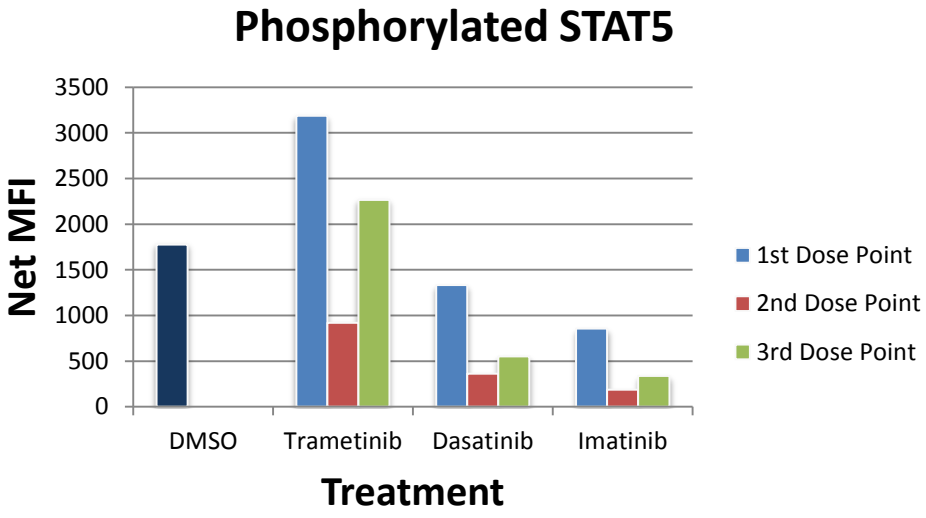


Figure 4.2 Multi FDA-Approved Drug Screen. K562, KCL-22- WT, and - IR cells were screened simultaneously against various FDA approved kinase inhibitors. **a.)** MTS proliferation assays and cell death % vs concentration of inhibitor. Max concentration was set to 110% Cmax values to insure drug activity **b.)** Heat map of Cmax values for respective inhibitor (C Max only). Green: 100-60%-, Black: 50%, Red: 40-0% CELL VIABILITY. N=3









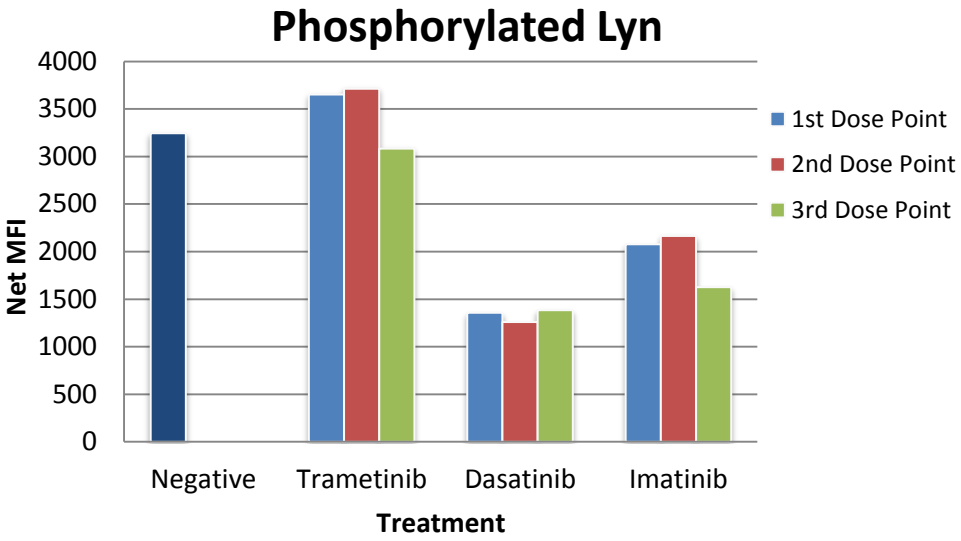


Figure 4.3 Select Kinase Inhibitors Influence on Active Proteins within KCL-22IR. Cells were treated with increasing concentrations of dasatinib, imatinib, and trametinib. Dose concentrations were as follows: 1st Dose Point = 50% cMax, 2nd Dose Point = 75% cMax, 3rd Dose Point = 100% cMax of respective drug. Negative treatment corresponds with cells that were only treated with DMSO, which all compounds were dissolved in, n=1.

4.4 Discussion

Since imatinib was FDA approved for the treatment of CML over 10,000 patent applications for kinase inhibtors have been filed (Akritopoulou-Zanze, 2009). This trend has not declined, and in fact continues to grow. Unfortunately, the number of drug patents outnumbers the number of drugs that successfully make it to market by a factor of twenty. Many factors make it extremely difficult for one drug to be approved for clinical use. On average, it takes a pharmaceutical company twelve years to get a new drug from the research lab to the patient (FDA Special Consumer Report, 2013).

Realizing this bottleneck in drug development, many have realized how imperative it is that molecules that are already FDA approved be explored for potential utility in diseases that are considered "off label". Our results demonstrate support for such a rationale. Screening the KCL-22IR cells along with the WT and K562 cells against various small molecule inhibitors revealed potential candidate compounds for drug resistant and non drug resistant CML. For example, although KCL-22IR cells are highly insensitive to imatinib and dasatinib, they displayed sensitivity when treated with SNS-032, temsirolimus, and trametinib. All of which are being used to treat other cancers besides CML. Both the WT and IR KCL-22 cells demonstrated increased sensitivity towards SNS-032, compared to K562 cells, which indicates that the WT and IR cells respond to changes made within cyclin protein activation. However, cyclin proteins serve many roles in cell growth and development, and inhibition of these proteins can easily cause death in any rapidly proliferating cancer (Galderisi, 2003). This was not the case in our studies, since K562 cells demonstrated cellular proliferation at a faster rate than both WT and IR KCL-22 cells (data not shown). These findings suggest two conclusions. Cyclin activity is already at a minimal within WT and IR KCL-22 cells or SNS-032 is cross-reacting with other key molecules present inside the KCL-22 cells. Looking at figure 4.1 we can see that SNS-032 sensitivity is reduced in IR cells compared to the WT KCL-22 cells, which indicates that other factors present within the IR cells that are not present within the WT cells are influencing cellular proliferation to a greater extent. From the last chapter, it was noted that in comparison to WT KCL-22 cells, the IR KCL-22 cells displayed increased activation in ERK1/2 and JAK3, and decreased activation levels in BCR-Abl, STAT5, and Raf-1. Our results demonstrate a unique finding in that although KCL-22IR cells displayed higher levels of JAK3 activation, they were less sensitive to JAK3 selective inhibitors such as EP009 and CP-690,550. However, both KCL-22 cell lines were more sensitive overall to

these inhibitors when compared to K562 cells. These results suggest that both KCL-22 cells utilize the JAK/STAT pathway to some degree, and that WT KCL-22 cells, despite not having high levels of JAK3 activation, are more sensitive to inhibition of JAK/STAT signaling. Other interesting finding involved the increased inhibition of proliferation due to MEK1/2 inhibition by trametinib. Although the KCL-22IR cells demonstrated significantly increased ERK1/2 activity, they were still more tolerant to trametinib treatment, compared to both KCL-22WT and K562 cells. These results suggest that drug resistance may be conferred when there is no particular pathway dependency, and multiple players besides a BCR-Abl mutant may be contributing to drug resistance.

Lastly, our results also demonstrated that cell death in KCL-22IR cells is associated with decreased levels in ERK1/2 activity, which was inhibited with increasing concentrations trametinib, as seen in Figure 4.2. It is also worthy to mention that decreased activity in ERK1/2, secondary to trametinib treatment, resulted in overall increased STAT5, p70S6K, and Akt activation. The reverse was also seen when STAT5, p70S6K, and AKT activity were inhibited by dasatinib treatment. However, when looking at figure 4.1 we can see that KCL-22R cells are significantly tolerant to high concentrations of dasatinib, and therefore the results suggest that STAT5, p70S6K, and AKT activity are not crucial for the survival of KCL-22R cells. In addition, although lyn activity is greater in KCL-22IR cells in comparison to WT KCL-22 cells figure 3.4, its inhibition via high concentrations of dasatinib are also not necessary for cell survival.

In conclusion these results demonstrate how small to large scale drug screens can be utilized as a tool in investigating the proteomic landscape of a particular drug resistant cancer. Secondly, these results prove how small molecule inhibitors can be used to treat cancers that they were not originally intended to treat. Utilizing such drug screening methods within the clinical

setting is of utter importance for the progression of cancer treatment, especially within patients who have failed initial treatment options. Which in our proposed model we highlight a potential pathway used by KCL-22IR cells **(Figure 4.4)**

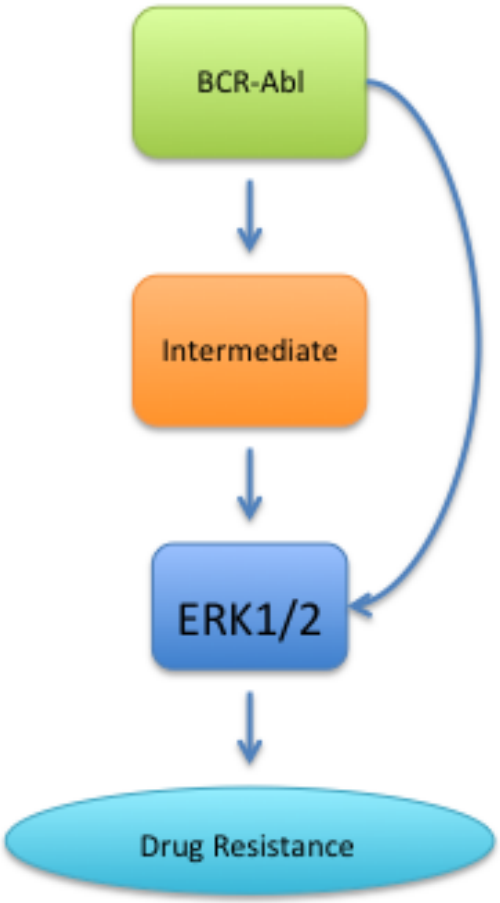


Figure 4.4 Schematic of How KCL22-IR Cells Depend on ERK1/2 Activity to Circumvent Drug Sensitivity. Within the KCL22-IR cells, inhibition of MEK1/2 (an ERK1/2 mediator) induces cell death. Inhibition of other active proteins such as Akt, p70S6K, Lyn, and JAK3 does not induce cell death. Therefore the KCL22-IR cells rely on activated ERK1/2 and possibly another intermediate in order to circumvent drug induced cell death.

Chapter V: Genetic Alterations in Drug Resistant Chronic Myeloid Leukemia

5.1 Introduction

Until now we have been concerned with the phenotype of drug resistant CML. The picture would not be complete if we were to ignore what lies beneath the surface, such as the genetic aberrations that are largely responsible for cancer promoting molecules. In recent years, data acquired from global efforts at sequencing genomes of multiple organisms and individuals with diseases has led to a vast amount of information regarding the genetic mutations that drive cancer (Kim, 2013). In common tumors (colon, breast, brain, and pancreas) there exists an average of 33 to 66 genes with somatic mutations that can be labeled as potential culprits responsible for damaged protein products. Depending on the extent and significance of such damaged proteins determines whether or not these genetic aberrations will give rise to a tumor promoting protein. The genome is a large set of information to navigate, and most sequencing efforts are interested in analyzing only the exome. The exome is the collection of exons in the human genome. And exome sequencing generally refers to the collection of exons that encode proteins (Vogelstein, 2013).

Due to advances in sequencing technology the cost to sequence an entire exome has dramatically decreased. With such changes in affordability the generation of sequence variation data is increasing at an exponential rate. To date, approximately 15-million single nucleotide variations and one million short indels of the human population have been cataloged as a result of the International HapMap Project and the ongoing 1000 Genomes Project. Bioinformatic tools such as PROVEAN, SIFT, PolyPhen, MAPP, PANTHER, and ANNOVAR are being developed in order to computationally predict the functional effect of sequence variations found within sequencing data. Tools such as PROVEAN (Protein Variation Effect Analyzer) carry out it's function by utilizing a unique algorithm that predict functional impact for all classes of protein sequence variations (including insertions, deletions, and multiple substitutions). In detail,

PROVEAN utilizes pairwise sequence alignment scores in order to generate a "PROVEAN Score". Change in alignment scores can be easily interpreted as a possible impact factor for protein function. PROVEAN measures the similarity of a protein variant to it's functional homologous counterpart before and after the introduction of the variant. Next it quantifies the degree of impact of protein variant by analyzing variant frequency and chemical properties of amino acid change. If the reference amino acid residue is found to be conserved and the variant sequence alignment generates a low score (<-2.5), the variant will be assigned as having a deleterious effect on protein function (Choi, 2012). Programs similar to PROVEAN, mentioned earlier, perform similar functions in determining alterations within exome data.

Identifying and understanding genetic aberattions is extremely important for moving towards targeted personalized medicine. Along with this, identifying genetic aberrations that may coincide with drug treatment failure can potentially help limit costs and unnecessary suffering for the cancer patient. It is with this objective that we aimed to use our highly drug resistant CML cell line, KCL-22IR, as a model for how the genomic landscape can change as a cancer evolves.

5.2 Materials and Methods

5.2.1 Purifcation of Genomic DNA: Utilized Qiagen DNeasy Blood & Tissue Kit according to manufacture's protocol. DNA concentration was measured using NanoDrop 3000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA), and was verified using 1% agarose gel (Ultra-pure Agarose with 1x TBE) containing 0.002% EtBr.

