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Proteogenomic Insights Into The Nature Of Chemoresistance In Chronic Myelogenous Leukemia

Brett Noel
Purdue University

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Is approved by the final examining committee:

Dr. Laurie Parker
Chair

Dr. Christine Hrycyna

Dr. Tony Hazbun

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PROTEOGENOMIC INSIGHTS INTO THE NATURE OF CHEMORESISTANCE IN CHRONIC MYELOGENOUS LEUKEMIA

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of

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To everyone who has influenced me, guided me, and pushed me to succeed.

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ABSTRACT

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Chronic Myelogenous Leukemia (CML) is a hematopoietic cancer caused by the formation of the Philadelphia chromosome, a reciprocal translocation of the *ABL1* oncogene on chromosome 9, and the breakpoint cluster region gene (*BCR*) chromosome 22, resulting in the BCR-ABL oncoprotein. BCR-ABL is a constitutively active tyrosine kinase that regulates cell growth and differentiation, leading to uncontrolled cell proliferation by deregulating downstream signaling pathways.

A revolution in cancer treatment was ushered in with the introduction of imatinib mesylate (Gleevec®), an ATP-competitive inhibitor specific to BCR-ABL which traps the oncoprotein in its inactive conformation, leading to its degradation. Imatinib validated small-molecule kinase inhibitors as promising candidates for cancer therapy. However, a small but significant portion of CML patients is intrinsically resistant to imatinib, and others develop resistance over time. Mutations in the kinase domain preventing drug binding are partially responsible for resistance, but in many patients, no mutations are present. Second-generation BCR-ABL TKIs nilotinib and dasatinib have

been developed which inhibit BCR-ABL even in the presence of kinase domain mutations conferring resistance to imatinib, but resistance to both second-generation tyrosine kinase inhibitors (TKIs) has been reported. We have developed lines of K562 cells resistant to imatinib (K562-IR), nilotinib (K562-NR), and dasatinib (K562-DR), respectively. We used a proteogenomic approach, bridging transcriptomics with proteomics to attempt to unravel the mechanisms underlying TKI-resistance and identify potential biomarkers of TKI-resistance in CML. We identified significant increased protein expression in proteins involved in cell proliferation, and a reduction of the proteins involved in protein translation across all three TKI-resistant cell lines, and also identified novel fusion genes present in our TKI-resistant cell lines that could potentially serve as biomarkers of acquired resistance.

CHAPTER 1. INTRODUCTION

1.1 The Cell and Cancer

1.1.1 Cellular Signaling Controls Cell Functions

The cell is basis of all human life, and constantly adapts to its environment. The cell reacts to external or internal stimuli through intricate molecular signaling pathways that can take the form of protein-protein interactions, ligand-receptor interactions, post-translational modifications, or protein-DNA interactions, and others, all of which can effect downstream gene expression, allowing the cell to properly respond to stimuli. The ability to sense and react to stimuli ensures proper cell differentiation and proliferation, and allows the cell to undergo apoptosis before attaining deleterious properties. This process continues to form tissues, and organs, and the cells comprising these tissues and organs communicate through cell-cell interactions composed of molecular signals. Molecular signaling is critical to maintain cellular homeostasis. Cells must regulate the influx and efflux of proteins, ATP, and ions, the internal pH of the cell, and neutralize free radicals, which can damage DNA. Cellular proliferation is also regulated by molecular signaling events that are coordinated throughout the cell cycle. A system of molecular checks and balances ensures that cells proliferate properly, and undergo the process of apoptosis, or programmed cell death, if they cannot maintain the proper

functions of that cell. Regulation of many processes at once requires integration of many signaling inputs, and a coordinated response of outputs that allows the cell to maintain its properties and perform its functions. While this process is unfathomably dynamic, it is also very delicate.

1.1.2 Cell Signaling and Mutation

Genetic mutation has wide-ranging effects on the ability of the cell to sense and adapt to its environment, and is necessary for the progress of evolution to proceed. There are many types of mutations, which can have varying phenotypic consequences ranging from null consequences to developmental abnormalities and organismal death. A loss-of-function mutation produces an inactive gene product, and is often recessive in diploid organisms because another copy of the gene is present and can compensate for the loss of function[1]. A gain-of-function mutation strengthens the effect of the gene product, and is known as an activating mutation[2]. Dominant-negative mutations are mutations that cause the mutant gene product to have an antagonistic effect on the wild-type gene. Dominant-negative mutations have been implicated in oncogenesis, such as the mutations found in the tumor-suppressing protein p53[3]. Mutations in p53 have been found in many tumors, and inhibition of p53 function has been shown to increase invasiveness, metastasis, and overall tumor growth by changing the activation of signaling networks in the cell[4].

Genetic mutations can arise in many ways, such as errors in DNA replication, or through external environmental factors such as exposure to UV light from the sun causing DNA damage[1, 2, 4-7]. A frameshift mutation arises from an insertion or deletion of a nucleotide or nucleotides in the gene encoding a protein, and changes the ensuing amino acid sequence by shifting the codon sequence produced, leading to dramatic changes in the composition of the protein eventually translated from the mRNA produced by the gene[8]. A nonsense mutation occurs when a mutation in a gene encodes for a premature stop codon, leading to the translation of a truncated protein[9]. Missense mutations occur when a point mutation a gene changes the codon produced, and the resulting amino acid sequence is altered only at the codon affected by the missense mutation[5, 10]. There are also neutral and silent mutations. Neutral mutations occur when alteration of the genetic code encodes for a chemically similar amino acid, such as the substitution of an arginine residue for a lysine residue[11]. A silent mutation refers to a mutation producing the same amino acid, despite the presence of a mutated nucleotide residue[12].

The mutation of an amino acid residue can disrupt the balance of complex molecular signaling networks by encoding for an inactive protein, an overactive protein, or a protein that otherwise alters the ability of the cell to sense and react to its environment such as the ability of the protein to bind other proteins or DNA. Mutation can also occur at the gene level, producing mutated proteins with the ability to effect cell growth.

Rearrangements of whole chromosomes can even occur, leading to novel genetic products. The generation of a novel gene due to the juxtaposition of two genes normally found in distant positions on the same chromosome or entirely different chromosomes is known as chromosomal translocation[13-19], which can result in genetic products that can be translated into mutant fusion proteins if open reading frames are preserved. Conversely, a situation that results in the deletion of a gene is known as an interstitial deletion[20], whereas the reversal of gene orientation is known as an inversion[21]. Mutations at the chromosomal level are equally capable of leading to disease pathologies as mutations at the amino acid level, particularly when fusion proteins are produced or critical genes are deleted. The consequences of mutation are variable, but many diseases arise from mutations altering the ability of genes important in molecular signaling to maintain homeostasis.

1.1.3 Genetic Mutation and Cancer

The term cancer refers to a collection of diseases characterized by uncontrolled cellular proliferation, resistance to apoptosis, evasion of growth suppression, increased angiogenesis, enabling replicative immortality, invasion and metastasis, reprograming of energy metabolism, and the ability to avoid immune detection[22]. The underlying cause of cancer is genomic instability, which compromises the ability of the cell to sense and properly react to its environment. Mutation of genes involved in the regulation of a variety of cellular processes, such as cell cycle regulation, cell proliferation, or DNA

damage response can cause oncogenesis[22]. The mutation of one protein in a complex signaling system can have wide-ranging effects that drive unchecked proliferation. A notable example of this is the mutation of the protein B-RAF, which occurs in about 66% of human melanomas. The protein RAS binds to a serine-threonine kinase RAF, activating RAF. RAF phosphorylates MEK, which phosphorylates a MAP kinase, which phosphorylates several transcription factors that activate transcription of genes critical to driving cell proliferation. One of these genes is the kinase EphA2, which phosphorylates p120RasGAP, which in turn downregulates wild-type RAS as a negative feedback mechanism to control the rate of cell proliferation[23-25]. However, mutant RAS is not downregulated, and the signaling cascade can proceed unchecked. Many other targets of the MAPK pathway are also affected, including repression of apoptotic signals, cell-cycle checkpoints, and cell division machinery. Despite the fact that cancer is caused by genomic instability, there are currently no methods to directly target the genetic instability underlying cancer progression, we must overcome this limitation by targeting the protein products of genomic instability.

1.1.4 Therapeutic Strategies in Cancer

Initial attempts to treat cancers using chemicals were considered largely successful at the time. The first use of chemicals in cancer therapy (chemotherapy) was in 1942, when Louis Goodman and Alfred Gilman used nitrogen mustards; a derivative of the mustard gas used a chemical warfare agent in World War I, to induce the regression of a

tumor in a patient being treated for non-Hodgkins lymphoma[26]. This was the first demonstration that cancer could be treated with pharmacological agents. Sidney Farber expanded on the discoveries of Goodman and Gilman in 1948, when he used the antifolate derivatives aminopterin and methotrexate to treat children with acute lymphoblastic leukemia (ALL)[27]. Because ALL is a disease of white blood cells, it is possible to directly measure the effect of chemotherapy by simply drawing blood and counting the depletion of cells over the course of treatment, and his highly controversial report in the New England Journal of Medicine piqued interest in the development of new pharmacological agents as anti-cancer therapies. At the time, little was known about the molecular biology of cancer. Accordingly, many cancer treatments developed then would be deemed barbaric by today's standards due to the fact that none of the treatments selectively targeted cancer cells. Other strategies for treating patients with cancer were based on the rationale that by developing treatments biased toward rapidly proliferating cells, one could kill all cancer cells before killing the patient. The evolution of cancer treatments progressed with the realization that disruption of the incorporation of nucelosides into a new strand of DNA would cause cells to undergo apoptosis, and the drug 6-mercaptopurine, which eventually became incorporated into DNA as a 6-thioguanine nucleotide, was discovered to be highly effective at treating leukemias[28]. With new therapies came undesirable side effects, and new forms of therapy targeting diverse aspects of cancer hallmarks were conceived. Vinca alkaloids were next developed, which inhibit microtubule polymerization and consequently inhibit mitosis leading to cell death[29]. Vinca alkaloids were followed by taxanes such

as paclitaxel, which inhibit the breakdown of microtubules leading to inhibition of cell division, topoisomerase I inhibitors such as camptothecin, which prevents DNA re-ligation during DNA synthesis leading to apoptosis, platinum-containing cancer drugs such as cisplatin, which induces irreparable DNA damage by cross-linking to DNA leading to apoptosis, and alkylating agents such as cyclophosphamide, which inhibit DNA synthesis by cross-linking DNA strands leading to apoptosis[29-33]. All of these therapies were effective to an extent, but the side effects of untargeted therapies, such as fatigue, loss of appetite, sexual dysfunction, neuropathy, and organ damage were devastating for many cancer patients. Better therapies were needed to directly treat the many different causes of cancer.

In the 1990's it was recognized that the growth of some breast cancers was partially dependent on the hormone estrogen. Estrogen binds to a receptor (estrogen receptor, ER) on breast cells and activates an intracellular molecular signaling cascade promoting breast cancer cell proliferation. To combat this critical signaling event, Tamoxifen, a drug developed in the 1960's as a potential emergency contraceptive was repurposed as an anti-estrogen compound. Tamoxifen is a prodrug that is metabolized by cytochrome P450 isoforms CYP2D6 and CYP3A4 in the liver to yield the active metabolites 4-hydroxytamoxifen and N-desmethyl-4-hydroxytamoxifen, which bind the ER on breast cancer cells with high affinity, blocking estrogen from binding to its receptor. The ER/4-hydroxytamoxifen complex also recruits repressors of cell proliferation, and modulates

the expression of genes driving cell proliferation[34]. The introduction of a molecule that blocked molecular signaling as a breast cancer treatment was a crucial step toward recognizing the need for targeted therapies in cancer treatment. An understanding of the underlying molecular machinery driving cancer progression was sorely lacking, and without a fundamental understanding of the driving forces behind cell proliferation, the development of targeted therapies was inefficient and ineffective.

1.1.5 Molecular Signaling and Cancer

Cancer is a disease that is characterized by uncontrolled cell proliferation, and the proliferation of healthy cells is tightly controlled by a system of checks and balances. Highly intertwined molecular signaling networks communicate by receiving and integrating signals from extracellular growth factors, cell-cell contact, and other intracellular signaling networks to regulate cell proliferation, protein synthesis, DNA synthesis, energy metabolism, and apoptosis[2, 24, 25, 35-38]. Disruption of these signals can cause an imbalance in the downstream signaling events, which are reliant on the information obtained from upstream signals. An imbalance in a signaling network propagates throughout the network and cells that cannot correct for imbalances can attain the characteristic properties of cancer, such as unregulated cell growth. A prominent molecular signaling mechanism is the enzyme-catalyzed transfer of a phosphate group from the active site of an enzyme known as a kinase to the desired target on a protein substrate[39]. Phosphorylation is a reversible process used to

control cell differentiation, proliferation, and apoptosis, and the 520 kinases make up approximately 2% of the human genome. There are two major classes of protein kinases, those that preferentially phosphorylate serine and threonine residues, and those that preferentially phosphorylate tyrosine residues. Serine/threonine kinases comprise approximately 80% of the protein kinases in the human genome, and although tyrosine kinases comprise about 20% of human protein kinases, tyrosine residues represent only about 1.8% of the phosphorylated residues in the human body[40]. Despite the dramatic difference in abundance, tyrosine kinases are tremendously important in cell signaling.

Tyrosine kinases are composed of two different sub-classes of protein kinases. Non-receptor tyrosine kinases (NRTK) which are free to roam about the intracellular space of the cell, and receptor tyrosine kinases (RTK), membrane-bound kinases activated by a ligand binding to the extracellular surface of the cell, which transduce extracellular signals into intracellular signals.[40-43]. Most RTKs exist as monomeric subunits, but others consist of multimeric units that work together to convert extracellular signals to intracellular signals. For example, the insulin receptor (IR) is a tyrosine kinase that plays a critical role in the regulation of glucose homeostasis. During transcription, alternative splicing leads to two isoforms of the gene, IR-A and IR-B. Downstream post-translational modification of the IR-A and IR-B protein products leads to α and β subunits, which can form homodimers or heterodimers to produce the insulin receptor complex. The IR

complex can bind insulin, Insulin-like Growth Factor I (IGF-I), and Insulin-like Growth Factor II (IGF-II). Binding of the ligand causes a conformational change in the dimer, resulting in autophosphorylation of the intracellular tyrosine residues on the β chain of the tyrosine kinase domain[44]. Phosphorylation of the tyrosine residues recruits adaptor proteins such as insulin receptor substrate (IRS) proteins, SH2-B domains, and phosphatases such as PTP1B to the tyrosine kinase domain, which regulate the downstream processes required to maintain glucose homeostasis[45]. In contrast, NRTKs are unbound and are free to move around the intracellular space freely. A well-known example of a NRTK is Ableson protein tyrosine kinase 1, also known as c-Abl. c-Abl has many diverse functions in the cell, such as cytoskeleton remodeling in response to extracellular stimuli, cell motility and adhesion, receptor endocytosis, autophagy, DNA damage response and apoptosis. c-Abl is typically found in the cytoplasm, but can be shuttled into the nucleus through protein-protein interaction with 14-3-3 scaffold proteins, and upon nuclear translocation, c-Abl is able to bind DNA and is involved in the DNA damage response, and activation of pro-apoptotic pathways if the damage to DNA is too much for the cell to survive[46].

Tyrosine kinases are ubiquitously expressed in many cell types, and have been prominently implicated in the progression of cancer. Janus kinase 2 (JAK2) is a NRTK in the cytokine type II receptor family that is critical to IL-3 receptor signaling, driving proliferation, differentiation, and apoptosis. JAK2 phosphorylates the STAT transcription

factors in the cytoplasm, and consequently the STAT transcription factors translocate to the nucleus and activate transcription of genes such as SOCS3, a negative regulator of RasGRP. RasGRP is a negative regulator of RAS, so the JAK2/STAT pathway indirectly activates the RAS pathway[47]. In several myeloproliferative disorders, JAK2 contains an activating mutation where the valine at the 617th amino acid position is mutated to a phenylalanine (V617F). This mutation allows for constitutive activation of JAK2 kinase activity, which drives unregulated cell proliferation[48]. Possibly the most famous example of tyrosine kinase involvement in cancer progression is the fusion protein BCR-ABL, produced by the chromosomal translocation of the breakpoint cluster region gene on chromosome 22 with the c-Abl tyrosine kinase on chromosome 9. The resulting chromosome is known as the Philadelphia Chromosome, and is found in 95% of all chronic myelogenous leukemia patients. The resulting fusion protein replaces exon 1 from the c-Abl gene with the BCR gene, resulting in a constitutively active form of c-Abl. The BCR-ABL oncoprotein is then free to drive unregulated cell proliferation through activation of the RAS/MAPK, JAK2, PI3K, and CRKL pathways[13, 15-17, 19, 46, 49-51]. The ability of tyrosine kinases to disrupt many signaling networks at once makes them prime candidates for pharmacological inhibition, and the development of specific tyrosine kinase inhibitors marked a turning point in the ability of physicians to treat cancer patients.

1.1.6 Chronic Myelogenous Leukemia Pathology

Chronic Myelogenous Leukemia (CML) is a hematopoietic cancer caused by the formation of the Philadelphia Chromosome, a reciprocal translocation of the 3' end of the gene encoding the *ABL1* tyrosine kinase on chromosome 9, and the 5' region of the breakpoint cluster region gene (*BCR*) chromosome 22, resulting in the BCR-ABL oncogene. The protein encoded by *BCR-ABL* is a constitutively active 210 kDa tyrosine kinase that regulates cell growth and differentiation, leading to uncontrolled cell proliferation by deregulating downstream signaling pathways[19, 46, 52]. The structure of the fusion protein lends insight into its constitutive nature. While the exon encoding the tyrosine kinase activity of ABL remains intact, the loss of an N-terminal myristoylation site removes an ABL autoinhibition site, resulting in increased kinase activity and unregulated propagation of downstream cell proliferation signals. Antisense oligonucleotides and knockout mouse models have demonstrated that BCR-ABL is a critical mutation on the path to develop CML, and thus represents an excellent target for innovative treatment strategies[53].

The BCR-ABL fusion protein drives oncogenesis through multiple prominent signaling pathways. BCR-ABL activates the JAK/STAT pathway through activation of STAT5, a transcription factor necessary for CML development. STAT5 is a known substrate of JAK2, and knockout of STAT5 prior to BCR-ABL mediated CML induction prevented the development of CML in a mouse model. Interestingly, knockout of JAK2 had no effect on

the development and maintenance of CML, suggesting BCR-ABL is capable of activating STAT5 directly[50]. BCR-ABL also activates the Phosphoinositide 3-Kinase (PI3K) pathway through the activation of GRB2/GAB2 and CBL adaptor proteins, and pharmacological inhibition of PI3K signaling incudes apoptosis in primary CML cells[54]. However, BCR-ABL is perhaps most commonly associated with the activation of Src-family kinases, kinases prominently involved in cell growth, differentiation, and motility. The SRC-family kinases HCK and LYN are strongly activated in the presence of BCR-ABL and along with FYN, another SRC-family kinase, are required for BCR-ABL mediated CML induction[42, 55-58].

BCR-ABL-mediated CML progression is not mechanistically limited to deregulated cell proliferation, but BCR-ABL also inactivates apoptosis pathways in a two-pronged manner. BCR-ABL mediated STAT5 regulation increases the expression of BCL-X_L and BCL-2, anti-apoptotic genes that inhibit apoptosis by inhibiting cytochrome C release from the mitochondria, preventing caspase activation and thus inhibiting apoptosis[15].

Constitutively active BCR-ABL also activates the PI3K/AKT pathway, which phosphorylates the pro-apoptotic BCL2-associated Death promoter (BAD), promoting it's association with 14-3-3 scaffolding proteins, sequestering BAD in the cytoplasm and preventing BAD from activating pro-apoptotic genes in the nucleus[54, 59, 60].

The progression of CML occurs in three phases. Most patients are diagnosed in the chronic phase (CML-CP), in which most patients experience no symptoms and a

quiescent population of hematopoietic stem cells, termed Leukemic Stem Cells (LSCs), lies dormant in the bone marrow. Although it is yet unknown what molecular events lead quiescent cells to gain self-renewal capabilities and begin proliferating uncontrollably, CML can then progress through an accelerated phase (CML-AP), or directly to blast phase (CML-BP)[52, 61]. CML-AP is defined as the presence of any of the following symptoms: 10-19% myeloblasts present in the blood or bone marrow, platelet count <100,000 related to therapy, platelet count >1,000,000 and unresponsive to therapy, >20% basophils in the blood or bone marrow, cytogentic evolution unrelated to the presence of the Philadelphia Chromosome, increased splenogamy or white blood cell count and unresponsiveness to therapy. Accelerated phase signals an imminent progression to blast-phase, and an increased likelihood of drug resistance. Blast phase is characterized by rapid cell proliferation brought on by inhibition of tumor suppressor proteins, genomic instability, and shortened telomeres, resulting in >20% myeloblasts or lymphoblasts in the blood or bone marrow, large clusters of blasts in the biopsy, and the development of chloroma (a solid focus of tumor outside the bone marrow). Once a patient reaches blast phase, prognosis is bleak and the patient generally has a short time to live. It is critical to diagnose and treat CML patients before blast phase is initiated[62, 63].

1.1.7 Treatment of CML Using Tyrosine Kinase Inhibitors (TKIs)

Prior to the introduction of imatinib, CML was treated with a combination of cytarabine and interferon-α therapy, or radiation therapy followed by allogenic stem cell transplantation (the transfer of stem cells from a healthy donor to the patient after radiation or chemotherapy). These treatment options were moderately effective, with the median 8-year survival rate between 42-65% from 1983-2000[64].

The introduction of imatinib mesylate (Gleevec®) changed everything. Imatinib inhibits BCR-ABL by binding its kinase domain and trapping BCR-ABL in an inactive conformation, leading to its degradation and subsequent apoptosis of the cell. With a 7-year overall survival rate of 86% and an event free survival rate of 81%, imatinib was a huge success, and demonstrated that small molecule ATP-competitive inhibitors could be highly effective cancer treatments. Since then, TKIs have been approved for BTK, ALK, VEGFR, FGFR, KIT, SRC-family kinase, JAK, among other targets, however none have been as successful as imatinib. For many CML patients, a daily dose of imatinib transformed a deadly disease into a manageable condition, as BCR-ABL is the product of a fusion gene found only in cancer cells making it a highly specific target for a small molecule inhibitor. However, approximately 4% of CML patients are intrinsically resistant to imatinib, and 15-25% develop resistance to imatinib over time[52].

Over 100 BCR-ABL point mutations have been identified, accounting for approximately 70% of acquired resistance to imatinib[65]. Many of these mutations prevent the binding of imatinib in the ATP-binding pocket of BCR-ABL, leading to imatinib resistance. Second-generation BCR-ABL TKIs nilotinib and dasatinib have been developed which inhibit BCR-ABL even in the presence of several kinase domain mutations conferring resistance to imatinib, but resistance to both second-generation TKIs has been reported[66].

1.2 Imatinib, Nilotinib, and Dasatinib

1.2.1 Imatinib

Imatinib, originally termed STI571, is a methylpiperazine derivative that was developed by Novartis in the early 1990's. In collaboration with Dr. Brian Druker, imatinib entered clinical trials and demonstrated remarkable efficacy in inducing apoptosis in CML cells. Imatinib works by outcompeting ATP to bind the ATP-binding pocket of BCR-ABL when in an inactive conformation, inhibiting its catalytic activity and driving apoptosis by inhibiting downstream signaling pathways necessary for cell proliferation. Imatinib inhibits BCR-ABL with high specificity, targeting only c-KIT and PDGFRα and PDGFRβ[67]. Because BCR-ABL is present in almost all CML cells, imatinib is an ideal treatment option for patients with CML. In June 1998, Dr. Brian Druker and colleagues initiated a phase I clinical trial in collaboration with Novartis deemed International Randomized Study of Interferon versus STI571 (IRIS) designed to assess the safety and efficacy of imatinib in

human patients who had failed interferon-α therapy. The results were striking. At 18 months, the rates of Complete Cytogenic Response (CCyR, defined as 0% Ph+ cells) were 76% in the imatinib group compared to only 15% in the group treated with a combination of interferon-α and cytarabine; at 60 months 97% of patients treated with imatinib achieved a CCyR[68]. At 18 months 100% of patients who achieved a Major Molecular Response (MMR; defined as a reduction in levels of *BCR-ABL* transcript to ≤ 0.1% of the average level of BCR-ABL transcript in 30 untreated CP-CML patients) remained in chronic phase, not progressing to accelerated phase or blast phase. Despite the success of imatinib, 15-25% of patients are either intrinsically resistant to imatinib or acquire resistance over time. BCR-ABL kinase domain mutations are a common mechanism conferring resistance to imatinib. Over 50 kinase domain mutations have been identified, many of which prevent the binding of imatinib to the kinase domain[69]. To combat imatinib resistance second-generation tyrosine kinase inhibitors have been developed, which inhibit several variants of mutant BCR-ABL.

1.2.2 Nilotinib

Nilotinib is a second-generation BCR-ABL inhibitor that inhibits most BCR-ABL mutants with the exception of the T315I, Y253H, E255V/K, and F359V mutations, which confer moderate resistance to imatinib. Nilotinib is more selective than imatinib, and binds BCR-ABL in imatinib-sensitive CML 20-50 times more tightly than imatinib, and 3-7 times more tightly in imatinib-resistant CML[70, 71]. In a phase II clinical trial of imatinib-

resistant patients, 46% of patients achieved a CCyR, and 56% achieved a MMR. At 24 months there was an 87% overall survival rate, with a 64% event-free survival observed[70, 72]. As with imatinib, patients eventually become resistant to nilotinib, and there are limited options to combat nilotinib resistance[73].

1.2.3 Dasatinib

Dasatinib is an ATP competitive TKI with dual activity against both BCR-ABL and SRC-family kinases, specifically SRC, LCK, LYN, YES, FYN, and FRK. Dasatinib inhibits BCR-ABL more potently than nilotinib or imatinib and its activity against the SRC- family kinases, which have been implicated in TKI resistance, make it an advantageous treatment option for patients resistant to both imatinib and nilotinib[74-76]. In a phase II clinical trial of imatinib-resistant or imatinib-intolerant CML patients, dasatinib induced a CCyR in 53% of patients at 24 months[77]. Although dasatinib inhibits BCR-ABL more potently than nilotinib and imatinib, it does not effectively inhibit BCR-ABL containing the T315I, E255K/G, F317L, and Q252H mutations, and dasatinib is associated with the development of further mutations in the kinase domain, which may result in CML resistance to all currently FDA-approved TKIs[13, 74-76, 78-80]. Despite the success of first- and second-generation TKIs, there is clearly a need to develop third-generation TKIs that effectively inhibit TKI-resistant BCR-ABL mutations, and explore alternative avenues conferring resistance.

1.2.4 Next-Generation CML Treatment

There is an unmet need to effectively inhibit BCR-ABL mutants resistant to first-and second-generation TKIs. The most notorious mutation commonly found in patients resistant to imatinib, nilotinib, and dasatinib is the T315I mutation, also known as the gatekeeper mutation. The T315I mutation is significant because it removes a critical oxygen residue necessary for first- and second-generation TKIs to bind to the kinase domain of BCR-ABL and effectively inhibit its kinase activity. Several experimental compounds have been proposed to meet this need, and are in varying stages of development.

Ponatinib is a novel tyrosine kinase inhibitor that is designed to inhibit the BCR-ABL T315I mutation, and was approved to treat patients with CP-CML, AP-CML, or BP-CML, as well as Ph+ Acute Lymphoblastic Leukemia (Ph+ ALL) in the United States and countries residing in the European Union. A phase II study of ponatinib yielded major cytogenic response in over half of adults with CP-CML, and a major hematological response in 50% of adults with BP-CML, and 34% of patients with Ph+ ALL, regardless of the presence or absence of the T315I mutation[81]. However, serious side effects such as vascular occlusion, heart failure, and hepatotoxicity have been observed in patients treated with ponatinib. Although ponatinib is a promising therapeutic option for patients resistant to other TKIs, it remains to be seen if patients will develop resistance due to other factors.

1.2.5 Acquired Resistance Due to Compensatory Signaling Pathway Activation

A commonly cited mechanism of resistance to imatinib and nilotinib is the over-activation of compensatory proliferative signaling pathways that allow CML cells to evade cell death. BCR-ABL activates several prominent signaling pathways, such as the SRC- signaling pathway, which includes LYN, a member of the SRC-family kinases. Knockdown of LYN using antisense RNA has restored imatinib and nilotinib sensitivity *in vitro*[15, 58, 82], however, dasatinib inhibits SRC-family kinase activity and patients lacking the T315I mutation still develop resistance to dasatinib[83], suggesting that the SRC-family kinase signaling pathway is not the only pathway compensating for BCR-ABL inhibition. In fact, constitutive ERK2 activation has been identified as a mechanism of *de novo* resistance to imatinib[84], and PI3K/AKT activation has also been described as a mechanism underlying BCR-ABL independent acquired imatinib resistance[54]. The study of BCR-ABL independent compensatory signaling pathway activation represents a promising therapeutic avenue, as pharmacological inhibition of these signaling pathways could be an effective strategy to mitigate BCR-ABL independent TKI-resistance.