5.2.2 Whole Exome Sequencing: Whole exome sequencing was performed at Otogenetics Corporation (Norcross, GA, USA). Exome sequencing was performed utilizing Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA). Genomic DNA was

subjected to multiple quality control checks performed by Otogenetics Corporation after which Illumina libraries were generated from fragmented gDNA using NEBNext reagents (New England Biolabs), and the libraries were subjected to exome enrichment using Agilent V4 (51 Mbp) kit. Libraries were tested for enrichment by qPCR and for size distribution using Agilent Bioanalyzer 2100. After libraries were confirmed, samples were sequenced on a Illumina HiSeq2000 at a 30x coverage. Sequence reads were aligned to the human genome reference sequence (build hg19) and variants called and annotated using the DNAnexus software package.

5.2.3 Patient DNA Sequencing: Patient gDNA was sent for sequencing after gene amplification was performed. Sequences were read using a 3130x/Genetic Analyzer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) in the Border Biomedical Research Center housed at The University of Texas at El Paso.

5.2.4 G-Banding Karyotype and SKY analysis: Chromosome analysis was conducted at the WiCell Research Institute, WiCell Cytogenetics Lab, Madison, WI, USA.

5.2.5 BCR-ABL Kinase Domain Mutation Analysis: Cells were sent to Blood Center of Wisconsin, Milwaukee, WI, USA, for interpretation of mutations identified within ABL kinase domain. Analysis was carried out using RT-PCR followed by DNA sequence analysis of ABL kinase domain covering amino acids 236-486. This region covers all the reported kinase domain mutations. Sensitivity of mutation detection is approximately 20%. The clinical significance of mutations present at <20% is not clear, according to BloodCenter of Wisconsin.

5.2.6 Patient Samples: 56-Patient samples were randomly collected from a cancer specimen repository. All patient samples were obtained under an approved Institutional Review Board Protocol. The patient sample repository consists mainly of leukemia/lymphoma cancer patients from within the Paso del Norte region. However, a small amount (less than 10%) of other cancers such as pancreatic, brain, lung, and kidney cancers were available in the repository.

5.2.7 Generation of Bioinformatics Pipeline: Pipeline was adapted from the PROVEAN and SIFT tool and was designed in collaboration with Dr. Ming-Ying Leung (Bioinformatics Program and Mathematical Department at The University of Texas at El Paso), and UTEP Bioinformatics Master candidate Joe Knapka. Primary functions of the pipeline were as follows:

1) Accept as input whole-exome sequence data as supplied by Otogenics Corp, in tabular comma-separated value format.

2) Compute protein variants for all isoforms of the genes affected by the SNPs and indels in the input data.

3) Compute the PROVEAN score for each protein variant.

4) Query the PubMed database to discover any publications associated with each variant, based on the dbSNP ID of the SNP (if provided) and the Human Genome Variation Society code for the corresponding protein variant.

5) Find any Gene Ontology terms associated with each gene affected by the SNPs and indels in the input data.

6) Assemble a final data set containing the input data with additional columns appended containing PROVEAN score, Gene Ontology terms, and links to any PubMed references.

7) Present a web service allowing sequence data to be submitted to the pipeline and results emailed to the submitter.

5.2.8 Gene Amplification: Gene amplification was employed using polymerase chain reaction (PCR). Primer sequences were obtained from NCBI primer blast tool, and were synthesized at The Midland Certified Reagent Co., Midland, Texas, USA.

Gene	Forward 5' to 3'	Reverse 5' to 3'
FLT4	GATTGGTTCTTTCCTGTCTCTGA	CCACAAATATCAATTTACAACCATTG
ERBB3	TGACTGATTCCCCCAACCTT	ATCCCCATGCTTCACTCCAC
TNFRSF14	CCCTCTCTTCTCAGGCACCGAGAG	GTGGAGCCCCCTCCCCAGGGCAC
NOTCH1	CCCTCGTTCTGTCGCCTG	CCATGGATGGCCAACACCAG
STAT5A	GTCCTTGTAAGCAGCCGCC	GCTGCCAACACTGAACTGAGA
PIK3CA	GATTGGTTCTTTCCTGTCTCTGA	CCACAAATATCAATTTACAACCATTG

5.2.9 Gene Mania: GeneMANIA locates other genes that are related to a set of input genes, using a very large set of functional associations. (GeneMANIA is actively developed at the University of Toronto, in the Donnelly Centre for Cellular and Biomolecular Research, in the labs of Gary Bader and Quaid Morris. GeneMANIA development was originally funded by Genome Canada, through the Ontario Genomics Institute (2007-OGI-TD-05) and is now funded by the Ontario Ministry of Research and Innovation).

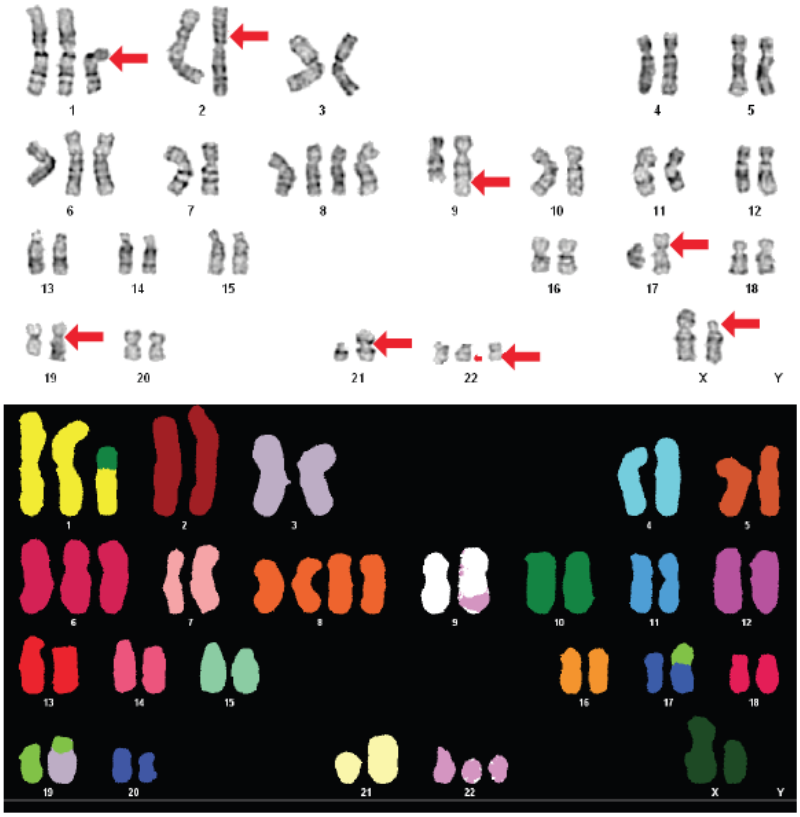
5.3 Results

5.3.1 Identification of T315I BCR-Abl Mutant Within KCL-22IR Cells

During cloning process of KCL-22IR cells, IR cells were sent for whole exome sequencing. Cells were also sent for SKY and G-banding Karyotyping analysis, along with in depth analysis of BCR-ABL kinase domain mutation analysis. Karyotyping revealed an abnormal karyotype with complex and numerical aberrations including the t(9;22) consistent with CML. The predominant clone (seventeen out of twenty cells) is shown in **(Figure 5.1a).** The predominant clone included eight structural abnormalities, an extra copy of the Philadelphia chromosome, trisomy 6, and tetrasomy 8. Two subclones were also identified with the same structural aberrations of the predominant clone. One subclone additionally had four copies of chromosome 6 and five copies of chromosome 8. The other subclone had four copies of chromosome 6, a structural aberration in one chromosome 6, and a derivative chromosome 18 resulting from an unbalanced translocation between chromosome 5 and 18 (these clones made up 5% of the population). Detailed karyotype results included:

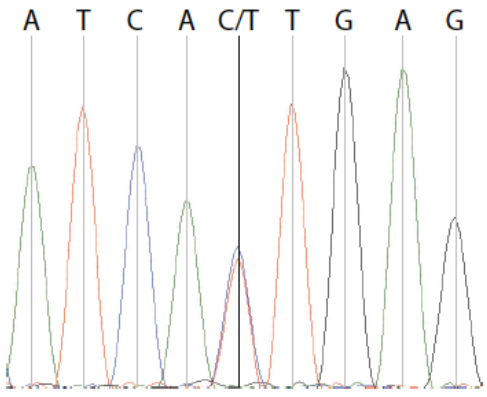
50~54,X,del(X)(p11.4p22.2),+der(1;10)(q10;p10),dup(2)(p13p21),+6,+8,+8,t(9;22)(q34;q11.2), der(17;19)(q10;q10),+19,der(19)t(3;19)(q25;q13.1),i(21)(q10),+der(22)t(9;22)[cp17]/. 55,idem,+6,+8[2]/. 51,idem,+6,add(6)(q13),der(18)t(5;18)(p13;p11.2)[1*]

a.)



b.)

KCL-22-R cell line



Bcr-Abl Kinase Domain

Figure 5.1 Karyotyping and ABL Kinase Domain Analysis. a.) T(9;22) identified along with trisomy 6, tetrasomy 8, and an extra Ph+ chromosome. **b.)** T315I mutation was detected in approximately an equal amount to the normal allele. Overall, karyotype was abnormal with significant alterations highlighted with red arrows.

BCR-Abl kinase domain analysis identified a mutation in the BCR-ABL kinase domain. The T315I mutation was detected in approximately an equal amount to the normal allele. Two minor variants were detected. One variant c.1087_1088delGA had a frameshift mutation starting at aspartate 363 and continued for 18 codons resulting in a truncated protein. The clinical significance of this mutation and whether it is responsible for imatinib resistance is unknown. However we are further investigating whether any of our clones possess this variant. A 35-bp insertion at the junction of exon 8 and 9 was detected that causes premature truncation of the protein that includes part of the kinase domain (Laudadio et al, J Mol Diagn 2008, 10:177-180). The insertion appears to be caused by alternative splicing of the 35-bp sequence from intron 8. This mutation is frequently found in imatinib-resistant patients and there is preliminary evidence that the mutation may cause imatinib resistance, computer predicted only, (Lee et al, Mol Cancer Ther 2008 7:3834-3841) but this has not been confirmed in the published literature.

5.3.2 Identification of Various Genetic Alterations within WT and IR KCL-22 Cells

Along with ABL kinase domain analysis, IR cells were sent to Otogenetics Corporation for whole exome sequencing. Generated IR clones from chapter one were sent for whole exome sequencing as well as WT KCL-22 cells. Sequencing results were analyzed utilizing a custom bioinformatics pipeline adapted (as described in methods) from the PROVEAN program. SIFT was also employed alongside PROVEAN in order to ensure validity of results. PROVEAN and SIFT programs together identified more than 100-deleterious (PROVEAN) and 100-damaging (SIFT) genetic variations within the KCL-22R (T315I) cells, as compared to the <80 variants found within the WT cells **(Table 2 Appendix).**

5.3.3 Clarification of Selected Genetic Variants within KCL-22IR Cells

Identified variants were narrowed down to a selected number based off of PROVEAN and SIFT scores. PROVEAN scores and SIFT scores were made stringent, by lowering PROVEAN score, in order to decrease likelihood of false interpretations **(Table 5.1).** Identified genes using this approach were then queried for biological associations using GeneMANIA. Utilizing this tool we were able to summarize associations amongst genes listed by our bioinformatics pipeline **(Figure 1 Appendix).** Next genes were chosen based on two criteria: 1.) They were not significantly involved within the same molecular pathways. 2.) Their variant must have occurred within a vital region of the protein, such as a DNA binding domain, tyrosine kinase domain, or other conserved region. Such rationale was used in order to obtain a broad set of genes involved in multiple pathways. Six genes were finally arrived at which were PIK3CA, FLT4, ERBB3, TNFRSF14, NOTCH1, B-Raf, and STAT5A. gDNA was obtained from KCL-22IR cells in culture and respective genes were amplified in order to verify findings obtained from whole exome sequencing and PROVEAN/SIFT scores **(Figure 5.2a).** Sequencing results demonstrated that gene variants were present within KCL-22IR genome, and were confirmed using both forward and reverse primer sets.

5.3.4 Identification of FLT 4 Variant within Random Patient Cancer Samples

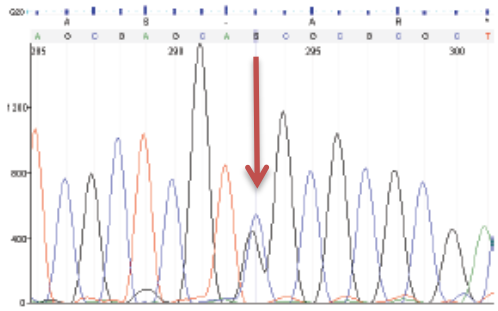
After clarifying variants identified through bioinformatics pipeline and whole exome sequencing, 56 random cancer patient samples were obtained (samples consisted mainly, 90%, of leukemia and lymphoma cancers, and the remaining cancers varied between brain, lung, and pancreatic cancers). GDNA was purified from these patients. The 6 identified gene variants were amplified in 10-patients to determine frequency of mutations. Of the 6-identified genes, nucleotide

alterations which led to a FLT4 H890Q variant was identified and confirmed within two of the first 10-patients. FLT4 was then the only gene used in screening the remaining 46 patient samples. Of the complete 56 random cancer patients, 13 patients (23% of patients) displayed the FLT4 H890Q mutation **(Figure 5.2b).**

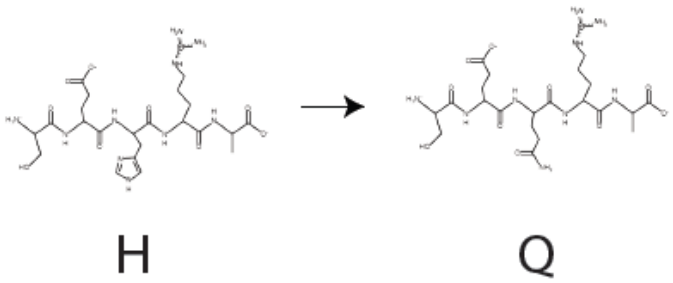
Table 5.1 Genomic Variants Identified as Potential Leads

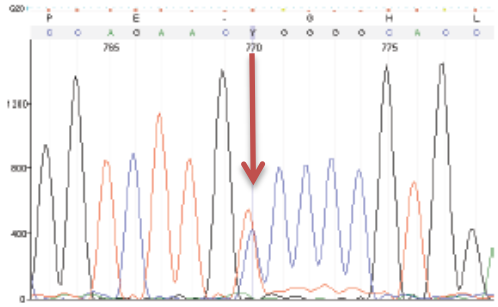
GENE	Variant	PROVEAN Score	Interpretation	SIFT Score	Interpretation
ABL1	T334I	-5.722	Deleterious	0.002	Damaging
ARID2	A1739V	-2.652	Deleterious	0.001	Damaging
AXL	R217H	-3.114	Deleterious	0.003	Damaging
BRAF	H608Y	-2.581	Deleterious	0.027	Damaging
BRCA1	E1038G	-5.783	Deleterious	0.044	Damaging
CDH1	P170L	-8.286	Deleterious	0.001	Damaging
ERBB3	G854S	-5.329	Deleterious	0	Damaging
FGFR4	T262K	-3.912	Deleterious	0.001	Damaging
	P683L	-9.146	Deleterious	0	Damaging
FLT4	H890Q	-5.137	Deleterious	0.045	Damaging
GNA11	Q209H	-4.814	Deleterious	0.007	Damaging
GPR124	C218F	-8.654	Deleterious	0	Damaging
	S535L	-3.463	Deleterious	0.002	Damaging
IKZF1	C150Y	-9.678	Deleterious	0.001	Damaging
IRS2	D1257H	-3.473	Deleterious	0.007	Damaging
KEAP1	L365Q	-4.939	Deleterious	0.001	Damaging
MST1R	M1196I	-3.868	Deleterious	0.009	Damaging
NOTCH1	G1195R	-7.398	Deleterious	0.023	Damaging
PDGFRB	R456S	-3.092	Deleterious	0.032	Damaging
PIK3CA	E545G	-5.866	Deleterious	0.001	Damaging
STAT5A	G431C	-7.99	Deleterious	0.001	Damaging
TET2	P50R	-3.105	Deleterious	0.002	Damaging
TNFRSF14	G174W	-6.046	Deleterious	0.001	Damaging
ZNF703	P284L	-2.815	Deleterious	0.355	Tolerated

a.)

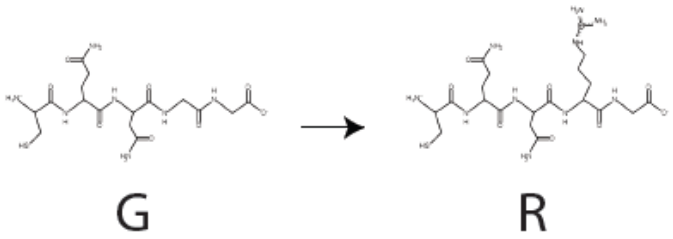


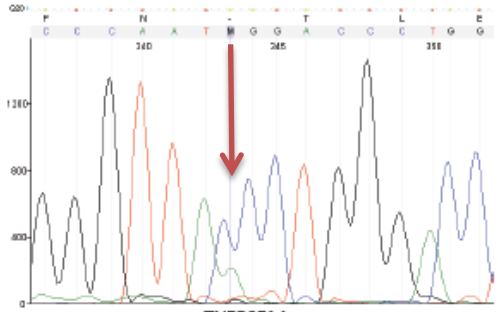
FLT4 Kinase Domain



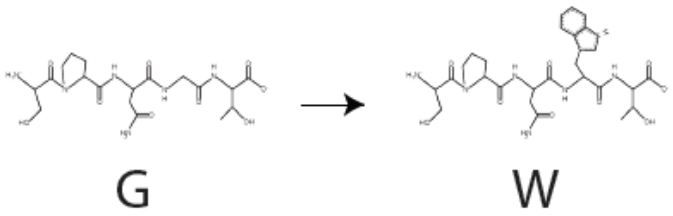


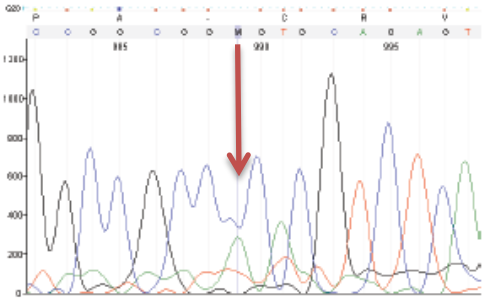
NOTCH1 EGF Domain



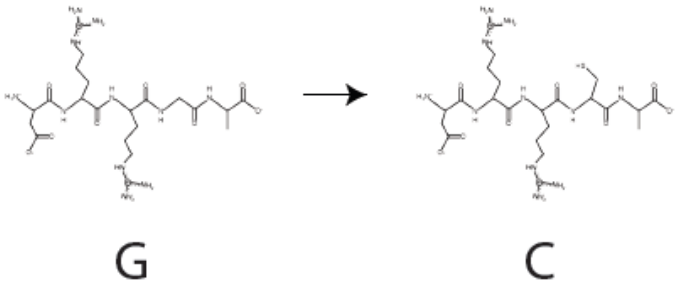


TNFRSF14

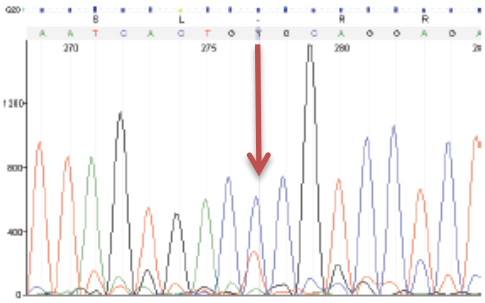




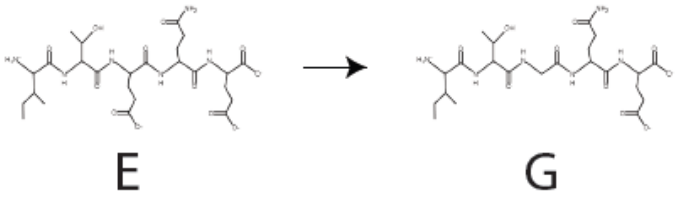
STAT5A DNA binding domain



G C



PIK3CA Helical Domain



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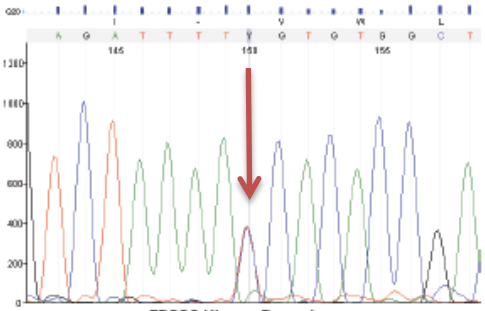
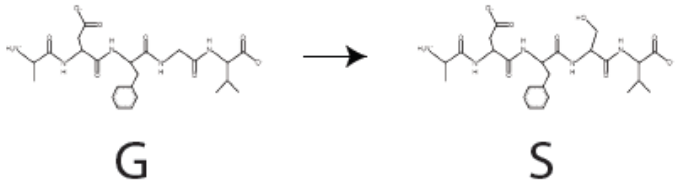
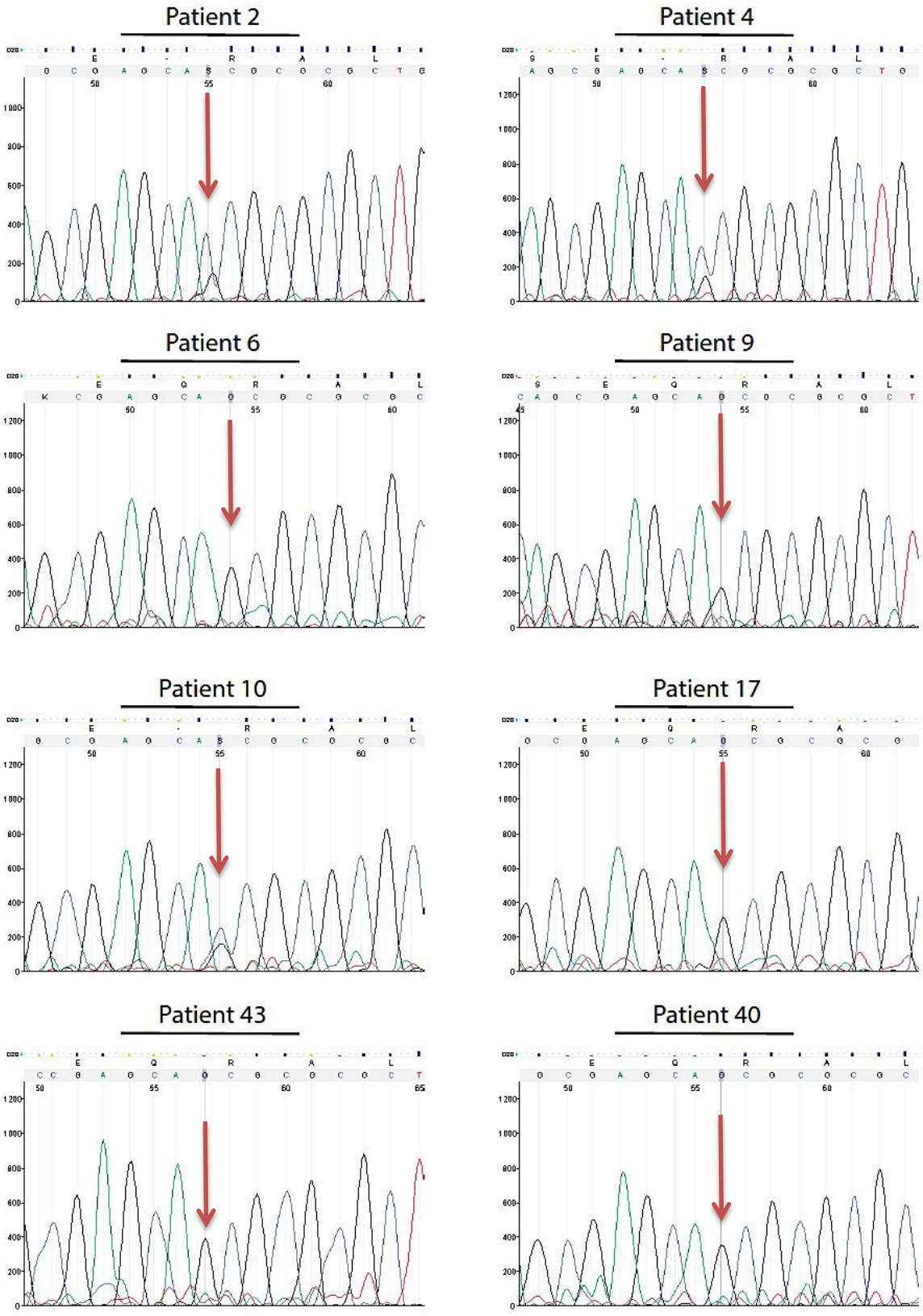


ERBB3 Kinase Domain



G S

b.)