1.2.6 Altered Expression of Drug Influx and Efflux Channels

The success of BCR-ABL inhibition is dependent upon the intracellular concentration of the inhibitor, and alteration of the expression of influx and efflux pumps can affect the internalization and accumulation of small-molecule inhibitors. Several studies have demonstrated the upregulation of the efflux pump multidrug-resistance gene (MDR-1)

and its translational product P-glycoprotein (PgP) in acquired resistance to imatinib. RNAi-mediated knockdown of PgP restored sensitivity to imatinib and increased intracellular concentrations of imatinib in K562 cells *in-vitro*, and pharmacological inhibition of PgP yielded similar results[85, 86]. In the other direction, other studies have demonstrated downregulation of influx pumps correlating with imatinib resistance. The best studied of these transporters is OCT1, an organic cation transporter responsible for transporting imatinib into the cell. Pharmacological inhibition of OCT1 resulted in reduction of imatinib uptake in CML cells, but the results of the study did not establish reduced OCT1 expression as a direct cause of imatinib resistance[87]. Subsequent studies have linked low OCT1 expression with reduced response to imatinib compared to patients that achieved a CCyR at 10 months following the onset of imatinib therapy[88], and patients with low expression of OCT1 receiving high doses of imatinib had much better rates of CCyR, MMR, and PFS than those with low expression of OCT1 receiving a standard dose of imatinib. Interestingly, patients with high expression of OCT1 responded to either dosage equally well. Taken together, the expression of drug influx and efflux transporters is a critical factor determining a patient's sensitivity to TKI therapy. There are many factors contributing to TKI-resistance. In this thesis we describe the use of transcriptomics and proteomics to identify potential mechanisms mediating TKI-resistance in a model of acquired resistance to imatinib, nilotinib, and dasatinib, respectively.

CHAPTER 2. IDENTIFICATION OF TRANSCRIPTOMIC DIFFERENCES BETWEEN WILD-TYPE CML CELLS AND CML CELLS RESISTANT TO TYROSINE KINASE INHIBITORS

2.1 Introduction

Despite the success of frontline CML treatments such as imatinib, 15-25% of CML patients will eventually develop resistance to therapy. In many cases, the roots of resistance can be traced to mutations in the kinase domain of BCR-ABL, preventing imatinib from binding to the ATP-binding pocket and inhibiting the catalytic activity of the kinase. However, approximately 30% of patients that develop resistance to imatinib do not exhibit any mutations preventing imatinib binding. Various explanations for BCR-ABL independent resistance have been proposed, such as the overexpression of BCR-ABL[49], compensatory signaling pathway activation[15, 82, 84, 89], downregulation of influx pumps[90], and upregulation of efflux pumps[18], but further study is required to identify potential biomarkers of TKI-resistance and novel mechanisms of resistance. Every CML patient has a unique biological response to any chemotherapy, and it is currently impossible to predict which TKI will most effectively inhibit BCR-ABL activity in an individual patient. Further, the current gold standard to measure the efficacy of a TKI in a CML patient is to measure BCR-ABL transcript level approximately 90 days after the onset of treatment. A goal in the Parker lab is to design novel peptide substrates capable of accurately reporting the kinase activity of a particular target. By measuring

the ability of each TKI to inhibit kinase activity, physicians will be able to make a more informed decision regarding TKI treatments on a patient-by-patient basis. Evidence asuggests that a 50% reduction of BCR-ABL activity at 28 days is equally predictive of treatment efficacy as measurement of BCR-ABL transcript levels at 90 days[91], and the development of a reliable method to test treatment efficacy based on the measurement of BCR-ABL activity would potentially improve patient survival rate and reduce the cost of therapy.

The Parker lab has developed a set of TKI-resistant CML cell lines based on the K562 immortalized myeloid cell line containing the BCR-ABL fusion protein, with the goal of elucidating novel biomarkers of drug resistance. To our knowledge no study has examined a cell line model of acquired TKI-resistance using next-generation sequencing. Here we describe the use of RNA sequencing (RNA-seq) to profile the transcriptomic differences between the wild type K562 CML cell line, and cell lines with evolved resistance to the TKIs imatinib, nilotinib, and dasatinib. The goal of this study is to identify potential biomarkers of acquired resistance to TKI-treatment *in vitro*, that may be able to be used to monitor resistance development in patient cells and/or suggest alternative avenues of therapy in response to TKI-resistance.

2.2 RNA-seq Profile of TKI-resistant K562 Cells

2.2.1 Materials and Methods

Generation of TKI-resistant Cell Lines. Dr. Steven Ouellette generated cells. K562 cells were purchased from ATCC and grown in Isocove's Modified Dulbecco's Media (IMDM) supplemented with 10% Fetal Bovine Serum (FBS) and 0.1% penicillin/streptomycin antibiotics in a humidified atmosphere maintained at 37°C and 5% CO_2. The cells were then divided into four groups, one treated with imatinib, one treated with nilotinib, and one treated with dasatinib, and one supplemented with 1:1,000 DMSO. Over the course of 90 days, the cells were incubated with escalating doses of TKI and gradually acquired resistance to the TKI in which they were incubated. The resulting cell lines were termed K562-IR (which is maintained in IMDM supplemented with 1µM imatinib), K562-NR (which is maintained in IMDM supplemented with 10nM nilotinib), and K562-DR (which is maintained in IMDM supplemented with 1nM dasatinib). Each cell line was split into three 10 cm tissue culture plates and passaged twice. When the density of the cells reached ~$7.5x10^5$ cells/mL media the cells were harvested and total RNA was isolated using an RNEasy kit from Qiagen. The RNA was then submitted to the University of Minnesota Genomic Core for RNA-seq analysis on an Illumina HiSeq 2500 sequencer using rapid mode with 50 base pair reads, and a total of 10 million reads per lane.

Differential Expression Testing. The resulting RNA-seq data were analyzed using the Galaxy platform from the University of Minnesota Supercomputing Institute. To

compute differential gene expression between conditions, the raw data were first groomed to remove adapter bases using FASTQ Groomer[92], and then aligned to the Human hg19 canonical reference genome using Tophat2[93]. Differential expression testing between the wild type K562 cells and K562-IR, K562-NR, and K562-DR cells, respectively, was computed using Cuffdiff, a transcript computation program available as a package through Cufflinks[94].

Novel Fusion Transcript Detection. After the raw RNA-seq data were aligned to the Human hg19_canonical reference genome using Tophat2, novel fusion transcripts were detected using DeFuse, as software package used to detect fusion transcripts from paired-end RNA-seq data[95].

Growth Inhibition Assay. K562, K562-IR, K562-NR, and K562-DR cells were transfected with 100nM of AXL siRNA (Cell Signaling #6263), 100nM ACP5 siRNA (Life Technologies #10032), or 100nM of CYP2S1 siRNA (Life Technologies #113728), respectively using a Neon Transfection system (Thermo Fisher Scientific) and incubated at 37°C in a 5% CO2 humidified atmosphere. The cells were seeded at 10,000 cells per well in 96-well plates and dosed with the indicated TKI concentrations or vehicle control (0.1% DMSO) 24 hours after transfection. The remaining cells were reserved for Western blot analysis. Plates were incubated for two days at 37°C in a 5% CO2 humidified atmosphere.

Following incubation, XTT reagent (ATCC) was added according to manufacturer's protocol and plates were incubated at 37°C in a 5% CO2 humidified atmosphere for 3 hours. Specific absorbance was read on a Biotek Synergy4 plate reader at 475 nm and non-specific absorbance read at 660 nm. Absorbance values at 660 nm were subtracted from 475 nm absorbance values before further data processing. To generate IC50 values, data were plotted in GraphPad Prism and curves were fit using non-linear four-parameter slope.

Western Blot Analysis. Cells were treated as described and pelleted by centrifugation. Media was removed, and cells were washed in ice cold PBS. After washing, lysis buffer (PhosphoSafe Extraction Buffer supplemented with 4 mM EDTA and Complete protease inhibitor (Roche)) was added to the cell pellet and cells were incubated on ice for 30 minutes. Cell pellets were vortexed, then centrifuged at 4° C for 20 minutes to remove insoluble cellular debris and lysate was assayed for protein content. 100 µg of protein was mixed with Laemmli protein gel loading buffer (BioRad) and incubated at 95° C for 5 minutes. Lysate were loaded onto a 10% Mini-Protean TGS gel 59 59 (BioRad) and separated at constant 150 V for 1 hour. Proteins were transferred to a PVDF membrane (Millipore) and blocked with 5% BSA in TBST (tris-buffered saline supplemented supplemented with 0.05% Tween-20). Primary antibodies (AXL (Cell Signaling, #8661), ACP5 (Sigma Aldrich SAB1401003), CYP2S1 (Novus Biologicals NBP2-32697,), GAPDH (Sigma Aldrich G8795) diluted 1:1000 in 5% BSA + TBST) were incubated with membrane

overnight at 4° C. Following 3 x 5 minute washes with TBST, the membrane was incubated with donkey anti-rabbit tagged with IRDye 800 or donkey anti-mouse tagged with IRDye 680(1:5000 in TBST) for 1 hours at room temperature. After 3 x 5 minute washes with TBST, the membrane was developed on a LICOR Odyssey infrared scanner.

2.2.2 Analysis of RNA-seq Results

2.2.3 Differential Expression Between K562 and K562-IR Cell Lines

The use of next-generation sequencing has been a powerful research tool to identify difference in gene expression in a variety of pathologies and developmental stages[96-98]. RNA-seq is a highly quantitative method to measure absolute transcript levels across samples without the need to normalize data to a set of standards, and the incredible sensitivity allows for a theoretical measurement of all transcripts present in a population of cells. We aimed to identify differences in transcription between wild type K562 cells and K562-IR cells in order to identify biomarkers for resistance, and potentially explain mechanisms of resistance to imatinib treatment. After analysis using Cuffdiff, we identified fifty-eight genes that were differentially expressed at a significant level ($p<0.05$), and had an absolute $\log_2$ fold change of >1, listed in Table 1 below.

Table 1. K562-IR genes found to have significant (p>0.05) differential expression with respect to parental K562 cells determined by RNA-seq. Differential expression was analyzed using Cufflinks on the Galaxy platform. Fold change is expressed in terms of log_2.

Gene	Fold Change (log_2)	p-value	Function
SIGLEC6	-4.37	1.00E-04	other
FCGRT	-3.292	5.00E-05	transmembrane receptor
PALM3	-3.184	5.00E-05	other
RASGRP4	-2.967	5.00E-05	other
CD33	-2.589	5.00E-05	other
TLE2	-2.527	5.00E-05	transcription regulator
ZNF433	-2.288	5.00E-05	other
B3GNT8	-1.78	2.25E-03	enzyme
PALM	-1.455	5.00E-05	other
CD320	-1.344	5.00E-05	other
NFIX	-1.303	5.00E-05	transcription regulator
RAB3A	-1.28	1.50E-04	enzyme
C19orf51	-1.222	5.00E-05	other
PPP1R14A	-1.209	5.00E-05	phosphatase
DLL3	-1.176	3.45E-03	other
CD37	-1.17	5.00E-05	other
CFD	-1.17	5.00E-05	peptidase
BRSK1	-1.151	2.00E-04	kinase
AURKC	-1.144	1.28E-02	kinase
TUBB4A	-1.137	5.00E-05	other
TYROBP	-1.071	6.10E-03	transmembrane receptor
FUZ	-1.022	8.60E-03	other
JUNB	1	5.00E-05	transcription regulator

Table 1 Continued.

EHD2	1.035	5.00E-05	other
ZNF626	1.039	3.50E-04	other
PRX	1.047	5.00E-05	other
EXOC3L2	1.08	5.00E-05	other
SERTAD1	1.084	5.00E-05	transcription regulator
ZNF134	1.094	5.00E-05	transcription regulator
HCN2	1.097	5.00E-05	ion channel
BBC3	1.128	5.00E-05	other
CNN1	1.192	5.00E-05	other
VSIG10L	1.309	5.00E-05	other
KCNC3	1.324	5.00E-05	ion channel
KLK1	1.357	5.00E-05	peptidase
GDF15	1.457	5.00E-05	growth factor
KCNN4	1.494	5.00E-05	ion channel
RRAS	1.534	5.00E-05	enzyme
ZNF91	1.631	5.00E-05	transcription regulator
TNFSF9	1.653	5.00E-05	cytokine
ZNF347	1.674	5.00E-05	other
ZNF211	1.711	5.00E-05	transcription regulator
SCN1B	1.734	5.00E-05	ion channel
KLK13	1.793	5.00E-05	peptidase
ZNF551	1.835	5.00E-05	other
GP6	1.876	5.00E-05	transmembrane receptor
F2RL3	1.935	5.00E-05	G-protein coupled receptor
PPP1R13L	1.941	5.00E-05	transcription regulator
ZSCAN18	1.953	5.00E-05	other
CLEC11A	2.175	5.00E-05	growth factor
TNNI3	2.553	5.00E-05	transporter

Table 1 Continued.

CYP2S1	2.616	5.00E-05	enzyme
SYT3	2.652	5.00E-05	transporter
AXL	2.784	5.00E-05	kinase
ZNF85	2.838	1.00E-04	transcription regulator
LRRC4B	3.301	5.00E-05	other
ACP5	3.472	5.00E-05	phosphatase
HKR1	3.767	5.00E-05	Other

The initial analysis of differential expression yielded some interesting results. Many of the genes displaying a high level of differential expression were transcription factors or encoded for transmembrane transport proteins, and several canonical signaling molecules were absent, such as members of the SRC-family kinases or AKT pathway. It is also noteworthy that many of the genes identified have not been described in cancer models, which may reflect their status as passenger genes, not directly responsible for any mechanism of resistance. Further study is warranted to determine what, if any, influence these genes have in imatinib resistance.

Several genes significantly differentially expressed genes are interesting candidates for further research. The most downregulated gene was SIGLEC6, a transmembrane receptor responsible for binding leptin. Two studies have associated increased SIGLEC6 with increased invasiveness, cell proliferation, and inhibition of apoptosis in gestational

trophoblastic disease and mucosa-associated lymphoid tissue lymphoma[99, 100], however we report a significant reduction in the transcription of SIGLEC6 in imatinib-resistant K562 cells. BRSK1 (-1.151 $\log_2$ fold change) a serine/threonine kinase involved in cell cycle regulation and the DNA damage response that has not been examined in any human leukemia models. Currently, the only description of BRSK1 expression levels in human cancer indicates that BRSK1 functions as a tumor suppressor in human breast cancer, and low levels of BRSK1 correlate with poor prognosis[101].

Aurora C (ARKC) has not been thoroughly investigated in human cancers, despite being a member of the Aurora Kinase family, of which Aurora A and Aurora B are known to be involved in oncogenesis. The few studies that have investigated ARKC's role in cancer have concluded that increased ARKC activity drives cancer progression[102, 103], however we observed a downregulation (-1.144 $\log_2$ fold change) of ARKC transcription.

The most highly upregulated gene, HKR1 (3.767 $\log_2$ fold change), is a Krüppel-type ZNF gene that has only been investigated in one study, which showed that exposure to cisplatin induced the transcription of HKR1[104]. Lastly, AXL is a receptor tyrosine kinase that has been implicated in imatinib resistance. Previous studies have demonstrated that AXL overexpression is associated with imatinib resistance through a scaffold function by stabilizing LYN kinase in an active conformation[105, 106], and the findings

of our RNA-seq experiment demonstrated a 2.784 $\log_2$ fold change for AXL transcripts, which supports previous reports.