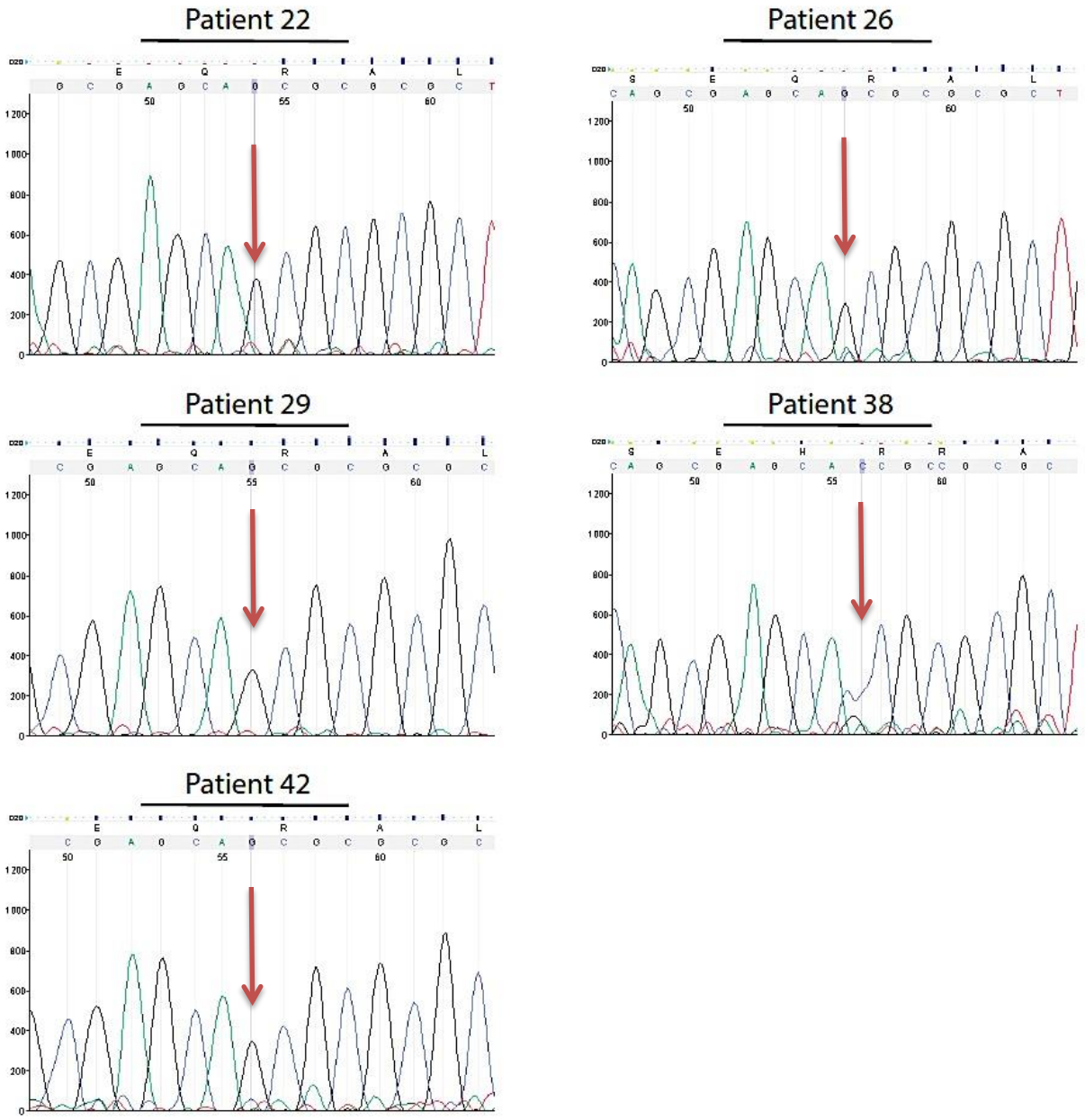


Figure 5.2 Chromatograph of Sequencing Results. a.) Variant clarification (red arrows) in KCL-22IR cells, and respective amino acid changes. **b.)** Patient variant clarification of FLT4 H890Q variant. Thirteen of the fifty-six patients displayed this FLT4 H890Q variant (red arrows). These results demonstrate that this mutation is not limited to a laboratory established cell model, but may also serve a role in clinical disease. The FLT4 H890Q variant occurs within the kinase domain of the protein, and is expected to be damaging to protein function as predicted by our bioinformatics program

5.4 Discussion:

Navigating the genomic landscape of cancer remains to be a challenging task. Improved technology and new bioinformatics tools such as PROVEAN are helping pave the way for a better understanding of the genetic underlying of cancer. However, we still have further to travel to fully utilize information obtained from a patient's genome in designing a personalized therapy. Such findings as the ones we have demonstrated serve as a "proof of concept" for how bioinformatics tools can be utilized to assess damaging mutations within a patient's exome. The results presented in **Figure 5.3** highlight how more than one mutation may be driving drug resistance in KCL-22R cells. After performing an analysis of the whole exomes of both KCL-22WT and IR cells, there were several fold more mutations present within the IR(T315I) versus the WT(Parent) KCL-22 cells. Such findings are significant for future clinical use of whole exome screening of cancer patients. For example, it would be advantageous for the clinician to identify genetic alterations that may lead to drug resistance before starting a patient on a treatment regime. Besides these findings, our results reveal numerous novel mutations (all with significant PROVEAN/SIFT scores). In the past, it was commonly held that such mutations were caused by exposure to the drug itself. However today that rational is losing support. Instead, novel mutations present within drug resistance cancers are being attributed to small populations of opportunistic cancer mutants that remain dormant, but become active in favoring conditions. Finding ways of identifying such minute populations of highly malignant cancer cells is of extreme importance. The mutations identified within Table3 may prove interesting when fully characterized with complete protein analysis, and may lead to data that would shine new light on how drug resistance is acquired within CML. Not only will these findings only apply to CML treatment, but will also prove useful towards understanding cellular mechanisms present in other cancers.

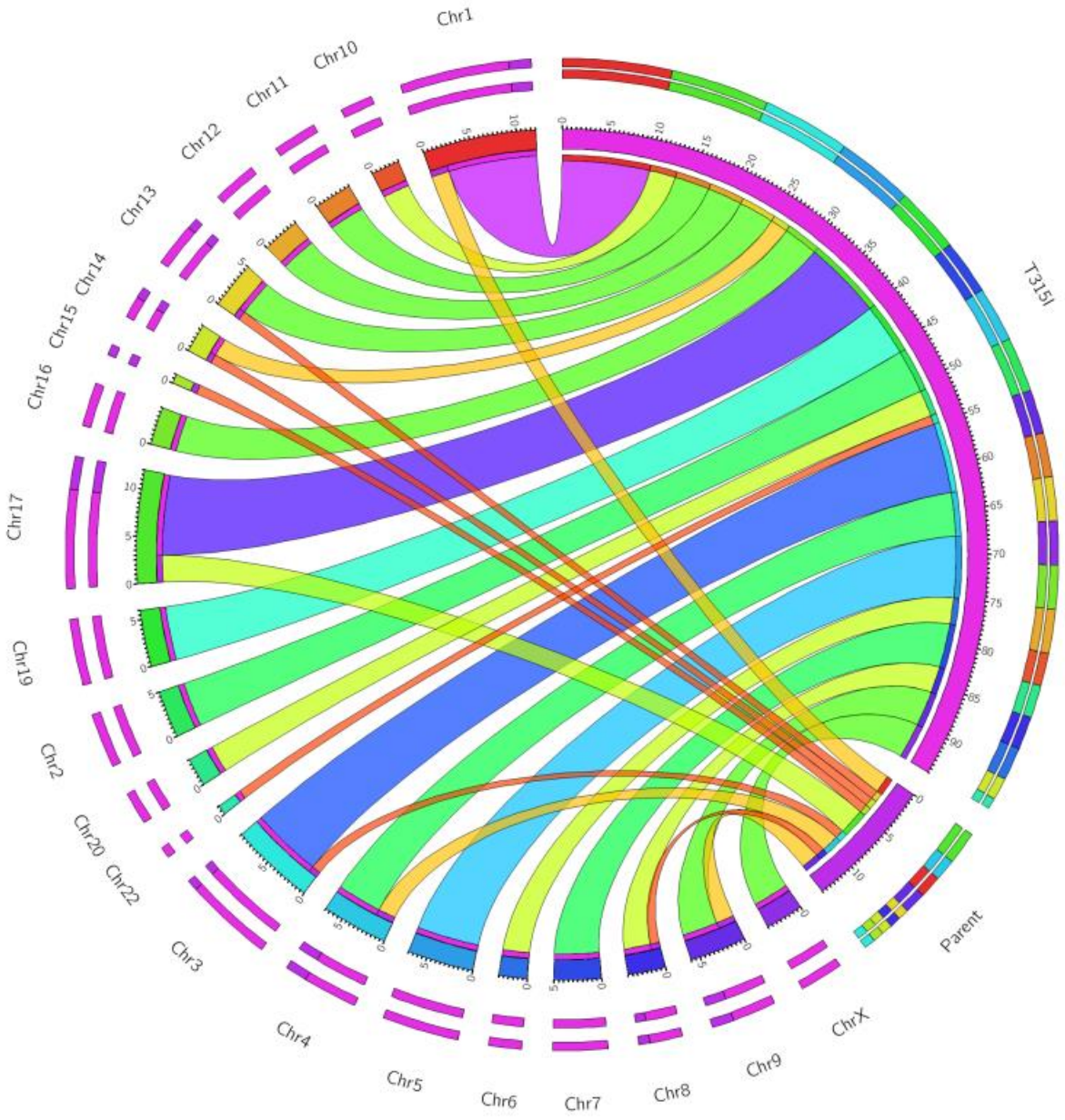


Figure 5.3 Circos Diagram of Genetic Alterations Present in Both Parent and T315I KCL-22 Cells. This diagram gives a graphic representation of the magnitude of genetic alterations seen in the KCL-22IR (T315I) cells in comparison to the parent (WT) cells. Larger ribbons indicate more mutations are present within respective (connecting) chromosome. Size of chromosome band indicates number of mutations overall (both WT and IR cells) found within chromosome. As shown, T315I resistant cells demonstrate a much larger array of mutations in comparison to the parental cells.

Chapter VI: Overview

In summary, our study has led to the generation of a T315I CML cell line, KCL-22IR, which shows high drug insensitivity towards first and second generation kinase inhibitors. Such a cell line will prove useful in future studies involving high throughput drug screening, and cell signaling studies investigating acquired drug resistance within cancer. Our study has also revealed non-CML cancer drugs efficacy in inhibiting T315I CML mutants. Such findings are significant, and demonstrate that drug resistant cancers can potentially be treated successfully if the cancer cells are screened against various drugs in vitro, ultimately saving the patient time and side effects. We also revealed novel activation states in key molecular pathways that coincide with CML drug resistance. Along with these findings, our study highlighted the protein ERK1/2 as being integral to T315I CML survival. For future directions, investigating the mechanisms of how ERK1/2 is regulated by BCR-Abl will be of interest. Our studies highlighted that activated Raf (an upstream modulator of ERK1/2) is not necessary for ERK1/2 activity. Identifying why this anomaly is occurring will prove interesting in future studies. ERK1/2 serves as a potential therapeutic target in our T315I drug resistant CML cell line, and can possibly be utilized in combination therapies. For example, *Khateb et al.* demonstrated that allosteric and ATP competitive inhibitors in combination can be utilized in overcoming BCR-Abl T315I CML, however the allosteric inhibitors reported within this study are not yet FDA approved. From our screening results we demonstrated that inhibition of mTOR can also induce cell death. One future aim would be to utilize FDA approved ERK1/2, MEK1/2, and mTOR inhibitors (such as Trametinib and Temsirolimus) in combination against our T315I drug resistant CML cell lines. Performing such experiments will prove useful for both scientists and clinicians in identifying a therapy that utilizes FDA approved compounds in combating drug resistant cancers. Finally, we have illustrated results from a bioinformatics pipeline that is capable of accurately predicting genetic variants from whole exome screening

results. We generated a list of over a hundred different variants impacting kinases, phosphatases, and transcription factors that are present in our T315I CML cell line. Future directions can be aimed at characterizing such variants, and their implications in the general patient population. As a "proof of concept", within our presented study we have already performed this by partially illustrating the abundance of a novel FLT4 H890Q mutation within leukemia patient samples. In conclusion, our study has illustrated how T315I drug resistant CML may rely on ERK1/2 activity for survival, and BCR-Abl may utilize multiple intermediates in the process of insuring constant ERK1/2 activity **(Figure 6.1)**.

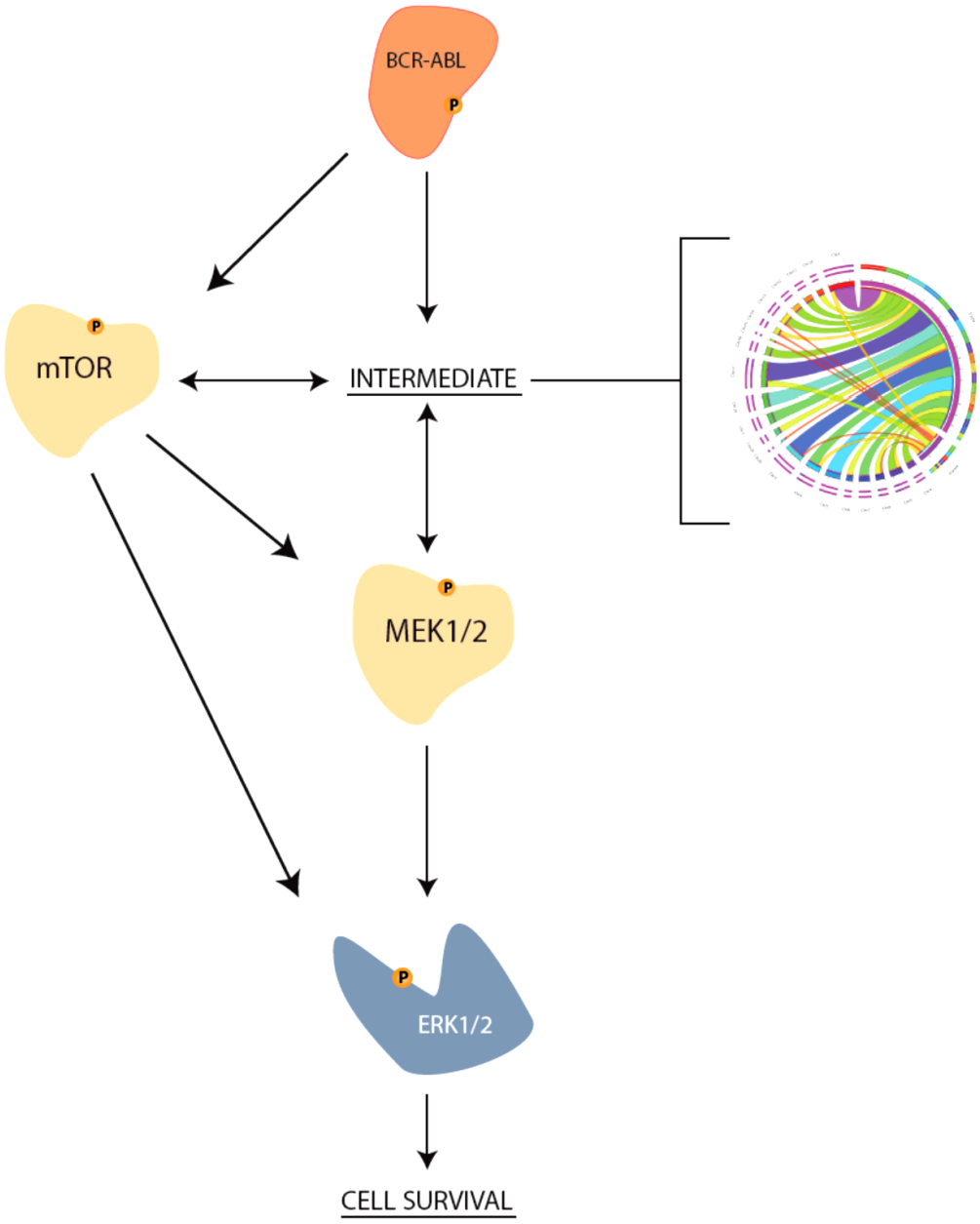


Figure 6.1 Schematic of BCR-Abl Modulation of MEK1/2 and ERK1/2 Activity within KCL22-IR T315I CML Cell Line. BCR-Abl utilizes MEK1/2 or ERK1/2 activity to ensure cell survival, and may be doing so either directly or through the utilization of an intermediate. Such an intermediate may be present within the multitude of genomic variants that were identified in our present study.