2.3.2 Differential Expression Between K562 and K562-NR Cell Lines

Nilotinib is another frontline tyrosine kinase inhibitor commonly prescribed to treat patients with Ph+ CML. Nilotinib inhibits BCR-ABL 10-30 times more potently than imatinib does, and also inhibits c-KIT, LCK, EPHA3, EPHA8, DDR1, DDR2, PDGFRB, MAPK11, and ZAK. Patients who have acquired resistance to imatinib can achieve some level of response to nilotinib, as a study found that 39% of patients with BP-CML achieved a hematological response and 27% had a cytogenic response to nilotinib after failing imatinib treatment[70]. This is possibly due to the fact that nilotinib inhibits all BCR-ABL kinase domain mutants except T315I, Y253H, E255K/V, and F359C/V[16, 70, 71], however, in a study of imatinib-resistance CML patients 23% of patients treated with nilotinib developed resistance within two years[14]. Interestingly, patients treated with nilotinib developed BCR-ABL mutations at a reduced rate compared with patients treated with imatinib[107], which suggests the possibility that resistance to nilotinib is dependent upon mechanisms other than target mutation, such as compensatory signaling pathway activation, BCR-ABL upregulation, a reduction in influx transport channels, or increase in efflux transport channels. We used RNA-seq to investigate

differential gene expression between the parental K562 cell line and the K562-NR cell line, which has evolved resistance to nilotinib. The results are shown in Table 2 below.

Table 2. K562-NR genes found to have significant (p>0.05) differential expression with respect to parental K562 cells determined by RNA-seq. Differential expression was analyzed using Cufflinks on the Galaxy platform. Fold change is expressed in terms of log_2.

Gene	Fold Change (log_2)	p-value	Function
BLOC1S3	-3.109	5.00E-05	other
ZNF257	-2.888	5.00E-05	other
EID2	-2.493	5.00E-05	other
ZNF429	-2.486	5.00E-05	other
TUBB4A	-2.422	5.00E-05	other
NFIX	-2.261	5.00E-05	transcription regulator
ZFP30	-2.216	5.00E-05	other
FOXA3	-2.12	5.00E-05	transcription regulator
HAUS8	-2.067	5.00E-05	other
ZNF433	-1.942	1.50E-04	other
C3	-1.876	5.00E-05	peptidase
GEMIN7	-1.856	5.00E-05	other
NLRP2	-1.832	5.00E-05	other
CCDC8	-1.753	5.00E-05	other
SCAMP4	-1.752	5.00E-05	other
ASF1B	-1.653	5.00E-05	other
ZNF607	-1.65	5.00E-05	other
ARRDC2	-1.636	5.00E-05	other
ZNF283	-1.619	3.00E-04	other
PALM3	-1.619	5.00E-05	other
SIGLEC6	-1.618	3.00E-04	other
ZSWIM4	-1.617	5.00E-05	other

Table 2 Continued.

RASGRP4	-1.608	5.00E-05	other
CCDC97	-1.601	5.00E-05	other
KISS1R	-1.596	1.80E-03	G-protein coupled receptor
ZNF550	-1.595	3.90E-03	other
ZNF616	-1.58	5.00E-05	other
ZNF816	-1.571	5.00E-05	other
SPHK2	-1.495	5.00E-05	kinase
VAV1	-1.477	5.00E-05	transcription regulator
ZNF788	-1.465	5.00E-05	other
ZNF30	-1.46	5.00E-05	other
ZNF324	-1.451	5.00E-05	other
EMR2	-1.442	5.00E-05	other
SIX5	-1.442	5.00E-05	transcription regulator
ZNF175	-1.44	5.00E-05	transcription regulator
ZNF8	-1.426	5.00E-05	other
FCGRT	-1.406	1.50E-04	transmembrane receptor
ZNF253	-1.387	2.00E-04	transcription regulator
SLC25A41	-1.38	2.75E-03	other
MIER2	-1.361	5.00E-05	other
LRG1	-1.334	5.00E-05	other
ZNF14	-1.322	5.00E-05	transcription regulator
ZNF567	-1.316	5.00E-05	other
MOB3A	-1.302	5.00E-05	other
EPOR	-1.295	5.00E-05	transmembrane receptor
LRRC8E	-1.288	5.00E-05	other
ZNF614	-1.279	5.00E-05	other
PALM	-1.276	5.00E-05	other
ZNF761	-1.26	5.00E-05	other
CD3EAP	-1.239	5.00E-05	other
TBXA2R	-1.234	5.00E-05	G-protein coupled receptor
PPAN	-1.211	5.00E-05	other
SCAF1	-1.2	5.00E-05	other
DPY19L3	-1.198	5.00E-05	other
ZNF181	-1.188	4.00E-04	other
SUGP1	-1.188	5.00E-05	other

Table 2 Continued.

PRR12	-1.162	5.00E-05	other
ITPKC	-1.16	5.00E-05	kinase
NFIX	-1.152	5.00E-05	transcription regulator
LDLR	-1.139	5.00E-05	transporter
PPP6R1	-1.139	5.00E-05	other
ZNF320	-1.123	5.00E-05	other
CD37	-1.116	2.90E-03	other
ZNF507	-1.109	5.00E-05	other
NUP62	-1.094	5.00E-05	transporter
NR1H2	-1.081	5.00E-05	ligand-dependent nuclear receptor
TICAM1	-1.073	5.00E-05	other
RASIP1	-1.069	5.00E-05	other
PIP5K1C	-1.059	5.00E-05	kinase
MYADM	-1.049	5.00E-05	other
KRI1	-1.023	5.00E-05	other
RDH13	-1.01	5.00E-05	enzyme
PAK4	1.01	5.00E-05	kinase
PLAUR	1.023	5.00E-05	transmembrane receptor
ZNF358	1.025	5.00E-05	other
UBE2S	1.026	5.00E-05	enzyme
CNN1	1.027	5.00E-05	other
TEAD2	1.036	5.00E-05	transcription regulator
FLT3LG	1.044	6.00E-04	other
ETHE1	1.054	5.00E-05	enzyme
KLF16	1.059	5.00E-05	other
GLTSCR2	1.061	5.00E-05	other
PNPLA6	1.073	5.00E-05	other
RPS15	1.076	5.00E-05	other
KDELR1	1.081	5.00E-05	transporter
KLK1	1.106	5.00E-05	peptidase
HOMER3	1.116	5.00E-05	other
HRC	1.127	5.00E-05	other
DOCK6	1.146	5.00E-05	other
ZNF626	1.151	7.00E-04	other

Table 2 Continued.

RPL28	1.159	5.00E-05	other
SCN1B	1.19	2.20E-03	ion channel
RPL13A	1.192	5.00E-05	other
AP2S1	1.21	5.00E-05	transporter
JUND	1.235	5.00E-05	transcription regulator
ECH1	1.301	5.00E-05	enzyme
DBP	1.338	5.00E-05	transcription regulator
PRX	1.348	5.00E-05	other
PEPD	1.355	5.00E-05	peptidase
LRRC4B	1.385	5.00E-05	other
C19orf79	1.404	3.00E-03	other
KCNN4	1.429	5.00E-05	ion channel
CERS1	1.43	5.00E-05	enzyme
ARHGAP33	1.482	5.00E-05	transporter
PPP1R13L	1.559	5.00E-05	transcription regulator
ZNF296	1.588	3.00E-04	other
TNFSF9	1.682	5.00E-05	cytokine
A1BG	1.764	5.00E-05	other
BBC3	1.781	5.00E-05	other
VSIG10L	1.815	5.00E-05	other
RRAS	2.01	5.00E-05	enzyme
CLEC11A	2.08	5.00E-05	growth factor
GDF15	2.083	5.00E-05	growth factor
APOC1	2.086	5.00E-05	transporter
APOE	2.208	5.00E-05	transporter
CYP2S1	2.246	5.00E-05	enzyme
TNNI3	2.251	5.00E-05	transporter
KLK13	2.437	5.00E-05	peptidase
ZNF85	2.464	3.50E-04	transcription regulator
SERTAD1	2.591	5.00E-05	transcription regulator
AXL	2.679	5.00E-05	kinase
PPP1R15A	2.768	5.00E-05	other
GADD45B	3.091	1.10E-03	other
ACP5	4.7	5.00E-05	phosphatase
RPL18A	4.803	5.00E-05	other

The RNA-seq results identified 126 genes that were significantly differentially expressed in the K562-NR cell line as compared to the K562 parental cell line. Interestingly, many of the genes that are the most significantly differentially expressed have not been characterized in leukemia, or other human cancers. BLOC1S3 encodes for a protein in the BLOC1 multi-protein complex, which regulates biogenesis of specific organelles in the endosomal-lysosomal system. Despite finding that BLOC1S3 has a $\log_2$ fold change of -3.109 in K562-NR cells as compared to K562 cells, no role in any human cancer has been described as of yet.

One very interesting finding is the downregulation of NFIX, a transcription factor critical to the regulation of differentiation in the hematopoietic system. The loss of NFIX perturbed normal gene expression in myeloid and lymphoid cells *in-vivo*, and NFIX has been demonstrated to interact with c-JUN in viral disease models[108, 109]. Furthermore, NFIX is a known target of miR-659-3p, a biomarker of improved progression-free survival in metastatic melanoma[110]. Taken together with our RNA-seq results, these studies suggest NFIX is an interesting candidate for further study of its role in nilotinib-resistant CML.

SERTAD1 is upregulated at a $\log_2$ fold change of 2.591 in K562-NR cells compared to K562 cells. SERTAD1 is a transcription factor interacting with the PHD-bromodomain-1, and stimulates E2F1/TFDP1 transcriptional activity, rendering the activity of cyclin

D1/CDK4 resistant to the inhibitory effects of CDKN2A/p16INK4A[111, 112]. Furthermore, SERTAD1 has been shown to inhibit the tumor suppressor protein PTEN in breast cancer cells, leading to cell survival and increased tumorigenesis by activating the PI3K/AKT cell proliferation pathway[113]. The significant upregulation in K562-NR cells and previously established role in tumorigenesis makes SERTAD1 a potential target for further studies examining the roots of nilotinib resistance in CML.

Another interesting set of genes that are significantly upregulated in K562-NR cells compared to K562 cells are APOE and APOC1, upregulated at a $\log_2$ fold change of 2.208 and 2.086, respectively. Members of the apolipoprotein family, APOE and APOC1 are primarily involved with binding, internalization, and catabolism of lipoproteins, and APOC1 has been implicated in the progression of pancreatic cancer[114], while increased expression of APOE is a biomarker of aggressive acute lymphoblastic leukemia and has been correlated with short progression-free survival in metastatic colorectal cancer[115, 116]. APOC1 is also overexpressed in gastric cancer, and treatment with the histone deacetylase inhibitor vorinostat dramatically reduced the transcription of APOC1 and induced cell death[117]. Many recent studies have investigated the use of vorinostat in acute myeloid leukemia, and it may be beneficial to examine its effects in CML as well.

Given the large raw data output from RNA-seq experiments, it can be difficult to determine if perturbation of a particular pathway may underlie resistance to TKI treatment. We used Ingenuity Pathway Analysis software (Qiagen) to establish pathways affected by nilotinib resistance, and found that the Eukaryotic Initiation Factor 2 pathway (EIF2) had a significant p-value ($p=3.02^{-3}$) and a z-score of 2. This indicates that the differential expression of the genes involved in this pathway predict that the EIF2 pathway will be activated in K562-NR cells. The genes differentially expressed in K562-NR cells that predict the activation of the EIF2 pathway are RPL18A, RRAS, RPL28, PPP1R15A, RPS15, and RPL13A, all of which are upregulated in K562-NR cells compared to K562 cells. Phosphorylation of EIF2 subunits inhibits protein synthesis and leads to apoptosis, but interestingly, PPP1R15A binds to PP1 and activates the EIF2 pathway through dephosphorylation of EIF2-α[117]. Treatment of T-cell Leukemia (T-ALL) with pegylated-human-arginase I (peg-Arg-I) induced decreased expression of PPP1R15A and subsequent dephosphorylation of EIF2, resulting in apoptosis in T-ALL bearing mice[118]. Targeting the EIF2 pathway may be a viable therapeutic option for patients resistant to nilotinib.

2.2.3 Differential Expression Between K562 and K562-DR Cell Lines

Dasatinib is a second-generation dual BCR-ABL/SRC inhibitor that binds approximately 325 times more tightly to wild type BCR-ABL than imatinib, and can overcome some of the limitations of imatinib and nilotinib by inhibiting SRC-family kinases whose

compensatory activation confers TKI-resistance. The results of the DASISION (Dasatinib versus Imatinib Study in Treatment-Naive CML-CP) clinical trials were recently published, in which 65% of the dasatinib-treated patients required BCR-ABL mutational analysis due to treatment issues (no cCCyR within 12 months; no MMR within 12 months; fivefold increase in *BCR-ABL1* transcript levels with loss of MMR; loss of CCyR), and surprisingly, only 9% of dasatinib-treated patients had developed BCR-ABL mutations[17]. It seems clear that further analysis of BCR-ABL independent dasatinib resistance is warranted. We developed a dasatinib-resistant K562 cell line (K562-DR) by culturing K562 cells in escalating concentrations of dasatinib, and performed RNA-seq on K562-DR cells to compare the transcriptomes of K562-DR cells with the parental K562 cells to identify potential mechanisms of resistance, and identify novel biomarkers of dasatinib resistance. The significantly differentially expressed genes in K562-DR cells as compared to K562 cells are shown in Table 3 below.

Table 3. K562-DR genes found to have significant ($p>0.05$) differential expression with respect to parental K562 cells determined by RNA-seq. Differential expression was analyzed using Cufflinks on the Galaxy platform. Fold change is expressed in terms of $\log_2$.

Gene	Fold Change (log_2)	p-value	Function
ZNF433	-3.861	2.70E-03	other
RASGRP4	-2.08	5.00E-05	other
ZNF257	-1.841	5.00E-05	other
ZNF93	-1.812	5.00E-05	transcription regulator
CD33	-1.725	5.00E-05	other
MYO1F	-1.435	5.00E-05	other
PALM3	-1.435	5.00E-05	other
ZNF559	-1.297	5.00E-05	other
B3GNT8	-1.279	3.75E-03	enzyme
CD320	-1.184	5.00E-05	other
TSKS	-1.165	5.00E-04	kinase
NFIX	-1.083	5.00E-05	transcription regulator
CALR	-1.07	5.00E-05	transcription regulator
DUS3L	-1.068	5.00E-05	other
C3	-1.024	5.00E-05	peptidase
ZNF776	1.011	5.00E-05	other
F2RL3	1.031	5.00E-05	G-protein coupled receptor
EXOC3L2	1.036	5.00E-05	other
LINC00085	1.05	5.00E-05	other
SIPA1L3	1.085	5.00E-05	other
ZNF91	1.088	5.00E-05	transcription regulator
ZNF600	1.178	5.00E-05	other
ZNF432	1.21	2.00E-04	other
VSIG10L	1.248	5.00E-05	other
CLEC11A	1.252	5.00E-05	growth factor
KLF1	1.26	5.00E-05	transcription regulator
ZNF347	1.261	5.00E-05	other
ZNF253	1.397	5.00E-05	transcription regulator
ZNF813	1.626	5.00E-05	other
ZNF551	1.772	5.00E-05	other
ZNF211	1.783	5.00E-05	transcription regulator
KLK1	1.84	5.00E-05	peptidase
KCNC3	1.861	5.00E-05	ion channel
KLK13	1.913	5.00E-05	peptidase

Table 3 Continued.

ZNF134	1.921	5.00E-05	transcription regulator
PPAP2C	2.012	5.00E-05	phosphatase
ZNF671	2.101	5.00E-05	other
PPP1R13L	2.138	5.00E-05	transcription regulator
KCNN4	2.157	5.00E-05	ion channel
SYT3	2.181	1.00E-04	transporter
ZSCAN18	2.305	5.00E-05	other
RPL18A	2.441	5.00E-05	other
AURKC	2.45	5.00E-05	kinase
TNNI3	2.457	5.00E-05	transporter
CYP2S1	2.475	5.00E-05	enzyme
HKR1	2.584	7.00E-04	other
AXL	2.766	5.00E-05	kinase
ACP5	2.907	5.00E-05	phosphatase
ZNF85	2.973	2.00E-04	transcription regulator
LRRC4B	3.057	5.00E-05	other

Dasatinib inhibits SRC-family kinases in addition to BCR-ABL, so one would expect a different set of genes to be differentially expressed in K562-DR cells as compared to K562-IR and K562-NR cells. As with the K562-IR and K562-NR cell lines, many members of the zinc-finger family (ZNF) of transcription factors are differentially expressed, however the vast majority of ZNF proteins are uncharacterized, and understanding their function in the context of TKI resistance would be a difficult task.

Ras guanyl nucleotide releasing protein 4 (RASGRP4) is a guanyl nucleotide exchange factor that activates RAS in mast cells, and is involved with platelet adhesion and cell

proliferation[119]. Interestingly, RASGRP4 is highly expressed in myeloid cells, and high expression of RASGRP4 has been observed in acute myeloid leukemia[120, 121]. Interestingly, the results of our RNA-seq experiment show RASGRP4 has a (log_2 fold change of -2.080 in K562-DR cells compared to K562 cells, potentially indicating that the mechanism of dasatinib resistance is independent of RAS activation. Further experimentation is needed to confirm our results, but RAS activation likely does not contribute to dasatinib resistance.

Another interesting finding from our K562-DR RNA-seq experiment is the downregulation of B3GNT8 (log_2 fold change of -1.279) compared to K562 cells. B3GNT8 is an enzyme that regulates the elongation of specific branch structures of multiantennary N-glycans, and is involved with the biosynthesis of N-glycan precursor proteins. The results of our RNA-seq experiment demonstrated a significant downregulation of B3GNT8, but several other studies have demonstrated B3GNT8 plays a significant role in cancer progression. B3GNT8 silencing reduces cell proliferation, migration, and metastasis in human glioma cells[122], and human laryngeal carcinoma cells[123]. Even more interesting is the fact that B3GNT8 was overexpressed in multidrug-resistant K562 cells, and knockdown of B3GNT8 increased sensitivity to adriamycin[124]. B3GNT8 has been suggested to increase resistance to chemotherapy by activating TGF-β signaling, which increases cell proliferation and resistance to apoptosis[123], however we report no change in TGF-β expression in K562-DR cells as

compared to K562 cells. This does not exclude the possibility that TGF-β activity is increased, however it is not possible to draw that conclusion based on transcriptomic data alone.

Analysis of the RNA-seq data comparing the genes expressed in our K562-DR cell line and K562 cells shows many of the significantly differentially expressed genes are upregulated in K562-DR cells despite having been reported to be downregulated in other cancers, and vice versa. The most differentially expressed gene in our RNA-seq results is LRRC4B, a protein regulating neurite outgrowth and dendrtitic segmentation in the developing brain. LRRC4B has been identified as a tumor suppressor in glioma carcinoma[36, 125], as overexpression of LRRC4 inhibited glioma tumorigenesis and induced apoptosis. We report an upregulation of LRRC4B (log_2 fold change of 3.057), which conflicts with reports that LRRC4B is a tumor suppressor. LRRC4B may be a passenger gene, and its upregulation may have no effect on TKI-resistance.

One possible explanation for dasatinib resistance is the upregulation of Aurora Kinase C (AURKC), which was found to have a log_2 fold change of 2.450. AURKC is a serine/threonine kinase that forms a complex with Aurora B kinase and plays a role in organizing microtubules in relation to the centrosome during mitosis. Several Aurora A and Aurora B inhibitors are currently undergoing clinical trials for a variety of human

leukemias, and treatment of imatinib-resistant CML patients with the Aurora A inhibitor MLN8237 was shown to significantly increase the efficacy of nilotinib, suggesting Aurora A activity may contribute to imatinib resistance[126]. AURKC has been noted to be upregulated in prostate cancer, epithelial cancer, colorectal cancer, and thyroid cancer[102, 103, 127, 128]. These findings support the hypothesis that AURKC activity may be involved in dasatinib-resistance in our K562-DR cell line by driving increased cell proliferation or increasing resistance to apoptosis, and further study is warranted to investigate AURKC's involvement in resistance.

RNA-seq comparison of the differentially expressed genes in the K562-DR cell line and K562 cell line revealed several differentially expressed genes that conflicted with reports from previous studies. It is important to remember that this study is based on TKI-resistant cell lines generated in a cell culture setting, and the resulting findings provide nothing more than a preliminary direction to explore. Further study of our results with human patient samples is necessary to validate any novel biomarkers or mechanisms of resistance.

2.3.4 Common Features of K562-TKIr Cell Lines

Treatment of CML is expensive, and the longer a patient fails to respond to therapy, the bleaker the prognosis. While it is important to understand the transcriptomic

differences between imatinib, nilotinib, and dasatinib resistant cell lines, it is equally important to identify any common themes linking resistance to imatinib, nilotinib, and dasatinib in order to suggest novel resistance mechanisms or biomarkers shared by resistant cell lines. We have identified 24 significantly differentially expressed genes that were identified in K562-IR, K562-NR, and K562-DR cell lines that could serve as potential biomarkers of TKI-resistance, or contribute to TKI-resistance mechanisms. Table 4 lists these genes below.

Table 4. K562-TKI-resistant cell line genes found to have significant (p>0.05) differential expression with respect to parental K562 cells determined by RNA-seq. Differential expression was analyzed using Cufflinks on the Galaxy platform. Fold change is expressed in terms of $\log_2$.

Gene	Fold Change (IR)	Fold Change (NR)	Fold Change (DR)	Function
ACP5	3.472	4.7	2.907	phosphatase
AURKC	-1.144	-0.689	2.45	serine/threonine kinase
AXL	2.784	2.679	2.766	tyrosine kinase
C3	-0.967	-1.876	-1.024	peptidase
CD33	-2.589	-0.711	-1.725	other
CYP2S1	2.616	2.246	2.475	enzyme
CLEC11A	2.175	2.08	1.252	growth factor
LRRC4B	3.301	1.385	3.057	other
KLF1	0.962	0.447	1.26	transcription factor

Table 4 Continued.

KLK1	1.357	1.106	1.84	peptidase
KLK13	1.793	2.437	1.913	peptidase
KCNN4	1.494	1.429	2.157	ion channel
JUNB	1	0.676	0.731	transcription factor
NFIX	-1.303	-2.261	-1.083	transcription factor
PPP1R13L	1.941	1.559	2.138	transcription factor
RASGRP4	-2.967	-1.608	-2.08	other
RRAS	1.534	2.01	0.937	enzyme
TLE2	-2.527	-1.451	-1.329	transcription factor
TNFSF9	1.653	1.682	0.87	cytokine
TNNI3	2.553	2.251	2.457	transporter
TUBB4A	-1.137	-2.422	-0.915	other
VSIG10L	1.309	1.815	1.248	other
ZNF85	2.838	2.464	2.973	trancription factor
ZNF433	-2.288	-1.942	-3.861	transcription factor

Based on our RNA-seq results we selected several candidate genes to assess their significance in TKI-resistance. We chose to ablate AXL as a positive control for our K562-IR and K562-NR cell lines, as it had previously been described as a mediator of imatinib and nilotinib resistance, but had not been previously described in dasatinib resistance[42, 105]. ACP5 and CYP2S1 were chosen for further investigation, as their upregulation were novel findings in a CML model.

We first had to confirm whether the increase in transcription correlated with protein expression. Figure 1 below confirms that protein expression of each gene is increased in each TKI-resistant cell line.

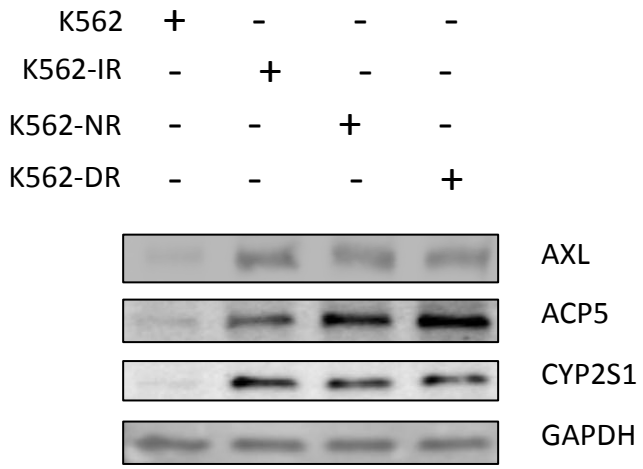

Figure 1. Protein expression is increased in TKI-resistant cell lines. Cells were grown to $7.5x10^5$ cells/mL and processed for western blot analysis. AXL, ACP5, and CYP2S1 protein expression was increased in all TKI-resistant cell lines.

AXL is part of the TAM family of receptor tyrosine kinases and is activated upon binding to the extracellular ligand GAS6. Upon binding, AXL initiates a signaling cascade influencing cell migration, aggregation, and proliferation[129, 130]. AXL is known to be integral to the proliferation, metastasis, and contributes to drug resistance in breast, lung, prostate, ovarian, and many other cancers[131-134]. Because of its high expression and contribution to drug resistance in so many cancers, AXL is an enticing drug target for small-molecule inhibitors. However, AXL has been shown to contribute to imatinib and nilotinib resistance in CML through a LYN-dependent scaffolding

function[42, 105]. We found AXL transcription to be upregulated in K562-IR (log_2 fold change of 2.784), K562-NR (log_2 fold change of 2.679), and K562-DR (log_2 fold change of 2.766) cell lines when compared to the parental K562 cell line. Despite having been implicated in imatinib and nilotinib resistance, AXL has never been implicated in dasatinib resistance. AXL mediates nilotinib resistance through LYN, and because dasatinib inhibits the kinase activity of LYN, we sought to determine if knockdown of AXL using siRNA in K562-DR cells inhibited proliferation relative to the K562 parental cell line in the same way it does in the K562-IR and K562-NR cells, or if inhibition of AXL kinase activity could inhibit proliferation in K562-DR cells. The results of the experiment confirmed previous reports that knockdown of AXL in imatinib and nilotinib resistant cells resensitized the cells to imatinib and nilotinib in a significant manner. The K562-IR cells were equally sensitive to imatinib as the parental K562 cells, and the knockdown of AXL in K562 cells did not have any effect on the proliferation of K562 cells in response to imatinib (Figure 2A). This indicates that AXL is an important mediator of acquired imatinib-resistance. Similarly, the knockdown of AXL in K562-NR cells restored sensitivity to nilotinib in the same manner as seen in the K562-IR cells (Figure 2A & 2B). However, the resensitization in the K562-DR cell line was modest. The proliferation of K562 cells and K562-DR cells was largely unaffected by AXL knockdown (Figure 2C), which may be indicative that the role of AXL in K562-DR cells is not as vital to TKI-resistance as it is in K562-IR and K562-NR cells, where it acts as a scaffold to stabilize LYN and drive proliferation[42, 105]. Dasatinib inhibits LYN kinase, so it is unlikely that the upregulation of AXL mediates resistance through LYN activation. We can conclude that

AXL does not affect dasatinib resistance, which may proceed through a different set of compensatory mechanisms than imatinib and nilotinib resistance.

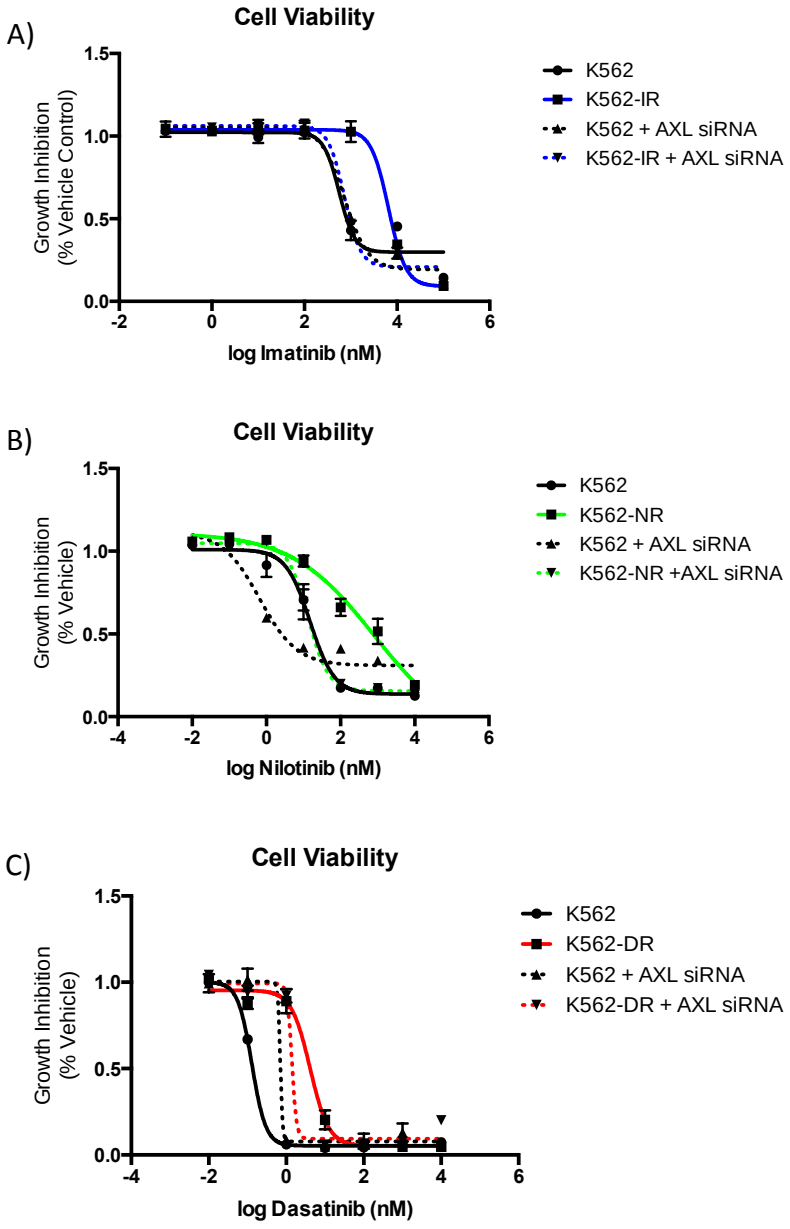

Figure 2. Knockdown of AXL Preferentially Sensitizes K562-IR Cells to imatinib, and K562-NR cells to nilotinib, but does not preferentially sensitize K562-DR cells to dasatinib. Cells were transfected with 100nM AXL siRNA for and seeded in 96-well

plates for 24 hours before treatment with the indicated inhibitor (n=3). A) Knockdown of AXL resensitizes K562-IR cells to imatinib. B) Knockdown of AXL resensitizes K562-NR cells to nilotinib. C) Knockdown of AXL modestly resensitizes K562-DR cells to dasatinib.