Literature Cited

Akritopoulou-Zanze I, Hajduk PJ.(2009) Kinase-targeted libraries: design and synthesis of novel, potent, and selective kinase inhibitors. Drug Discov. Today 2009; 14:291-297.

Apperley JF. (2007) Part I: mechanisms of resistance to imatinib in chronic myeloid leukaemia. Lancet Oncol. 2007 Nov;8(11):1018-29.

Apperley JF. (2007) Part II: management of resistance to imatinib in chronic myeloid leukaemia. Lancet Oncol. 2007 Dec;8(12):1116-28.

Arena S, Benvenuti S, Bardelli A. (2005) Genetic analysis of the kinome and phosphatome in cancer. Cell Mol Life Sci. 2005 Sep;62(18):2092-9.

Aster, J.C., Blacklow, S.C. & Pear, W.S. (2011) Notch signaling in T-cell lymphoblastic leukaemia/lymphoma and other haematological malignancies. The Journal of Pathology, 223, 262-273.

Bartram CR, de Klein A, Hagemeijer A, et al. (1983) Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature, 1983;306:277-280.

Bixby D., Talpaz M. (2011) Seeking the causes and solutions to imatinib-resistance in chronic myeloid leukemia. Leukemia. 2011. Jan;25(1):7-22. Doi:10.1038/leu.2010.238.

Chai SK, Nichols GL, Rothman P. (1997) Constitutive activation of JAKs and STATs in BCR-Abl-expressing cell lines and peripheral blood cells derived from leukemic patients. J Immunol. 1997 Nov 15;159(10):4720-8.

Chatenoud Lilian, Bertuccio P, Bosetti, C, Levi F, Negri E, Vecchia C (2010). Childhood Cancer Mortality in America, Asia, and Oceania, 1970 Through 2007. Cancer. 2010 Nov 1;116(21):5063-74. doi: 10.1002/cncr.25406.

Chen E, Staudt LM, Green AR. (2012) Janus kinase deregulation in leukemia and lymphoma. Immunity. 2012 Apr 20;36(4):529-41. doi: 10.1016/j.immuni.2012.03.017.

Choi Y, Sims GE, Murphy S, Miller JR, Chan AP (2012) Predicting the Functional Effect of Amino Acid Substitutions and Indels. PLoS ONE 7(10): e46688.

Chow EJ, Puumala SE, Mueller BA, Carozza SE, Fox EE, Horel S, et al. (2010) Childhood cancer in relation to parental race and ethnicity: a 5-state pooled analysis. Cancer 2010;116:3045-53.

Chung E, Kondo E. (2011) Role of Ras/Raf/MEK/ERK signaling in physiological hematopoiesis and leukemia development. Immunol Res (2011) 49:248-268. doi: 10.1007/s12026-010-8187-5

Cilloni D, Saglio G. (2012) Molecular pathways: BCR-ABL. Clin Cancer Res. 2012 Feb 15;18(4):930-7. doi: 10.1158/1078-0432.CCR-10-1613.

Coleman M, Marshall C, Olson M. (2004) RAS and RHO GTPases in G1-phase cell-cycle regulation. Nature Reviews Molecular Cell Biology 5, 355-366; doi:10.1038/nrm1365.

Comert M, Baran Y, Saydam G. (2013) Changes in molecular biology of chronic myeloid leukemia in tyrosine kinase inhibitor era. Am J Blood Res. 2013 Aug 19;3(3):191-200.

Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science, 1990;247:824-830.

Danial N, Pernis A, Rothman P (1995) JAK-STAT signaling induced by the v-abl oncogene. Science 29 September 1995; Vol. 269 no. 5232 pp.1875-1877 doi: 10.1126/science.7569929.

de Klein, A., van Kessel AG, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, Spurr NK, Heisterkamp N, Groffen J, Stephenson JR (1982). A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature, 300(5894):765−767.

Deeren D. (2013) Unravelling survival pathways: The road to next-generation chronic myeloid leukaemia drugs? Leuk Res. 2013 Nov;37(11):1438-9. doi: 10.1016/j.leukres.2013.09.004.

Deininger, Michael, Goldman, John, Melo, Junia (2000). The molecular biology of chronic myeloid leukemia. Blood, 15 November 2000, Volume 96, Number 10: 3343-3356.

de Groot RP, Raaijmakers JA, Lammers JW, Jove R, Koenderman L. (1999) STAT5 activation by BCR-Abl contributes to transformation of K562 leukemia cells. Blood. 1999 Aug 1;94(3):1108-12.

Donato NJ, Wu JY, Stapley J, Gallick G, Lin H, Arlinghaus R, Talpaz M. (2003) BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. Blood. 2003 Jan 15;101(2):690-8.

Dorshkind, Kenneth (2010). Not a split decision for human hematopoiesis. Nat Immunol. 2010 Jul;11(7):569-70. doi:10.1038/ni0710-569.

Doulatov, Sergei, Notta, F., Laurenti, E., Dick, J.(2012) Hematopoiesis: A human perspective. Cell Stem Cell. 2012 Feb 3;10(2):120-36. doi: 10.1016/j.stem.2012.01.006.

Dreesen O, Brivanlou AH. (2007) Signaling pathways in cancer and embryonic stem cells. Stem Cell Rev. 2007 Jan;3(1):7-17.

Drexler HG, MacLeod RA, Uphoff CC. (1999) Leukemia cell lines: in vitro models for the study of Philadelphia chromosome-positive leukemia. Leuk Res. 1999 Mar;23(3):207-15.

Enver T, Greaves M.(1998) Loops, lineage, and leukemia. Cell. 1998 Jul 10;94(1):9-12.

Faderl, S., Talpaz, M., Estrov, Z., O'Brien, S., Kurzrock, R., Kantarjian, H.M, (1999). The biology of chronic myeloid leukemia. NEJM, 341, 164-172.

Farnault, Laure, Sanchez C., Baier C., Treut T., Costello RT. (2012) Hematological malignancies escape from NK cell innate immune surveillance: mechanisms and therapeutic implications. Clin Dev Immunol. 2012;2012:421702. doi:10.1155/2012/421702.

Fenouille, Nina et al. (2010) Persistant activation of the Fyn/ERK kinase signaling axis mediates imatinib resistance in chronic myelogenous leukemia cells through upregulation of intracellular SPARC. Cancer Res December 1, 2010 70; 9659.

Friedman AD. (2007) Transcriptional control of granulocyte and monocyte development. Oncogene. 2007 Oct 15;26(47):6816-28.

Galderisi U, Paolo Jori F, Giordano A (2003) Cell cycle regulation and neural differentiation. Oncogene 22, 5208-5219.

Gozgit JM, Wong MJ, Moran L, Wardwell S, Mohemmad QK, Narasimhan NI, Shakespeare WC, Wang F, Clackson T, Rivera VM. (2012) Ponatinib (AP24534), a multitargeted pan-FGFR inhibitor with activity in multiple FGFR-amplified or mutated cancer models. Mol Cancer Ther. 2012 Mar;11(3):690-9. doi: 10.1158/1535-7163.MCT-11-0450.

Greaves M. (2009) Darwin and evolutionary tales in leukemia. The Ham-Wasserman Lecture. Hematology Am Soc Hematol Educ Program. 2009:3-12. doi: 10.1182/asheducation-2009.1.3.

Green AS, Chapuis N, Lacombe C, Mayeux P, Bouscary D, Tamburini J. (2011) LKB1/AMPK/mTOR signaling pathway in hematological malignancies: from metabolism to cancer cell biology. Cell Cycle. 2011 Jul 1;10(13):2115-20.

Greuber EK, Smith-Pearson P, Wang J, Pendergast AM. (2013) Role of ABL family kinases in cancer: from leukaemia to solid tumours. Nat Rev Cancer. 2013 Aug;13(8):559-71. doi: 10.1038/nrc3563.

Grippo JF, Zhang W, Heinzmann D, Yang KH, Wong J, Joe AK, Munster P, Sarapa N, Daud A. (2013). A phase I, randomized, open-label study of the multiple-dose pharmacokinetics of vemurafenib in patients with BRAF V600E mutation-positive metastatic melanoma. Cancer Chemother Pharmacol. 2013 Nov 1.

Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G. (1984) Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell, 1984;36:93-99.

Gu L, Vogiatzi P, Puhr M, Dagvadorj A, Lutz J, Ryder A, Addya S, Fortina P, Cooper C, Leiby B, Dasgupta A, Hyslop T, Bubendorf L, Alanen K, Mirtti T, Nevalainen M (2010) Stat5

promotes metastatic behavior of human prostate cancer cells in vitro and in vivo. Endocrine-Related Cancer, 2010 (17) 481-493.

Hanahan, Douglas, Weinberg, Robert (2011). Hallmarks of Cancer: The Next Generation. Cell. 2011; Vol. 144, Issue 5, 646-674.

Hansen J, Palmfeldt J, Vang S, Corydon TJ, Gregersen N, Bross P. (2011) Quantitative proteomics reveals cellular targets of celastrol. PLoS One. 2011;6(10):e26634. doi: 10.1371/journal.pone.0026634.

Hantschel O, Grebien F, Superti-Furga G. (2012) The growing arsenal of ATP-competitive and allosteric inhibitors of BCR-ABL. Cancer Res. 2012 Oct 1;72(19):4890-5. doi: 10.1158/0008-5472.CAN-12-1276.

Havelange V, Demoulin JB. (2013) Review of current classification, molecular alterations, and tyrosine kinase inhibitor therapies in myeloproliferative disorders with hypereosinophilia. J Blood Med. 2013 Aug 9;4:111-21. doi: 10.2147/JBM.S33142.

Horne SD, Stevens JB, Abdallah BY, Liu G, Bremer SW, Ye CJ, Heng HH. (2013) Why imatinib remains an exception of cancer research. J Cell Physiol. 2013 Apr;228(4):665-70. doi: 10.1002/jcp.24233.

Howlader N, Noone AM, Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer Statistics Review (2013) National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2010/, based on November 2012 SEER data submission, posted to the SEER web site, April 2013.

Hoyle M, Rogers G, Moxham T, Liu Z, Stein K. (2011) Cost-effectiveness of dasatinib and nilotinib for imatinib-resistant or -intolerant chronic phase chronic myeloid leukemia. Value Health. 2011 Dec;14(8):1057-67. doi: 10.1016/j.jval.2011.07.006.

Huang W, Hu L, Bei L, Hjort E, Eklund EA. (2012) The leukemia-associated fusion protein Tel-platelet-derived growth factor receptor β (Tel-PdgfRβ) inhibits transcriptional repression of PTPN13 gene by interferon consensus sequence binding protein (Icsbp). J Biol Chem. 2012 Mar 9;287(11):8110-25. doi: 10.1074/jbc.M111.294884.

Huber R, Pietsch D, Gunther J, Welz B, Vogt N, Brand K (2012) Regulation of monocyte differentiation by specific signaling modules and associated transcription factor networks. Cell Mol Life Sci. 2013 Mar 24.

Hunter, Melissa, Avalos, Belinda. (1998). Phosphatidylinositol 3'-Kinase and SH2-Containing Inositol Phosphatase (SHIP) Are Recruited by Distinct Positive and Negative Growth-Regulatory Domains in the Granulocyte Colony-Stimulating Factor Receptor. J Immunol 1998; 160:4979-4987.

Ihle JN, Nosaka T, Thierfelder W, Quelle FW, Shimoda K. (1997) JAKs and Stats in cytokine signaling. Stem Cells. 1997;15Suppl 1:105-11; discussion 112.

Iwasaki, Hiromi, Akashi, Koichi (2007) Myeloid lineage commitment from the hematopoietic stem cell. Immunity. 2007 Jun;26(6):726-40.

Kabarowski JH, Allen PB, Wiedemann (1994) A temperature sensitive p210 BCR-ABL mutant defines the primary consequences of BCR-ABL tyrosine kinase expression in growth factor dependent cells. EMBO J. 1994 December 15; 13(24): 5887–5895.

Kapeller R, Cantley LC. (1994). Phosphatidylinositol 3-kinase. Bioessays. 1994 Aug;16(8):565-76.

Karaman M, Herrgard S, Treiber D, Gallant P, et al. (2008). A quantitative analysis of kinase inhibitor selectivity. Nat Biotech 26;(1) 2008. Doi:10.1038/nbt1358.

Khateb M, Ruimi N, Khamisie H, Najajreh Y, Mian A, Metodieva A, Ruthardt M, Mahajna J. (2012) Overcoming Bcr-Abl T315I mutation by combination of GNF-2 and ATP competitors in an Abl-independent mechanism. BMC Cancer. 2012 Nov 27;12:563. doi: 10.1186/1471-2407-12-563.