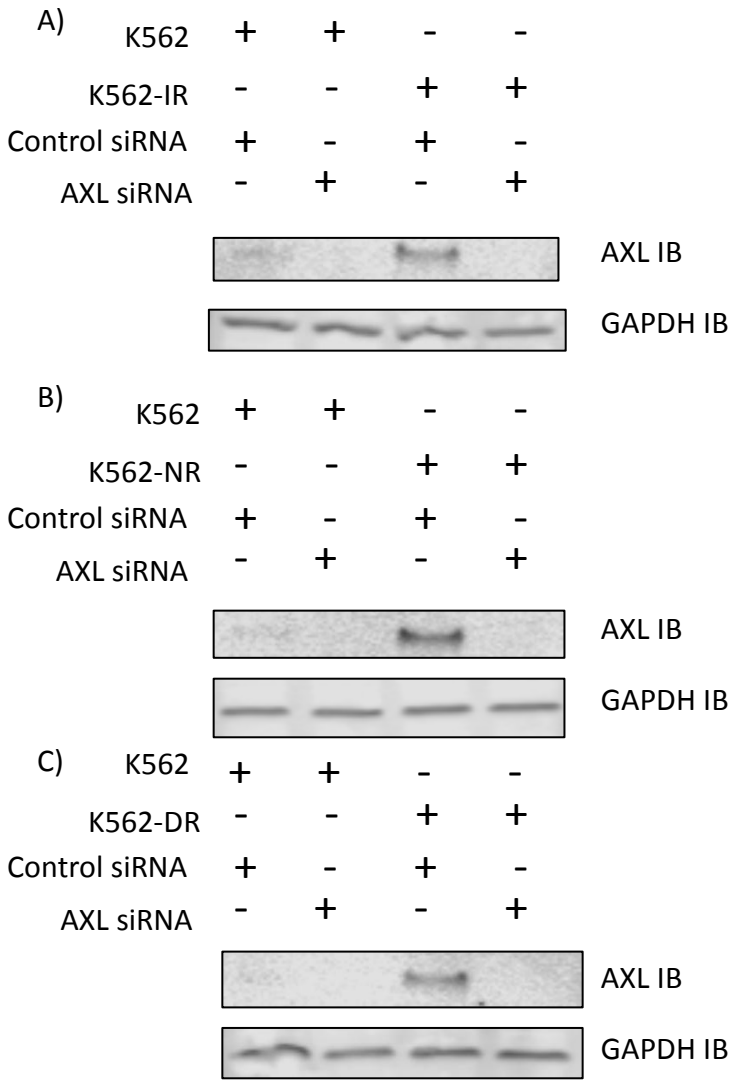

Figure 3. Confirmation of AXL Knockdown for XTT Assay. Cells were transfected with 100nM AXL siRNA and dosed with the indicated inhibitors 24 hours later. Seventy-two

hours post-transfection $1x10^6$ cells were lysed and subjected to western blot analysis. A) Knockdown of AXL was successful in K562 and K562-IR cells. B) Knockdown of AXL was successful in K562 and K562-NR cells. C) Knockdown of AXL was successful in K562 and K562-DR cells.

Tartrate-resistant acid phosphatase 5 (ACP5) is the only acid phosphatase not inhibited by L(+)tartrate. Bone resorption and osteoclast differentiation are directed by ACP5 through modulation of focal adhesion kinase (FAK) but ACP5 is also a primary driver of metastasis and invasiveness in several cancers, and upregulation has been correlated with poor prognosis. Overexpression of ACP5 drives invasiveness and metastasis in hepatocellular carcinoma and human melanoma[135, 136], and it is highly upregulated in gastric cancer, breast cancer, and bone metastases from breast cancer patients[137-140]. ACP5 expression has not been studied in any human leukemia, but the significant upregulation of ACP5 transcription in all three TKI-resistant cell lines compared to the parental K562 cell line (log_2 fold change of 3.472, 4.700, and 2.907 in K562-IR, K562-NR, and K562-DR cells, respectively) along with the many reports of increased ACP5 levels coinciding with increased invasiveness and metastasis in other cancers led us to validate the effect of upregulated ACP5 in TKI-resistance in CML. Interestingly, we observed a pronounced sensitivity to imatinib and nilotinib (Figure 4A & 4B), but a negligible resensitization to dasatinib (Figure 4C). The knockdown of ACP5 in K562-DR cells and K562 cells had almost no effect on the proliferation of either cell line in the presence of

dasatinib, indicating that ACP5 is not likely a primary driver of dasatinib-resistance (Figure 4C). In both K562-IR and K562-NR cells, knockdown of ACP5 had a similar effect as the knockdown of ACP5 in K562 cells, suggesting that the upregulation of ACP5 may be correlated with acquired resistance to imatinib and nilotinib, respectively (Figure 4A & 4B). ACP5 is a marker of poor prognosis in other cancers, but the specific mechanism by which ACP5 mediates oncogenesis is unclear[135, 136, 138, 141]. Future studies may determine ACP5's role in cancer pathogenesis and chemoresistance.

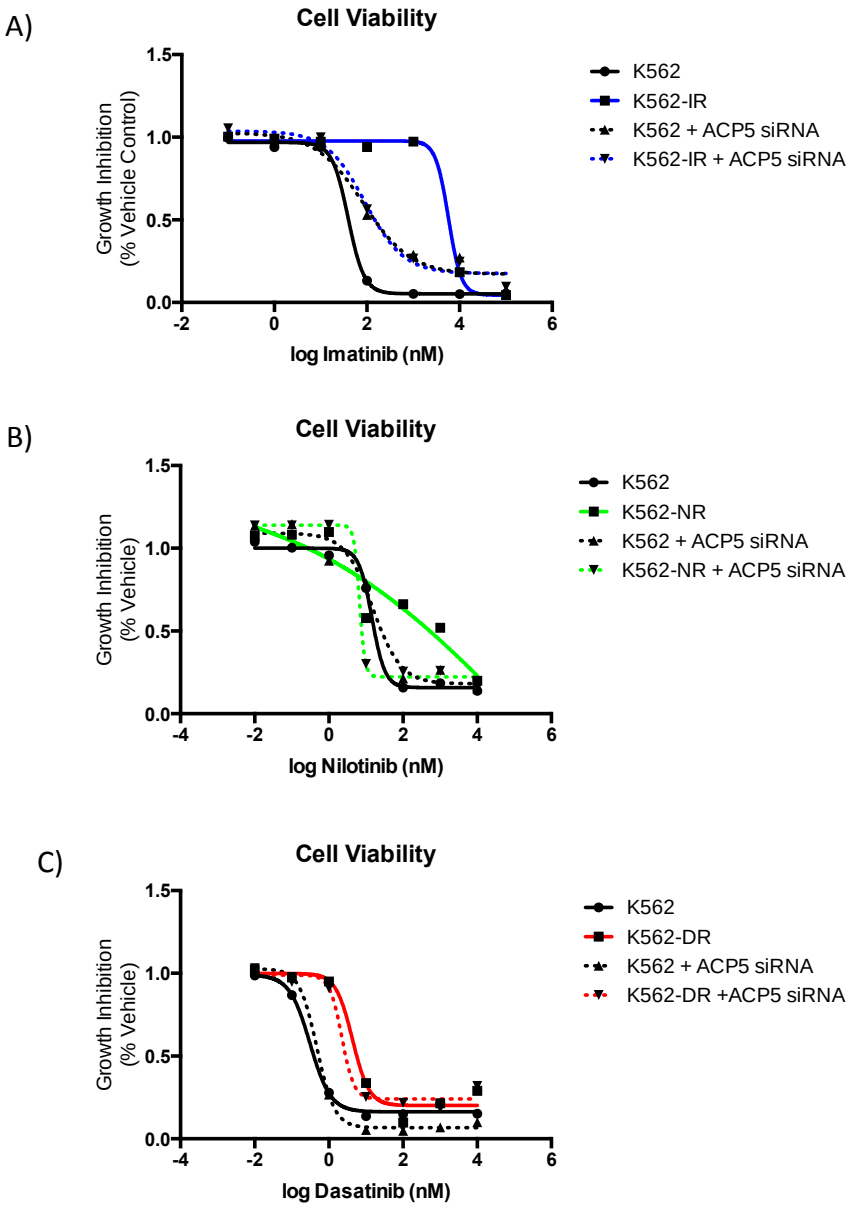

Figure 4. Knockdown of ACP5 Preferentially Sensitizes K562-IR Cells to imatinib, and K562-NR cells to nilotinib, but does not preferentially sensitize K562-DR cells to dasatinib. Cells were transfected with 100nM ACP5 siRNA for and seeded in 96-well plates for 24 hours before treatment with the indicated inhibitor (n=3). A) Knockdown of ACP5 resensitizes K562-IR cells to imatinib. B) Knockdown of ACP5 resensitizes K562-

NR cells to nilotinib. C) Knockdown of ACP5 does not resensitize K562-DR cells to dasatinib.

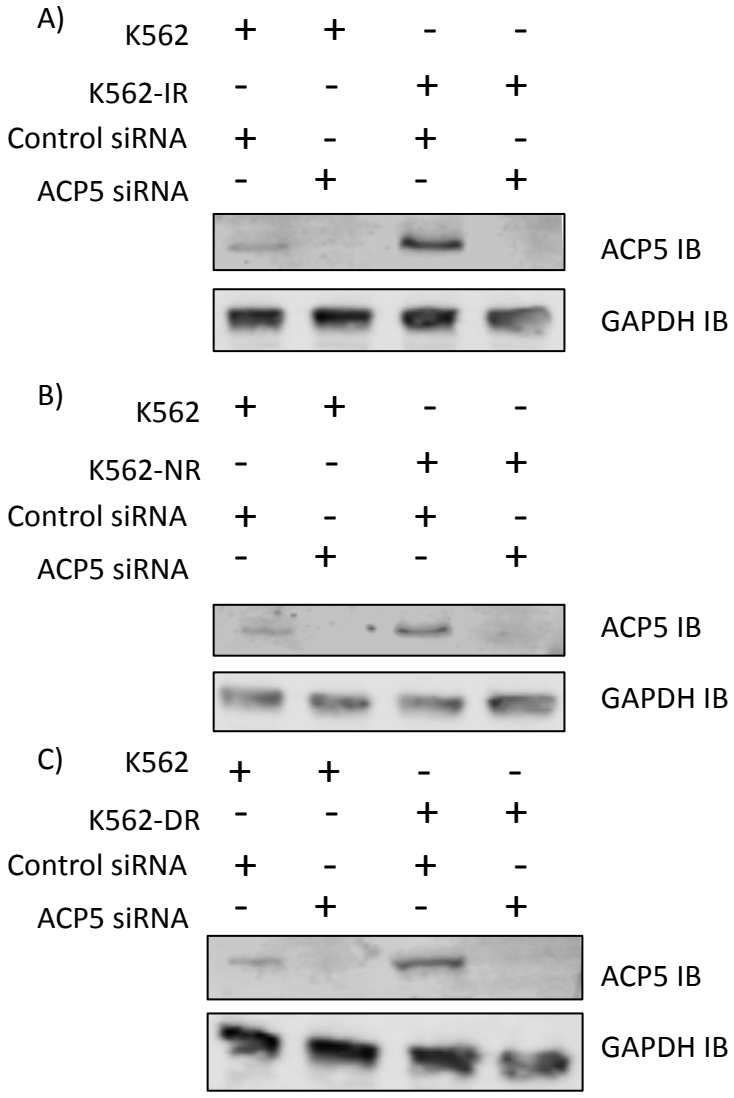

Figure 5. Confirmation of ACP5 Knockdown for XTT Assay. Cells were transfected with 100nM ACP5 siRNA and dosed with the indicated inhibitors 24 hours later. Seventy-two hours post-transfection the cells were lysed and subjected to western blot analysis. A)

Knockdown of ACP5 was successful in K562 and K562-IR cells. B) Knockdown of ACP5 was successful in K562 and K562-NR cells. C) Knockdown of ACP5 was successful in K562 and K562-DR cells.

Maintaining consistent levels of TKI in the cell is a critical factor affecting the efficacy of TKI treatment. CYP2S1 is a cytochrome p450 isoform that is upregulated in colorectal cancer and its high expression is a marker of poor prognosis in colorectal cancer. It has also been shown to inactivate benzothiazole antitumor compounds in breast and colorectal cells, and the ablation of CYP2S1 resulted in increased sensitivity to chemotherapy [142, 143]. In all three of our K562-TKI resistant cell lines we observed a marked increase in the transcription of CYP2S1 (log_2 fold change of 2.616, 2.246, and 2.475 in K562-IR, K562-NR, and K562-DR cells, respectively). We hypothesized that increased CYP2S1 activity may reduce TKI activity, allowing for drug resistance to evolve, and siRNA-mediated knockdown of CYP2S1 would restore TKI-sensitivity in the K562-TKI resistant cells. CYP2S1 knockdown yielded mixed results. In the K562-IR cell line, the knockdown of CYP2S1 dramatically increased the sensitivity of K562-IR cells to imatinib, but also increased the resistance of K562 cells to imatinib, suggesting that the increase in CYP2S1 transcription is not a mechanism by which K562 cells acquire imatinib-resistance (Figure 6A). The knockdown of CYP2S1 in K562-NR cells did not have an appreciable affect on the sensitivity of K562 or K562-NR cells to nilotinib, and we conclude the CYP2S1 does not confer significant resistance to nilotinib (Figure 6B).

Knockdown of CYP2S1 did increase the sensitivity of both K562 cells and K562-DR cells to dasatinib, further illustrating the differences in compensatory mechanisms contributing to acquired resistance to dasatinib as compared to acquired imatinib and nilotinib resistance (Figure 6C). Given the kinase inhibition profile of dasatinib, it is not surprising that the knockdown of a variety of different transcripts would yield results differing from those of cells treated with imatinib or nilotinib.