Keeshan K, Cotter TG, McKenna SL. (2002) High Bcr-Abl expression prevents the translocation of Bax and Bad to the mitochondrion. Leukemia. 2002 Sep;16(9):1725-34.

Kim J, Sohn I, Son DS, Kim DH, Ahn T, Jung SH. (2013) Prediction of a time-to-event trait using genome wide SNP data. BMC Bioinformatics. 2013 Feb 19;14:58. Doi:10.1186/1471-2105-14-58.

Kirken, RA, Rui H, Malabarba MG, Farrar WL. (1994) Identification of interleukin-2 receptor-associated tyrosine kinase p116 as novel leukocyte-specific Janus Kinase. J Biol Chem. 1994 Jul 22;269(29):19136-41.

Knight ZA, Lin H, Shokat KM. (2010) Targeting the cancer kinome through polypharmacology. Nat Rev Cancer. 2010 Feb; 10(2):130-7. Doi: 10.1038/nrc2787.

Kroll ME, Stiller CA, Murphy MF, Carpenter LM. (2011). Childhood leukaemia and socioeconomic status in England and Wales 1976-2005: evidence of higher incidence in relatively affluent communities persists over time. Br J Cancer. 2011 Nov 22;105(11):1783-7. Doi:10.1038/bjc.2011.415.

Kubonishi I, Miyoshi I (1983) Establishment of a Ph1 chromosome-positive cell line from chronic myelogenous leukemia in blast crisis. Int J Cell Cloning. 1983 Jun;1(2):105-17.

Kumar P, Henikoff S, Ng PC. (2009). Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc. 2009;4(7):1073-81. doi: 10.1038/nprot.2009.86.

Laudadio J, Quigley N, Henley D, Hubbard R (2008) Abl kinase domain pseudoexon insertion is not uncommon in BCR-Abl transcripts. J Mol Diagn. Sep 2008; 10(5):475-476.

Lee HK, Braynen W, Keshav K, Pavlidis P. (2005) ErmineJ: tool for functional analysis of gene expression data sets. BMC Bioinformatics. 2005 Nov 9;6:269.

Lee TS, Ma W, Zhang W, Giles F, Cortes J, Kantarjian H, Albitar M (2008) BCR-Abl alternative splicing as a common mechanism for imatinib resistance: evidence from molecular dynamics simulations. Mol Cancer Ther. 2008 Dec; 7(12):3844-41. Doi: 10.1158/1535-7163.MCT-08-0482. Epub 2008 Dec 3.

Lema, Carolina, Varela-Ramirez A, Aguilera R (2011) Differential nuclear staining assay for high-throughput screening to identify cytotoxic compounds. Curr Cell Biochem 2011 1(1):1-14.

Leonard W. J. and O'shea J. J. (1998): JAKs and STATs: biological implications. Annu. Rev. Immunol., 16,293-322.

Leung AY, Man CH, Kwong YL. (2013) FLT3 inhibition: a moving and evolving target in acute myeloid leukaemia. Leukemia. 2013 Feb;27(2):260-8. doi: 10.1038/leu.2012.195.

Levy, D.E., Darnell Jr., J.E., (2002) Stats: transcriptional control and biological impact. Nat. Rev. Mol. Cell. Biol. 3, 651-662.

Liu KD, Gaffen SL, Goldsmith MA. (1998) JAK/STAT signaling by cytokine receptors. Curr Opin Immunol. 1998 Jun;10(3):271-8.

Loh ML, Zhang J, Harvey RC, Roberts K, Payne-Turner D, Kang H, Wu G, Chen X, Becksfort J, Edmonson M, Buetow KH, Carroll WL, Chen IM, Wood B, Borowitz MJ, Devidas M, Gerhard DS, Bowman P, Larsen E, Winick N, Raetz E, Smith M, Downing JR, Willman CL, Mullighan CG, Hunger SP. (2012) Tyrosine kinome sequencing of pediatric acute lymphoblastic leukemia: a report from the Children's Oncology Group TARGET Project. Blood. 2013 Jan 17;121(3):485-8. doi: 10.1182/blood-2012-04-422691.

Lugo TG, Pendergast AM, Muller AJ, Witte ON. (1990) Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science, 1990;247:824-830
.

Luo C, Laaja P. (2004) Inhibitors of JAKs/STATs and the kinases: a possible new cluster of drugs. Drug Discov Today. 2004 Mar 15;9(6):268-75.

Mackay H, Twelves C. (2007) Targeting the protein kinase C family: are we there yet? Nature Reviews Cancer 7, 554-562 (July 2007). doi:10.1038/nrc2168.

Mahon FX. (2012). Is going for cure in chronic myeloid leukemia possible and justifiable? Hematology Am Soc Hematol Educ Program. 2012;2012:122-8. doi: 10.1182/asheducation-2012.1.122.

Marin D. (2013) Management of the new patient with CML in chronic phase. Curr Hematol Malig Rep. 2013 Mar;8(1):37-42. doi: 10.1007/s11899-012-0151-0.

Martin GS (2001) The hunting for SRC. Nat Rev Cancer 4:470-480.

Mathers CD, Loncar, D (2006). Projections of Global Mortality and Burden of Disease from 2002 to 2030. Plos Med 3(11): e442. doi:10.1371/journal.pmed.0030442.

Mauro MJ. (2013) Striving to achieve safe, permanent treatment discontinuation in chronic myeloid leukemia. Leuk Res. 2013 Nov;37(11):1395-403. doi: 10.1016/j.leukres.2013.07.029.

McArthur GA. (2012) The coming of age of MEK. Lancet Oncol. 2012 Aug;13(8):744-5. doi: 10.1016/S1470-2045(12)70317-0.

Melo JV, Barnes DJ. (2007) Chronic myeloid leukaemia as a model of disease evolution in human cancer. Nat Rev Cancer. 2007 Jun;7(6):441-53.

Miranda MB, Johnson DE (2007). Signal Transduction pathways that contribute to myeloid differentiation. Leukemia (2007) 21, 1363-1377; doi:10.1038/sj.leu.2404690.

Murati A, Brecqueville M, Devillier R, Mozziconacci MJ, Gelsi-Boyer V, Birnbaum D. (2012) Myeloid malignancies: mutations, models and management. BMC Cancer. 2012 Jul 23;12:304. doi: 10.1186/1471-2407-12-304.

Murphy, K., Travers, P., Walport, M. (2008). Janeway's Immunobiology (7th ed.). New York, NY: Garland Science, Taylor & Francis Group, LLC, an informa business.

Nagy Z. S., LeBaron M. J.,Ross J. A.,Mitra A.,Rui H., Kirken R. A. (2009) STAT5 regulation of BCL10 parallels constitutive NFκB activation in lymphoid tumor cells. Mol. Cancer 8, 67

Nelson EA, Walker SR, Weisberg E, Bar-Natan M, Barrett R, Gashin LB, Terrell S, Klitgaard JL, Santo L, Addorio MR, Ebert BL, Griffin JD, Frank DA. (2011) The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. Blood. 2011 Mar 24;117(12):3421-9. doi: 10.1182/blood-2009-11-255232.

Nowell P, Hungerford D (1960). A minute chromosome in human chronic granulocytic leukemia. Science, 1960; 132:1497.

O'Hare T, Deininger MW, Eide CA, Clackson T, Druker BJ. (2011) Targeting the BCR-ABL signaling pathway in therapy-resistant Philadelphia chromosome-positive leukemia. Clin Cancer Res. 2011 Jan 15;17(2):212-21. doi: 10.1158/1078-0432.CCR-09-3314.

O'Hare T, Shakespeare WC, Zhu X, Eide CA, Rivera VM, Wang F, Adrian LT, Zhou T, Huang WS, Xu Q, Metcalf CA 3rd, Tyner JW, Loriaux MM, Corbin AS, Wardwell S, Ning Y, Keats JA, Wang Y, Sundaramoorthi R, Thomas M, Zhou D, Snodgrass J, Commodore L, Sawyer

TK, Dalgarno DC, Deininger MW, Druker BJ, Clackson T. (2009). AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. Cancer Cell. 2009 Nov 6;16(5):401-12. doi: 10.1016/j.ccr.2009.09.028.

Oda T, Heaney C, Hagopian JR, Okuda K, Griffin JD, Druker BJ. (1994) Crkl is the major tyrosine-phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia. J Biol Chem. 1994 Sep 16;269(37):22925-8.

O'Shea JJ, Plenge R. (2012) JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. Immunity. 2012 Apr 20;36(4):542-50. doi: 10.1016/j.immuni.2012.03.014.

Panjarlan, Shoghag, R. Lacob, S. Chen, J. Engen, T. Smithgall (2013) Structure and Dynamic Regulation of Abl Kinases. The Journal of Biological Chemistry Vol. 288, No. 8, pp. 5443-5450, doi:10.1074/jbc.R112.438382.

Pavlu J, Szydlo RM, Goldman JM, Apperley JF. (2011) Three decades of transplantation for chronic myeloid leukemia: what have we learned? Blood. 2011 Jan 20;117(3):755-63. doi: 10.1182/blood-2010-08-301341.

Pearson G, Robinson F, Gibson TB, XU BE, Karandikar M, Berman K, Cobb M. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocrine Reviews 22(2): 153-183.

Pendergast AM, Quilliam LA, Cripe LD, Bassing CH, Dai Z, Li N, Batzer A, Rabun KM, Der CJ, Schlessinger J, et al. (1993) BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. Cell. 1993 Oct 8;75(1):175-85

Polak R, Buitenhuis M (2012) The PI3K/PKB signaling module as key regulator of hematopoiesis: implications for therapeutic strategies in leukemia. Blood. 2012 November 7, 2011; 119:911-923. doi: 10.1182/blood-2011-07-366203.

Qattan M, Demonacos C, Krstic-Demonacos M. (2012) Roadmap to personalized medicine. Croat Med J. 2012 Aug;53(4):294-7.

Radich JP, Dai H, Mao M, Oehler V, Schelter J, Druker B, Sawyers C, Shah N, Stock W, Willman CL, Friend S, Linsley PS. (2006) Gene expression changes associated with progression and response in chronic myeloid leukemia. Proc Natl Acad Sci U S A. 2006 Feb 21;103(8):2794-9.

Radich JP. (2007) The Biology of CML blast crisis. Hematology Am Soc Hematol Educ Program. 2007:384-91.

Radivoyevitch T, Hlatky L, Landaw J, Sachs RK. (2012). Quantitative modeling of chronic myeloid leukemia: insights from radiobiology. Blood. 2012 May 10;119(19):4363-71. Doi:10.1182/blood-2011-09-381855.

Rassi FE, Khoury HJ. (2013) Bosutinib: a SRC-ABL tyrosine kinase inhibitor for treatment of chronic myeloid leukemia. Pharmgenomics Pers Med. 2013 Aug 5;6:57-62.

Rix U, Hantschel O, Dürnberger G, Remsing Rix LL, Planyavsky M, Fernbach NV, Kaupe I, Bennett KL, Valent P, Colinge J, Köcher T, Superti-Furga G. (2007) Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets. Blood. 2007 Dec 1;110(12):4055-63.

Ross JA, Nagy ZS, Cheng H, Stepkowski SM, Kirken RA. (2007) Regulation of T cell homeostasis by JAKs and STATs. Arch Immunol Ther Exp (Warsz). 2007 Jul-Aug;55(4):231-45.

Ross JA, Spadaro M, Rosado D, Cavallo F, Kirken R, Pericle F (2013) Inhibition of JAK3 with a novel, selective and orally active small molecule induces therapeutic response in T-cell malignancies. Leukemia (23 October 2013); doi:10.1038/leu.2013.309.

Rowley JD (1973). A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining [letter]. Nature, 1973;243:290-293.

Rüdiger Hehlmann , Andreas Hochhaus , Michele Baccarani (2007) Chronic myeloid leukaemia. The Lancet, Volume 370, Issue 9584, 28 July–3 August 2007, Pages 342–350.

Rumjanek VM, Vidal RS, Maia RC. (2013) Multidrug resistance in chronic myeloid leukemia: How much can we learn from MDR - CML cell lines? Biosci Rep. 2013 Sep 26.

Schindler, C., Levy, D.E., Decker, T., 2007. JAK-STAT signaling: from interferons to cytokines. J. Biol. Chem. 282, 20059-20063.

Scholar, Eric (2007). Chronic Myelogenous Leukemia, In xPharm: The Comprehensive Pharmacology Reference, edited by S.J. Enna and David B. Bylund, Elsevier, New York, 2007, Pages 1-5, ISBN: 9780080552323.

Scott E, Hexner E, Perl A, Carroll M. (2010) Targeted signal transduction therapies in myeloid malignancies. Curr Oncol Rep. 2010 Nov;12(6):358-65. doi: 10.1007/s11912-010-0126-z.

Scott LM, Rebel VI. (2012) JAK2 and genomic instability in the myeloproliferative neoplasms: a case of the chicken or the egg? Am J Hematol. 2012 Nov;87(11):1028-36. doi: 10.1002/ajh.23243.

Sinclair A, Latif AL, Holyoake TL. (2013) Targeting survival pathways in chronic myeloid leukaemia stem cells. Br J Pharmacol. 2013 Aug;169(8):1693-707. doi: 10.1111/bph.12183.

Small D. (2006) FLT3 mutations: biology and treatment. Hematology Am Soc Hematol Educ Program. 2006:178-84.

Smithgall T, Briggs S, Schreiner S, Lerner E, Cheng H, Wilson M.(2000) Control of myeloid differentiation and survival by Stats. Oncogene. 2000; 19, 2612-2618.

Staerk J, Constantinescu SN. (2012) The JAK-STAT pathway and hematopoietic stem cells from the JAK2 V617F perspective. JAKSTAT. 2012 Jul 1;1(3):184-190.

Sun C, Wang L, Huang S, Heynen G, Prahallad A, Robert C, Haanen J, Blank C, Wesseling J, Willems S, Zecchin D, Hobor S, Bajpe P, Lieftink C, Mateus C, Vagner S, Grernrum W, Hofland I, Schlicker A, Wessels L, Beijersbergen R, Bardelli A, Nicolantonio F, Eggermont A, Bernards R (2014) Reversible and adaptive resistance to BRAF (V600E) inhibition in melanoma. Nature 508, 118-122. Doi:10.1038/nature13121.

Sweet KL, Hazlehurst LA, Pinilla-Ibarz J. (2013) The one-two punch: Combination treatment in chronic myeloid leukemia. Crit Rev Oncol Hematol. 2013 Aug 19. doi:pii: S1040-8428(13)00168-6. 10.1016/j.critrevonc.2013.07.017.

Swords R, Freeman C, Giles F. (2012) Targeting the FMS-like tyrosine kinase 3 in acute myeloid leukemia. Leukemia. 2012 Oct;26(10):2176-85. doi: 10.1038/leu.2012.114.

Talpaz M, Hehlmann R, Quintas-Cardama A, Mercer J, Cortes (2013) Re-emergence of interfereon-α in the treatment of chronic myeloid leukemia. Leukemia (2013) 27, 803–812; doi:10.1038/leu.2012.313

Texas Department of State Health Services, "Cancer data have been provided by the Texas Cancer Registry, Cancer Epidemiology and Surveillance Branch, Texas Department of State Health Services, 211 E. 7th Street, Suite 325, Austin TX 78701, http://www.dshs.state.tx.us/tcr/default.shtm.

Tian SS, Tapley P, Sincich C, Stein RB, Rosen J, Lamb P. (1996) Multiple signaling pathways induced by granulocyte colony-stimulating factor involving activation of JAKs, STAT5, and/or STAT3 are required for regulation of three distinct classes of immediate early genes. Blood. 1996 Dec 15;88(12):4435-44.

U.S. Cancer Statistics Working Group (2013). United States Cancer Statistics: 1999–2010 Incidence and Mortality Web-based Report. Atlanta: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute; 2013. Available at: www.cdc.gov/uscs.

Vera J, Rateitschak K, Lange F, Kossow C, Wolkenhauer O, Jaster R. (2011) Systems biology of JAK-STAT signalling in human malignancies. Prog Biophys Mol Biol. 2011 Aug;106(2):426-34. doi: 10.1016/j.pbiomolbio.2011.06.013.

Vivanco I, Sawyers CL. (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer. 2002 Jul;2(7):489-501.

Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW. (2013) Cancer genome landscapes. Science. 2013 Mar 29;339(6127):1546-58. doi: 10.1126/science.1235122.

Wagner E, Nebreda A. (2009) Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer. 2009 Aug;9(8):537-49. doi:10.1038/nrc2694.

Wang K, Li M, Hakonarson H. (2010). ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010 Sep;38(16):e164. doi: 10.1093/nar/gkq603.

Warsch, Wolfgang, Walz C, Sexl V. (2013) JAK of all trades: JAK2-STAT5 as novel therapeutic targets in BCR-ABL1 chronic myeloid leukemia. Blood 26 September 2013: 2167-2175.

Weisberg E, Sattler M, Ray A, Griffin JD. (2010) Drug resistance in mutant FLT3-positive AML. Oncogene. 2010 Sep 16;29(37):5120-34. doi: 10.1038/onc.2010.273.

Wilhelm S, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, Schwartz B, Simantov R, Kelley S. (2007) Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. Nat Rev Drug Discov. 2006 Oct;5(10):835-44. Review. Erratum in: Nat Rev Drug Discov. 2007 Feb;6(2):126.

William A Sands, Mhairi Copland, Helen Wheadon (2013) Targeting self-renewal pathways in myeloid malignancies. Cell Commun Signal. 2013; 11: 33. Published online 2013 May 15. doi: 10.1186/1478-811X-11-33.

Yao R, Cooper GM. (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science. 1995 Mar 31;267(5206):2003-6.

Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Mahfouz R, Behm FG, Raimondi SC, Relling MV, Patel A, Cheng C, Campana D, Wilkins D, Zhou X, Li J, Liu H, Pui CH, Evans WE, Naeve C, Wong L, Downing JR. (2002). Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell. 2002 Mar;1(2):133-43.

Younes A, Samad N. (2011) Utility of mTOR inhibition in hematologic malignancies. Oncologist. 2011;16(6):730-41. doi: 10.1634/theoncologist.2010-0318.

Appendix

Table 1 List of FDA Approved Drugs Screened against KCL-22 Cell Line.

DRUG	USAGE	TARGET
Dasatinib	CML, Endometrial Clear Cell Carcinoma (Phase2), Prostate Cancer (Phase2).	Abl, Src, c-Kit (D816V/WT)
Imatinib	CML, Gastrointestinal stromal tumor, Liposarcoma	PDGFR, c-Kit, v-Abl
Sorafenib	Desmoid Tumor, Hepatopulmonary Syndrome, Hepatocellular Carcinoma, Renal Cell Carcinoma	Raf-1, VEGFR2/FLK1, B-Raf (V599E), PDGFRb, FLT3, c-Kit, VEGFR2, FGFR1
Vemurafenib	Neoplasms, Multiple Myeloma (Phase2)	SRMS, ACK1, B-Raf (V600E), C-Raf, KHS1(MAP4K5), Lck, BRK, NEK11, BLK, Lyn B, Yes1, WNK3, Src
Trametinib	Melanoma (Phase2), Cancer General	MEK1, MEK2
Pimozide	Antipsychotic Agent, Tourette's Disorder	Dopaminergic receptors, STAT5
Sunitinib	Breast Cancer (Phase1), Melanoma, Genitourinary Cancer	PDGFRb, VEGFR2, FGFR1, EGFR
CP 690 550	Rheumatoid Arthritis	JAK3, JAK2, JAK1, ROCK2, LCK,
Nilotinib	CML, Gastrointestinal Stromal Tumors	Bcr-Abl
Gefitinib	Non-Small-Cell Lung Cancer, Lung Cancer	EGFR, ErbB2, ErbB4, c-Src
Lapatinib	HER2+ Metastatic Breast Cancer, Recurrent Thyroid Cancer, Breast Cancer, Breast Neoplasms	EGFR, ErbB2, ErbB4, c-Src

Temsirolimus	Cervical Adenocarcinoma, Ovarian Clear Cell Cystadenocarcinoma, Neuroblastoma, Endometrial Adenocarcinoma	mTOR
SNS-032	B-Lymphoid Malignancies (Phase1), Tumors (Phase1)	CDK9/CyclinT, CDK2/CyclinA, CDK2/CyclinE, CDK7/CyclinH, GSK-3a, CDK5/p35, CDK1/CyclinB, GSK-3b, CDK4/CyclinD
EP009	Experimentally demonstrated in T-ALL	JAK3
INCB018424	Primary Myelofibrosis, Metastatic Pancreatic Adenocarcinoma, Atypical CML, CLL	JAK1, JAK2
Ponatinib	(Suspended) Ph+ ALL, GIST, Adenocarcinoma of the Lung	Abl, PDGFRb, VEGFR2, FGFR1, c-Src, c-Kit

Table 2 Genomic Variants Identified in KCL-22 Imatinib Resistant (T315I) Cell Line.

T315I			
Gene	**Chromosome**	**Variant**	**Zygosity**
ABL1	9	T334I	Het
		A537_V538insGCEYL	Het
		E810G	Het
		R804_L805insGW*K	Het
		S1018P	Het
		T333I	Het
		T315I	Het
ALK	2	D1529E	Het
		K1491R	Het
		I1461V	Hom
APC	5	V1763D	Hom
AR	X	E81Q	Het
ARID2	12	A856V	Het
ASXL1	20	A654_T655insGHR*	Het
		P874L	Het
		L815P	Hom
ATM	11	N1635S	Hom
ATR	3	M211T	Het
		V2250A	Het
AURKA	20	F31I	Het
		I57V	Hom
AURKB	17	M299T	Hom
AXL	19	R217H	Het
		N266D	Hom
BCL2L2	14	Q133R	Hom
BCOR	X	V878A	Hom
BCORL1	X	F111L	Hom
BRCA1	17	A1502V	Het
		K1183R	Het
		E1038G	Het
		P871L	Het
		I180N	Het
BRCA2	13	N289H	Het
		N991D	Het
		V2466A	Hom

		T315I	
Gene	**Chromosome**	**Variant**	**Zygosity**
BRIP1	17	S919P	Hom
CCND3	6	S63A	Het
CDH1	16	P170L	Het
CHEK1	11	I437V	Hom
DOT1L	19	V1418L	Hom
EGFR	7	R521K	Het
ERBB2	17	A37T	Het
		I655V	Het
		P1170A	Het
ERBB3	12	G854S	Het
ETV1	7	S60G	Het
ETV5	3	A177P	Het
EWSR1	22	R603W	Het
		R615Q	Het
FANCA	16	G809D	Hom
		G501S	Hom
		T266A	Hom
FANCD2	3	N405S	Het
		P672L	Het
		V725A	Het
FANCE	6	R343Q	Het
		M487T	Het
FBXW7	4	N71D	Het
FGF23	12	T239M	Het
		R175W	Het
FGF4	11	S127R	Het
FGFR2	10	M71T	Het
FGFR3	4	V411M	Het
FGFR4	5	V10I	Het
		T150K	Het
		Q189K	Het
		G388R	Het
		P455L	Het

T315I			
Gene	Chromosome	Variant	Zygosity
		T361R	Het
		D363E	Het
		P136L	Hom
FLT3	13	T227M	Hom
FLT4	5	H890Q	Het
GNA11	19	Q209H	Het
GNA13	17	K42_T43ins	Het
GNAQ	9	Y101*	Het
		T96S	Het
GPR124	8	C218F	Het
		S535L	Het
		V806M	Het
		R963G	Het
		S698L	Het
IKBKE	1	A602V	Hom
IKZF1	7	A10T	Het
IL7R	5	I66T	Het
		V138I	Het
IRS2	13	R1259K	Het
		V1258L	Het
		D1257H	Het
		G1057D	Het
JAK3	19	L1060M	Het
KDM5A	12	M865T	Het
KDM5C	X	Y751H	Het
		G249A	Het
KEAP1	19	L365Q	Het
MCL1	1	E124K	Het
		E124K	Het
MEN1	11	T541A	Het
		W423*	Het
		T541A	Het
MLH1	3	L161S	Het
		L259S	Het

T315I			
Gene	**Chromosome**	**Variant**	**Zygosity**
MSH6	2	F1088_F1088del	Het
		G39E	Hom
MST1R	3	E1091D	Het
		M1090I	Het
		M1090I	Het
		E1091D	Het
		S1089G	Hom
MTOR	1	P1254T	Het
		M825I	Het
		S275T	Het
		S275T	Het
		M825I	Het
		P1254T	Het
MUTYH	1	I510L	Het
		I520L	Het
MYCN	2	V408M	Het
		V408M	Het
NOTCH1	9	G1195R	Het
		G1195R	Het
		T311P	Het
		H1190P	Het
NOTCH2	1	E38K	Het
		C19W	Het
		A14V	Het
NTRK1	1	G151R	Het
		G151R	Het
PDGFRA	4	S478P	Het
PDGFRB	5	R456S	Het
PHF14	7	K115R	Het
PIK3CA	3	E545G	Het
PIK3CD	1	T421A	Het
		M786I	Het
PIK3R1	5	M56I	Het

		T315I	
Gene	**Chromosome**	**Variant**	**Zygosity**
PIK3R2	19	S313P	Hom
PMS2	7	K541E	Het
		T485K	Het
PRKDC	8	I166_P167insYQIQF*	Het
PTPN13	4	C594Y	Het
PTPN14	1	Y165H	Het
PTPN23	3	L647_D648delinsFY	Het
		A944T	Het
		L1119M	Het
PTPRD	9	S887*	Het
RET	10	D489N	Het
RICTOR	5	S837F	Hom
RNF43	17	L418M	Het
		R117H	Het
		I47V	Het
SETD2	3	G1231E	Het
		M1080I	Het
SOCS1	16	L200I	Het
		A44T	Het
SOCS3	17	H4Q	Het
		T3R	Het
SPEN	1	A970V	Het
		L1091P	Het
		A2105_A2106ins	Het
		N2360D	Het
		Q2413*	Het
SPRED2	2	N321K	Het
SPRY2	13	P106S	Het
STAT3	17	Q829*	Het
STAT5A	17	G431C	Het
TET1	10	D162G	Het
		I1123M	Hom

		T315I	
Gene	**Chromosome**	**Variant**	**Zygosity**
TET2	4	P29R	Het
		F868L	Het
		I1762V	Het
		G1861_V1862insEWPWLQLM	Het
TET3	2	A163V	Het
		Q1163*	Het
TNFRSF14	1	Y26C	Het
		G174W	Het
TSC2	16	S1621L	Het
TSHR	14	R269S	Het
WISP3	6	Q74H	Het
ZNF217	20	T548I	Het
ZNF703	8	A28V	Het
		P284L	Het

Table 3 Genomic Variants Identified in KCL-22WT (Parent) Cell Line.