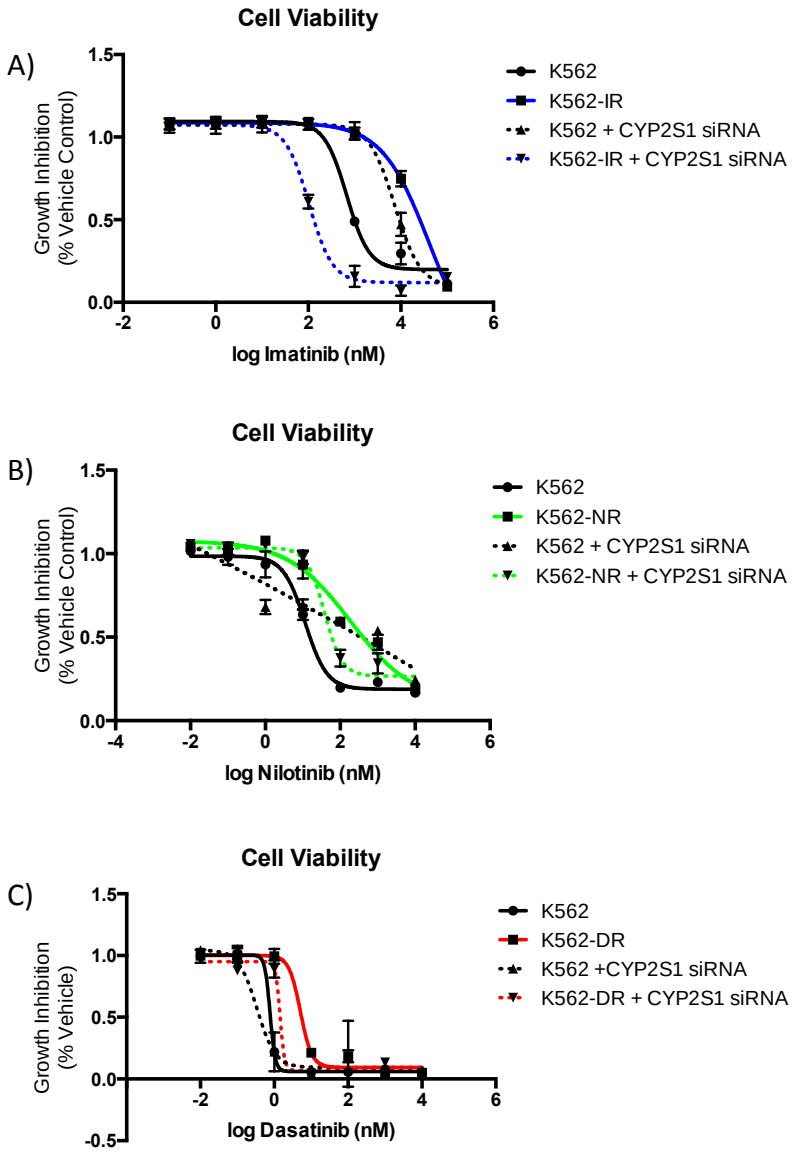

Figure 6. Knockdown of CYP2S1 Preferentially Sensitizes K562-IR Cells to imatinib, and K562-DR cells to dasatinib, but does not preferentially sensitize K562-NR cells to nilotinib. Cells were transfected with 100nM CYP2S1 siRNA for and seeded in 96-well plates for 24 hours before treatment with the indicated inhibitor (n=3). A) Knockdown of CYP2S1 resensitizes K562-IR cells to imatinib. B) Knockdown of CYP2S1 modestly

resensitizes K562-NR cells to nilotinib. C) Knockdown of CYP2S1 does not resensitize K562-DR cells to dasatinib.

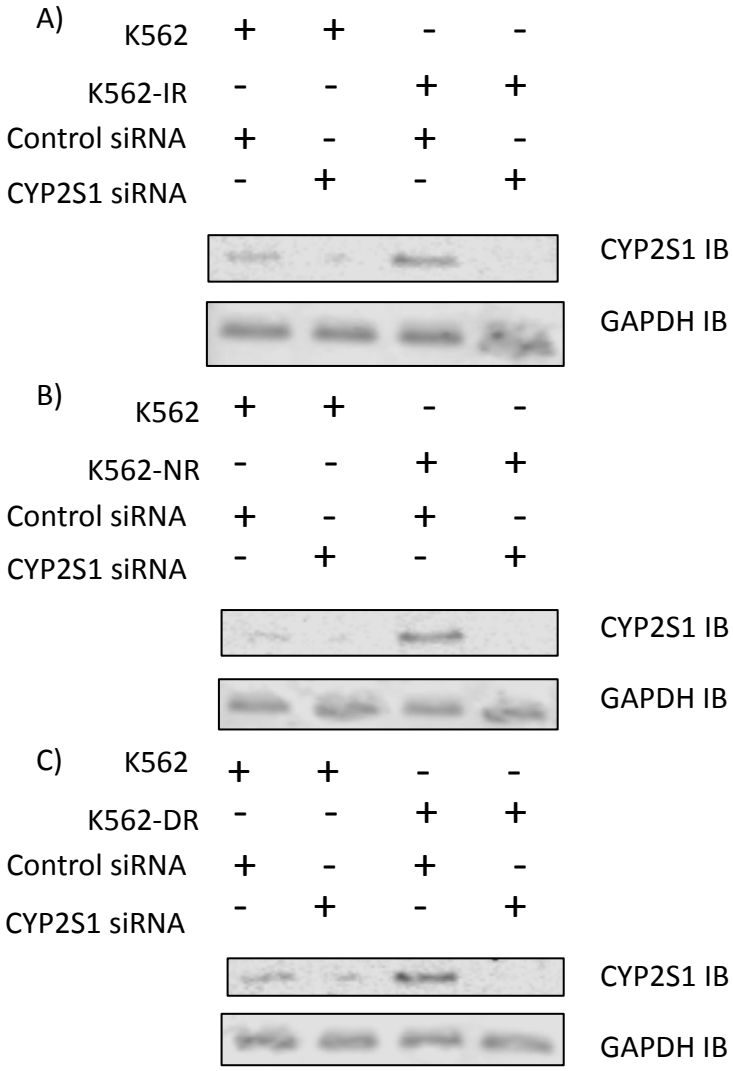

Figure 7. Confirmation of CYP2S1 Knockdown for XTT Assay. Cells were transfected with 100nM CYP2S1 siRNA and dosed with the indicated inhibitors 24 hours later. Seventy-two hours post-transfection the cells were lysed and subjected to western blot

analysis. A) Knockdown of CYP2S1 was successful in K562 and K562-IR cells. B) Knockdown of CYP2S1 was successful in K562 and K562-NR cells. C) Knockdown of CYP2S1 was successful in K562 and K562-DR cells.

2.3.5 Emergent Transcripts in the K562-TKI Resistant Cell Lines

The goal of this study is to identify novel biomarkers of imatinib, nilotinib, and dasatinib resistance, and elucidate potential mechanisms underlying the development of acquired resistance to TKI therapy. As a patient undergoes treatment with imatinib, nilotinib, or dasatinib, signaling pathways will be altered in response to the inhibition of the BCR-ABL signaling pathway, and compensatory pathways may be activated to drive cellular proliferation, division, or apoptosis. As these signaling pathways are perturbed and resistance is acquired, the expression of genes will be disrupted to reflect the restructuring of cellular signaling pathways and other cellular functions to accommodate sustained proliferation. One potentially useful approach to identifying if a patient is acquiring resistance to TKI therapy is to monitor the emergence or disappearance of mRNA transcripts that are associated with TKI resistance. We have identified six transcripts displaying a fragments per kilobase of transcript per million mapped reads (FPKM, which is an expression of the raw number of transcripts read in a given sample) of greater than ± 10 in the K562-TKI resistant cell lines in relation to the parental K562 cell line, reflected in Table 5 below.

Table 5. Emergence or Disappearance of mRNA Transcripts as Tyrosine Kinase Inhibitor Resistance is Acquired. Raw transcript measurements from RNA-seq analysis are shown in FPKM. The denoted change for K562-IR, K562-NR, and K562-DR cell lines is relative to the parental K562 cell line.

Cell Line	Gene	FPKM Change Relative to K562
K562-IR	CLC	-19.404
	GNG8	79.085
	MIR3940	8.49E+03
	MIR637	1.15E+04
	RLN3	12.707
	SGK110	13.447
	SNORD88A	1.07E+04
	SNORD88B	1.08E+04
K562-NR	SNORD88C	-1.09E+04
	ZNF350	-17.451
	GNG8	45.339
	IGFL2	10.626
	RLN3	43.822
	SNORD33	2.78E+04
	SNORD35A	7.22E+04
K562-DR	SNORD88C	-1.31E+04
	CREB3L3	15.809
	GNG8	40.468
	SGK110	17.982

The loss of CLC in K562-IR cells is somewhat intriguing, as it is a lysophospholipase associated with inflammation and has been implicated in myeloid differentiation[144],

but very little is known about its potential role in leukemia. RLN3 (expressed in K562-IR and K562-NR cells) and SGK110 (expressed in K562-IR and K562-DR cells) have never been described in any cancer studies, and SGK110, an SH3-domain binding serine/threonine kinase, has never been characterized in any capacity. Further study into the functions of these genes in TKI-resistance may yield promising results. The loss of ZNF350 expression in K562-NR cells is noteworthy, as methylation of the promoter region and subsequent loss of ZNF350 expression has been reported in tamoxifen-resistant breast cancer cells. Treatment with 5-aza-2'deoxycitidine significantly reduced promoter methylation, and the resulting increase in ZNF350 expression corresponded with tamoxifen re-sensitization in MCF-7 breast cancer cells[145]. Perhaps a similar event is the reason for ZNF350 transcriptional downregulation. The other novel transcript found in K562-NR cells, IGFL2, has been shown to be upregulated in Sezary syndrome, a rare form of CD4+ cutaneous T-cell lymphoma, although no causative explanation was reported[146]. CREB3L3 is a protein belonging to the cAMP-dependent family of transcription factors, which is activated by ER-stress in hepatocellular carcinoma. Activated CREB3L3 then promotes cell proliferation via c-JUN mediated activation of AP-1 target genes[147]. CREB3L3 is also upregulated in response to treatment with the putative antimetastatic agent silibinin, suggesting a possible role in drug-resistance in hepatocellular carcinoma[148].

The modest increase in transcript levels of GNG8 in K562-IR, K562-NR, and K562-DR cell lines is noteworthy because it is a known cofactor in the WNT/β-catenin signaling

pathways, and has also been implicated in chronic lymphocytic leukemia pathogenesis[149]. The WNT/β-catenin pathway is activated by BCR-ABL in CML pathogenesis, but TKI-mediated inhibition of BCR-ABL signaling abrogates WNT/β-catenin activity in imatinib-sensitive cells, and pharmacological inhibition of the WNT/β-catenin pathway in combination with imatinib treatment dramatically reduced the amount of leukemia stem cells in a mouse model of CML[150]. Recently, sustained WNT signaling was demonstrated in TKI-resistant CML stem cells via expression of the tumor necrosis factor ligands CD70 and CD27[151]. Activation of the WNT/β-catenin signaling pathway in imatinib-resistant CML has been reported in many studies, but has not been reported in nilotinib or dasatinib resistant CML patients. It is tempting to speculate that emergence of the GNG8 transcript may underlie activation of the WNT/β-catenin signaling pathway, conferring resistance to imatinib, nilotinib, and dasatinib, but further study is necessary to tease apart any potential involvement of GNG8 in WNT/β-catenin-mediated TKI-resistance.

The differential expression of miR3940, miR637, snORD33, snORD35A, snORD88A, snORD8B, and snord88C transcript levels are tremendous in comparison to the other emergent transcripts. miR3940 has been shown to be downregulated in non-small cell lung carcinoma[152], but those data were based on a correlation between decreased miR3940 expression and the increasing severity of tumor growth and no oncogenic role was established. However, miR637 has been demonstrated to have a significant role in

the inhibition of hepatocellular carcinoma progression through the disruption of STAT3 signaling, which rescues cells from apoptosis and promotes cell proliferation[153]. It is surprising that both miRNAs were found to be significantly overexpressed in the K562-IR cell line, when they were found to be significantly downregulated in lung and liver cancer tissue in the studies previously described. snORD88A, snORD88B, and snORD88C are small non-coding RNA's that function to modify the biogenesis of other small nuclear RNA's. Very little is currently known about the function of snORD88A, snORD88B, and snORD88C, but it is predicted that they can suppress gene expression in a miRNA-like manner[154]. Interestingly, transcription of snORD88C is lost in K562-NR and K562-DR cells, while it is significantly upregulated in K562-IR cells. Regardless, a complete understanding of the function of these transcripts is not always necessary to use as a benchmark for therapeutic performance, and miR3940, miR637, snORD88A and snORD88B could have potential as biomarkers of imatinib resistance.

2.3.6 Novel Fusion Transcripts in the TKI-Resistant Cell Lines

In addition to differential expression testing and the emergence or disappearance of transcripts in the K562-TKI resistant cell lines relative to the parental K562 cell line, the detection of novel fusion genes represents a novel biomarker avenue to detect emerging TKI-resistance. Fusion genes are known drivers of oncogenesis, and the detection of fusion transcripts has long been an indicator of prognosis in various

cancers. Chronic myelogenous leukemia is induced by the development of the BCR-ABL fusion gene, and oncogenesis can be spurred by the fusion of a gene to a strong promoter region, allowing for increased transcription of an oncogene, or elimination of an inhibitory residue, which induces a constitutively active oncogene. In every replicate from each of our conditions we identified transcription of the BCR-ABL fusion gene, which served as an internal positive control for gene fusion detection. Additionally, we identified several novel fusion genes in our TKI-resistant cells that have not been previously reported.

We used the DeFuse software to identify fusion genes present in our TKI-resistant cell lines in an effort to identify potential drivers of resistance or biomarkers that could be potentially used to detect impending TKI resistance at an early stage. Novel fusion genes detected in our RNA-seq results are listed in Table 6 below.

Table 6. Novel Fusion Genes Present in K562-TKI Resistant Cell Lines. Novel fusion genes identified from RNA-seq analysis in K562-IR, K562-NR, and K562-DR cells are shown. Fusion genes were identified using DeFuse software on the Galaxy platform through the Minnesota Supercomputing Institute.

Cell Line	Gene 1	Gene 2	Gene 1 Chromosome	Gene 2 Chromosome
K562-IR	ANAPC1	RMND5A	11	8
	RPL11	TCEB3	23	23
	CTSC	RAB38	8	8
K562-NR	RPL11	TCEB3	23	23
K562-DR	CTSC	RAB38	8	8
	RPL11	TCEB3	23	23

We were able to identify three fusion genes not previously described in a CML model, which were not present in the parental K562 cell line. ANAPC1-RMND5A had previously been reported in BT474 and SKBR3 breast cancer cells, and had been identified as a mutation driving oncogenesis[155], and could potentially play a role in imatinib resistance in our K562-IR model. The RPL11-TCEB3 fusion had not yet been described in CML cells, but its identification was not surprising, as they are found on the same locus. The detection of the RPL11-TCEB3 fusion transcript was not a read-through error, despite their proximity on the same locus. Interestingly, both RPL11 and TCEB3 have been described as tumor-suppressors in other cancers[156, 157]. Although it is outside our current scope, it would be interesting to determine whether the fusion of these genes results in a functionally inactive protein that undermines their ability to suppress tumorigenesis. The last fusion gene detected was CTSC-RAB38, which is found in approximately 20% of renal cell carcinoma patients[158].