		Parent	
Gene	**Chromosome**	**Variant**	**Zygosity**
ABL1	9	A841_L842insP	Het
		A846_A847insSRWP	Het
		L805_V806insMRK	Het
		L842_G843insAPSPRAWSWTAPRRCASPS	Het
		L860_G861insAPSPRAWSWTAPRRCASPS	Het
BLM	15	S517_S518insKPNLPMILIILT*	Het
		P521_G522insYMSLQ*	Het
BRCA2	13	E1441_N1459del	Het
		N1463_N1463del	Het
		E1465F	Het
		I1470_R1471insF	Het
		R1471_K1472insT*SKKMA*RRNI*WST	Het
		M1475_D1476delinsYCRLCRKLFV*	Het
		L1478_S1479ins**FRIS	Het
		T1483_D1484insC*RSKKH*FFQSNIQCKRCKCIP	Het
		K1489_K1489del	Het
		L1491_N1501delinsYR	Het
		G1529_K1530insN*	Het
		K1531_K1533delinsQRE*	Het
		A1535_V1542del	Het
		C1573_K1574insS*VSLI	Het
		L1576_A1579delinsF*HSKWKI	Het
		C1591_S1676del	Het
		S1682_S1682delinsF*NRR*Y	Het
GRIN2A	16	F1396_F1396delinsSCQTT	Het
		Q1398_G1399delinsPIT	Het
		S1501_N1502insLMLQIRIICTLPPGF*IPAAIDACTRKCLV	Het
		R1503_K1508delinsL	Het
		P1510_*1517delinsFX	Het
MUTYH	1	I383L	Het
		I509L	Het
		I417L	Het
PRKDC	8	K216_L217insLCSIQLRHICHLLCLQ*V	Het
		L217_P218insILPRFSV*	Het
		L220_A221insKEQGLRSSHET*	Het

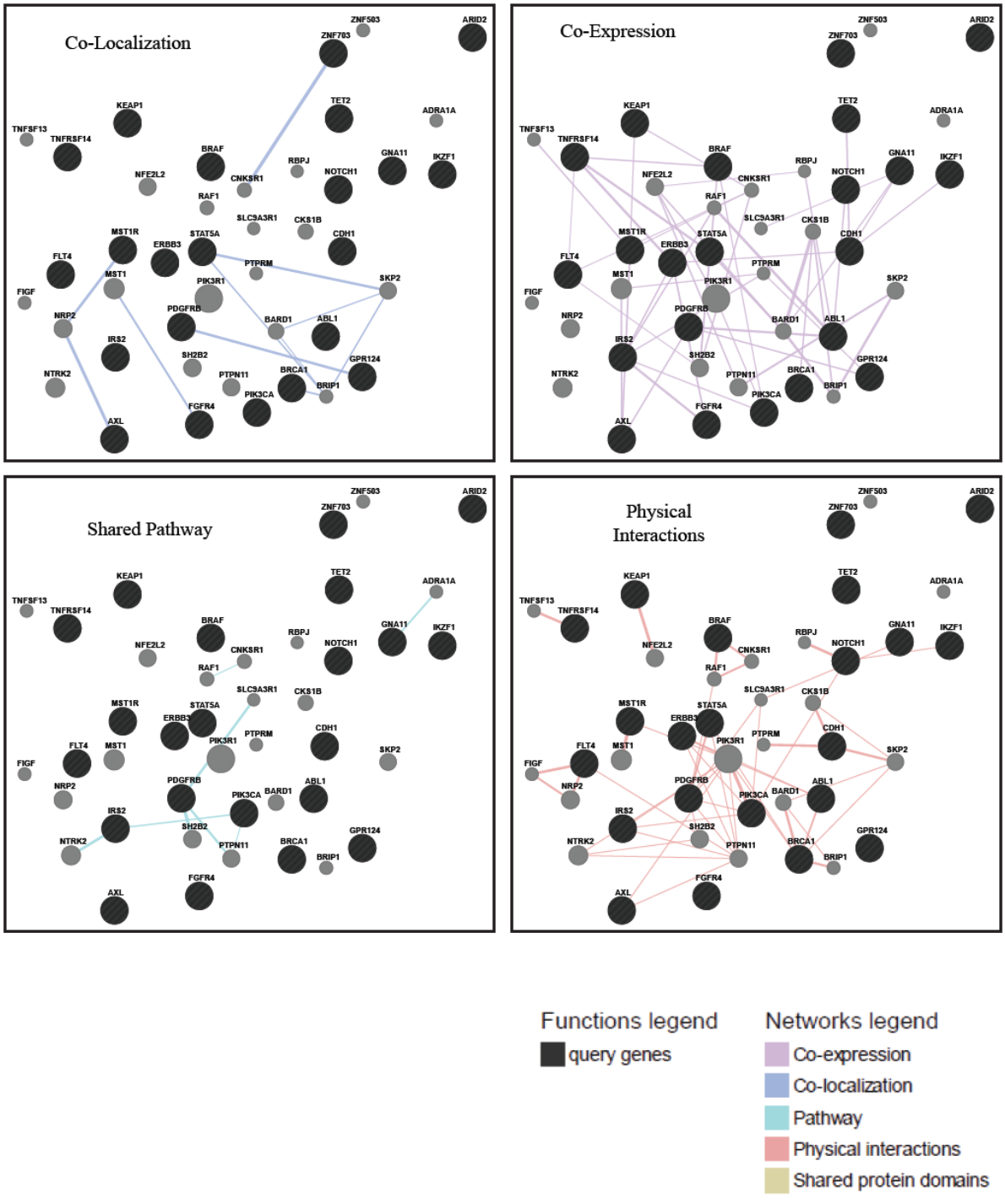
		Parent	
Gene	Chromosome	Variant	Zygosity
		F234_T235insWRIRRPPWD	Het
		K236_S237insRLGRW*	Het
		R246_E247insDY	Het
		E247_I248insTVSRGDF	Het
		I248_F249insPHDKRDLFRSLLAVHQLDQP*	Het
		P258_Q259insV	Het
		Q259_I260insRANRRV**AGDSHGVSAKA	Het
		L274_F275insEDEYKP*GFPGAP	Het
		A276_L277insSANSPVYQSDVT*	Het
		H278_C285del	Het
		K374_E387del	Het
		I389_I389del	Het
		C392_K393insL	Het
		Y799_L1198del	Het
		S1203_S1203del	Het
		N1205_L1206insQHPWQ	Het
		R264_Y265insYCYLEGPSFEHHVP	Het
PTPN13	4	A29V	Het
		C594Y	Het
		A29V	Het
		C594Y	Het
		A29V	Het
PTPN23	3	A1002T	Het
		A818T	Het
		L993M	Het
PTPRD	9	S887*	Het
		S890*	Het
RPTOR	17	V291M	Het
SPEN	1	L1090P	Het
		A2104_A2105insLQHRQGRGNLGW*	Het
		A2106_Q2107insSETQTRKWWLL*R	Het
		E2110_E2110delinsGSHT	Het
		S2125_P2126insRVLMKTVGSTQGPCL*	Het

Parent			
Gene	Chromosome	Variant	Zygosity
		P2688_L2692delinsSCF	Het
		S2769_V2953del	Het
		N2956_S3024del	Het
		G3029_G3029delinsSHTLSFPPLPLLACL	Het
		S3042_S3043insRRQRGPRRE*	Het
		T3044_A3148del	Het
		H3153_P3154insLDLQTG	Het
		P3154_P3155insLSLTPRFRGHKQKQARLPS	Het
		E3156_H3162delinsLCLSP*SLTFQSLFPLR	Het
		A3168_Q3173delinsCL	Het
		E3175_P3225del	Het
		G3229_P3254del	Het
		P3343_Q3357delinsS	Het
		Q3360F	Het
		A3362_P3364delinsTL	Het
		Q3449_Q3449delinsCCRSSRPVSSLRV	Het
		A3451_Q3478delinsC	Het
		P3481_K3482insW	Het
		E3541_P3545del	Het
		E3632_*3664delinsX	Het
		N2359D	Het
		A2066_Q2067insSETQTRKWWLL*R	Het
		E2072_E2072delinsAPLLR*Q	Het
		V2075_V2076insSQGRRSHGLSLLH*	Het
TET2	4	E1909_E1909delinsPMAKK*	Het
		H1912_G1913insGPCP*PQTPQ*L	Het
		G1913_L1914insLHMPSLGSQ	Het
TNK1	17	V598M	Hom
		V593M	Hom
TP53	17	P142_G143insQGALSEHC	Hom
		Q216_S217insLT	Hom
		S217_S217del	Hom
		S219_*235delinsX	Hom
		P169Q	Hom

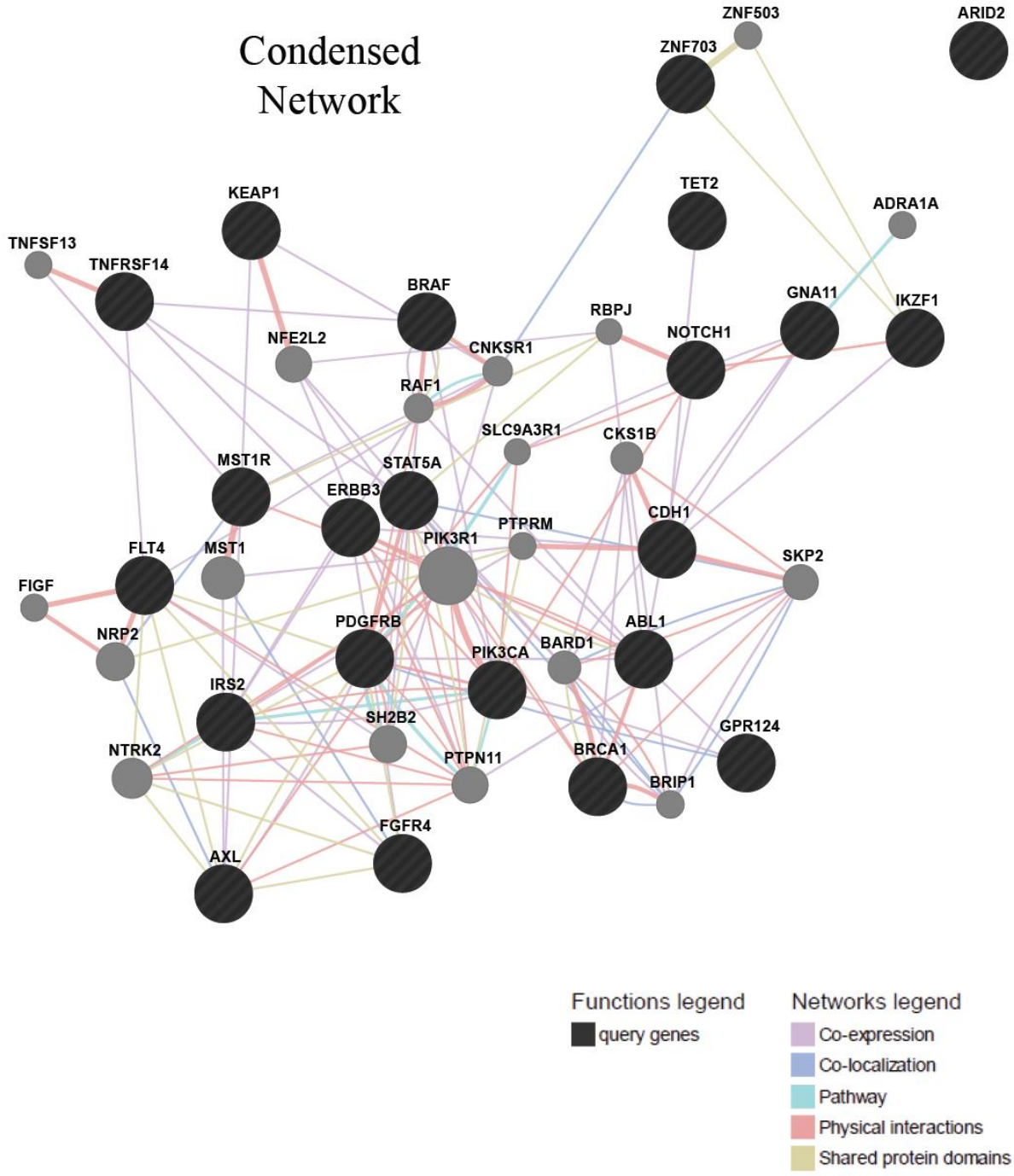
Parent			
Gene	Chromosome	Variant	Zygosity
		S171_R174del	Hom
		T180_S181insISPFRCYLTYDGV	Hom
		P262Q	Hom
		P270_N271insSEHC	Hom
		N179_T180del	Hom
		A232_K240delinsQ	Hom
		S244_S244del	Hom
		P309_N310insSEHC	Hom
		N310_N311insTTPAPLPSQRR	Hom
		N311_T312insHWME	Hom
		P72R	Hom
		S283_T284insV	Hom
		T284_K285insASRCSES*	Hom
		K285_R286insPAT*SP	Hom
		A287_A287delinsVS	Hom
		W338_*347delinsRX	Hom

Figure A1 Gene Mania Results of Associations Between Lead Genomic Variants.

a.)



b.)



Curriculum Vita

Derrick Oaxaca was born in El Paso, Texas. The second son of David Oaxaca and Yvette Jurado Oaxaca, he graduated from Montwood High School, El Paso, Texas, in the spring of 2008 and entered El Paso Community College in the summer. After completing core curriculum classes he transferred to The University of Texas as El Paso to pursue a bachelor's degree in biological sciences. While pursuing his degree, Derrick Oaxaca worked as an emergency medicine scribe between the years 2009 and 2012. One year prior to graduating, he began volunteering as an undergraduate research assistant in the lab of Dr. Robert Kirken, which ultimately led to him enrolling as a graduate student for the Graduate School at the University of Texas at El Paso in the fall of 2012, with plans to receive a Master's degree in Biological Sciences.

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