Functional studies are required to determine any contribution these novel fusion transcripts may have to TKI-resistance in CML, but their identification may allow them to be implemented as biomarkers of treatment failure. It may be possible to identify emergence of these novel fusion proteins using transcriptomics or mass spectrometry to monitor a patient's response to treatment, or predict if a patient will elicit a favorable response to a particular TKI prior to treatment.

2.3 Conclusions

We report a transcriptomics-based characterization of a cell-culture model of acquired resistance to imatinib, nilotinib, and dasatinib, respectively, in chronic myelogenous leukemia. Although there are differences in transcription profiles between the TKI-resistant cells, we report twenty-four genes all three cell lines significantly differentially expressed with respect to the parental K562 cell line. Additionally, we report the emergence and disappearance of transcripts in our TKI-resistant cell lines with respect to the parental cell line, with GNG8 a common emergent transcript. Finally, we report novel fusion transcripts unique to each imatinib, nilotinib, and dasatinib. This bioinformatics approach must be followed up with data from patient samples, but the results of our cell-culture model may serve as a guide to direct further research into the acquisition of resistance to imatinib, nilotinib, and dasatinib, and lead to the development of alternative or novel combinational chronic myelogenous leukemia therapies.

CHAPTER 3. IDENTIFICATION OF PROTEOMIC DIFFERENCES BETWEEN WILD-TYPE CML CELLS AND CML CELLS RESISTANT TO TYROSINE KINASE INHIBITORS

3.1 Introduction

Proteomics is the study of the proteins present in a particular sample, which extends the understanding of the physiological functions of the genes that transcribe them. Proteins are modified through posttranslational modifications that alter the structure and activity of the proteins and can affect the rates of degradation of proteins in a complex spatiotemporal manner. The constant changes in rates of degradation and synthesis of proteins makes proteomics a dynamic field much more complex than genomics or transcriptomics. Tyrosine kinase inhibitors enact cellular change by acting at the proteinlevel; therefore it is prudent to study the changes occurring at the cellular level at which most signaling occurs, the protein level. In this chapter I examine the proteomic differences between the parental K562 cell line and the K562-IR, K562-NR, and K562-DR cell lines, respectively, using Sequential Window Acquisition of all Theorectical Spectra mass spectrometry (SWATH-MS).

3.2 Materials and Methods

Generation of TKI-resistant Cell Lines. Described in Chapter 2.

Sample Preparation for Proteomic Analysis. K562, K562-IR, K562-NR, and K562-DR cell lines were thawed and grown in IMDM supplemented with 10% FBS and 0.1% penicillin/streptomycin. The cells were grown to a density of ~$7.5x10^5$ cells/mL, and $1x10^7$ cells of each cell line were centrifuged and washed three times with ice-cold PBS. The cells were then resuspended in lysis buffer (50mM ammonium bicarbonate pH 7.0, 4mM EDTA, phosphatase inhibitors (Roche)) and immediately incubated at 95°C for five minutes. The cells were then sonicated for 30 minutes in a water-bath and the insoluble fraction was separated from the cell lysate by centrifugation at $1.5x10^3$ RPM for 20 minutes at 4°C. Each sample was reduced (20mM DTT for 1 hour at 60° C), and alkylated (40mM IAA for 30 minutes at room temperature, protected from light). Reactions were quenched by adding DTT to a final concentration of 10mM. The samples were then trypsin digested overnight at 37° C at a ratio of 1:50 (w/w). Samples were then cleaned up using MCX stage-tips[143, 159] and resuspended in Triple TOF sample buffer (90% water/ 10% acetonitrile/ 0.1% formic acid) to a final concentration of 0.15 mg/mL and transferred to mass spectrometry sample vials (Eksigent).

SWATH-MS analysis. Eight hundred nanograms of each sample were loaded onto a 3 µm x 10 cm ChromXP™ C-18 chip column (particle diameter 3 µm, pore size 120Å) in

serial mode with a Eksigent 400 cHIPLC system. The run gradient was over 90 min with a gradient from 5% to 35% buffer B (buffer A: LC-grade water + 0.1% formic acid; buffer B: LC-grade acetonitrile + 0.1% formic acid) at a flowrate of 300 nl/min. The Eksigent 400 cHIPLC system was coupled to a TripleTOF 5600 (SCIEX) mass spectrometer. The acquisition parameters were as follows: one 50 msec MS scan (at >30K resolution), followed by 34 SWATH windows of 25 da each at 50 msec accumulation time for m/z 400-1250. The total cycle time was ~1.8 sec and each analysis was performed in duplicate.

Analysis of SWATH-MS Data Using DIA-Umpire. DIA files were first converted to centroided mzXMLs with the Sciex MS Data Converter. DIA Umpire signal extraction module was used to detect peptide fragment features based on m/z and retention time information and construct 'pseudospectra' for potential peptides[160]. The Q1 and Q3 level pseudospectrum MGF file outputs from DIA Umpire SE were converted to indexed mzXML and searched against a human swissprot reference database using the Comet and xtandem! native and xtandem! kscore algorithms via the trans proteomic pipeline (v4.8.0). Search outputs were converted to pep.xml files and peptide probability modeling was performed using the xinteract peptide prophet algorithm (using high mass accuracy binning, retention time information, and non-parametric decoy modeling). Search results corresponding to one raw file generated by the three separate algorithms were combined using the interprophetparser. The final, combined search result was

loaded into Skyline software to construct an peptide transition assay library of all peptide spectral matches with probability scores > 0.95. The five peptide fragments with highest intensities (excluding fragments within 3 residues of either the C- or N-terminus) were used to represent each peptide. The assay list was filtered to include only proteotypic (e.g., unique to a single protein sequence, non-degenerate) peptide sequences and decoys with shuffled sequence were appended to the list. This final transition assay list was extracted against each of the individual DIA files for targeted re-analysis and peptide quantification in Skyline. False discovery rates were estimated with the mprophet algorithm feature, and peptides with FDR < 0.01 were exported for statistical processing in MSSTATS (v3.2.2). Data were normalized to median signal intensities and protein inference was estimated using the Tukey's Median Polish based protein abundance modeling algorithm. Pairwise comparisons were calculated between experimental groups of interest, and Bonferonni adjusted p-values were used to control for multiple testing effects.

3.3 Proteomics as a Cancer Biomarkers

One of the primary limitations of transcriptomics-based biomarker identification is that the output does not reveal any information about the activity of the resulting protein, or even if the protein is ultimately translated. miRNA and other non-coding RNA play a substantial role in mRNA translation and protein synthesis[161-163], and dynamic changes in protein degradation rates further complicate predicting protein levels based

on transcriptomic data. There is a need to identify protein activity levels in disease pathology, as mRNA levels do not strongly correlate with protein levels, and it is impossible to determine the activity of a protein from its mRNA[164]. Recent advances in mass spectrometry-based proteomics have dramatically improved the ability to detect proteins and their post-translational modifications in complex protein mixtures, such as cell lysate. Mass-spectrometry based proteomics has traditionally been dominated by two complimentary modes of detection. Bottom-up discovery proteomics, or "shotgun proteomics", utilizes high-performance liquid chromatography in combination with a tandem mass-spectrometer to identify peptides from complex mixtures based on the spectral profile of the MS2 fragmentation pattern. Shotgun proteomics is useful for determining the proteins in a given sample, but is limited by the large *m/z* range over which the instrument is required to detect fragment ions. The resulting data is heavily biased toward the most abundant MS1 precursor peptides, and excludes low-abundance proteins from being identified. Targeted proteomics, or selected-reaction monitoring (SRM), is the monitoring of the products of a precursor ion at a specific *m/z* range in an effort to quantify a specific protein in a sample. Targeted proteomics requires the researcher to know the identity of their protein of interest in order to monitor its detection, whereas shotgun proteomics is useful for discovering proteins present in a sample. A recent advance in mass-spectrometry has effectively combined the advantages of SRM and shotgun proteomics into one method, allowing for unbiased global proteomic profiling.

3.3.1 Use of SWATH-Mass Spectrometry in Biomarker Research

Sequential Window Acquisition of All Theoretical Spectra (SWATH-MS) is a data-independent acquisition (DIA) mass-spectrometry method capable of detecting all MS2 fragment ions generated from all MS1 precursor ions eluting from an HPLC column in a given sample, thus generating a permanent digital library of all detectable fragment ion spectra within the instrument's dynamic range. In SWATH-MS experiments, the mass spectrometer steps through 2-4 second cycles in which all precursor ions eluting from the HPLC column at a given time are fragmented, and the mass spectrometer rapidly detects the resulting fragment ions. Unlike in Data Dependent Acquisition (DDA) methods, where only a set of the most abundant MS1 precursor ions are fragmented for MS2 analysis, DIA offers an opportunity to both identify and quantify an enormous number of proteins in a complex protein mixture, such as cell lysate, combining the level of peptide coverage afforded by shotgun proteomics with the sensitivity and quantification of SRM. SWATH-MS is a very versatile tool for cell biology research, having been used to identify 14-3-3 protein-binding partners, biomarkers of prostate cancer aggressiveness, and even cannabinoid metabolites found in urine[165-167]. Transcription of a gene does not significantly correlate with its translation or protein activity upon translation[164, 168, 169], and the bias of shotgun proteomics toward high abundance proteins makes the use of SWATH-MS an ideal solution to identify novel protein biomarkers of TKI-resistance in chronic myelogenous leukemia that may be present at lower abundances and undetectable using a conventional DDA approach. We used SWATH-MS to probe the proteome of K562, K562-IR, K562-NR, and K562-DR cell

lines for potential biomarkers of TKI resistance, and compare the protein composition of each cell line with their transcriptome.

3.4 Proteomic Changes in K562 Cells as Compared to K562-TKI Resistant Cells

We sought to compare the transcriptomic changes in K562 cells with evolved resistance to imatinib, nilotinib, and dasatinib with the proteomic changes in the same cell line to determine if the transcription levels correspond with the observed protein levels. The complex relationship between transcription and translation has been well documented, as miRNA and other factors effect the translation of mRNA into proteins and dynamic changes in the rates of protein degradation affecting protein levels after translation. SWATH-MS was used to quantify the proteins present in K562 cells and K562-TKIR cells. DIA-Umpire was then used to extract pseudo-MS spectra. DIA-Umpire is an open-source software program that allows for untargeted peptide identification from DIA mass spectrometry data without the use of a spectral library created from DDA data. DIA-Umpire works by incorporating several computer algorithms to detect all possible precursor fragment ions signals in MS1 and MS2, including unfragmented ions in MS2 data. The algorithm then computes the Pearson correlation coefficient of the retention time and peak apex of eluting fragment ions to determine the likelihood that a particular fragment ion corresponds to a precursor ion. The precursor-fragment groups are then grouped together as pseudo-MS/MS spectra that are searchable in conventional protein databases[160]. The Q1 and Q3 level pseudo-spectrum MGF file outputs from DIA Umpire SE were converted to indexed mzXML and searched against a

human swissprot reference database using the Comet and X!Tandem native and X!Tandem kscore algorithms via the trans proteomic pipeline (v4.8.0)[170]. Search outputs were converted to pep.xml files and peptide probability modeling was performed using the xinteract peptide prophet algorithm (using high mass accuracy binning, retention time information, and non-parametric decoy modeling). Search results corresponding to one raw file generated by the three separate algorithms were combined using the interprophetparser. The final, combined search result was loaded into Skyline software to construct a peptide transition assay library of all peptide spectral matches with probability scores > 0.95. The five peptide fragments with highest intensities (excluding fragments within 3 residues of either the C- or N-terminus) were used to represent each peptide. The assay list was filtered to include only proteotypic (e.g., unique to a single protein sequence, non-degenerate) peptide sequences and decoys with shuffled sequence were appended to the list. This final transition assay list was extracted against each of the individual DIA files for targeted re-analysis and peptide quantification in Skyline[171, 172]. False discovery rates were estimated with the mprophet algorithm feature, and peptides with FDR < 0.01 were exported for statistical processing in MSSTATS (v3.2.2)[171, 172]. Data were normalized to median signal intensities and protein inference was estimated using the Tukey's Median Polish based protein abundance modeling algorithm. Pairwise comparisons were calculated between experimental groups of interest, and Bonferonni adjusted p-values were used to control for multiple testing effects. The construction of the spectral library using DIA-Umpire and analysis of SWATH-MS data using Skyline was performed by Dr. Sarah

Parker. Across all of the TKI-resistant samples, we found 69 proteins that were significantly downregulated compared to the parental K562 cells ($p<0.05$), and 32 proteins that were significantly upregulated compared to the parental K562 cells ($p<0.05$). We chose to include proteins with a $\log_2$ fold change of ±2 in our report, and significantly differentially expressed proteins in K562-IR cells as compared to K562 cells are listed in Table 7 below.

Table 7. K562-IR proteins found to have significant ($p<0.05$) differential expression with respect to parental K562 cells determined by SWATH-MS. Differential expression was analyzed using Skyline, and statistics were compiled using MSStats.

Protein	$\log_2$ Fold Change	p-value
PHB2	2.463	4.76E-02
PURA	2.378	4.34E-05
CAST	-2.441	3.66E-02
AHNAK2	-2.524	3.39E-03
KRT6B	-2.716	3.32E-04
NUCKS1	-2.787	2.15E-03
CA1	-3.017	1.14E-04
KRT10	-3.229	2.71E-05
DSC1	-3.422	1.74E-02
KRT1	-3.515	7.16E-07
KRT14	-3.734	5.10E-03
KRT9	-3.976	7.52E-05
CALML5	-4.691	1.25E-03
DSG1	-5.044	5.64E-03
KRT2	-5.047	1.47E-03

Table 7 Continued.

KRT5	-5.167	5.70E-03
DSP	-6.283	2.35E-03
KRT16	-6.917	2.48E-02
JUP	-7.68	3.16E-02

One striking feature of the K562-IR cell line is the increased protein levels of only two proteins, and seventeen downregulated proteins. There are also six proteins that have a $\log_2$ fold change of -5.0 or more, which is more differentially expressed than any gene found in our RNA-seq experiments. Of note are the most positively and negatively differentially expressed proteins. PHB2 is a mediator of transcriptional regulation, and mutational studies have demonstrated its roles in driving ERK1/2 signaling, inhibition of apoptosis, release from the G1/S checkpoint in mitosis, and protein stabilization[173-175].

On the other hand, the significant loss of JUP, DSG1, and DSP, along with eight proteins members of the keratin-family is interesting; all eleven proteins have been shown to mediate cell to cell adhesion by forming desmosomes[176-178], the loss of which is a common feature of cancer[179]. Part of the desmosome complex is the metastasis-suppressing protein VE-cadherin, which forms a complex with JUP and several keratins to mediate cell-cell adhesion and prevent cell motility, however, loss of VE-cadherin has long been known to be a biomarker of CML development[180]. This is the first reported

loss of the remaining components of the desmosome complex, which may be indicative of their role in acquired imatinib resistance. A hallmark of the metastatic process in recurrent cancer is the epithelial-to-mesenchymal transition (EMT), which is characterized by a loss of E-cadherin, and upregulation of N-cadherin (EN-switch)[181]. N-cadherin forms a cytoplasmic complex with β-catenin, increasing Wnt-mediated β-catenin nuclear translocation and transcriptional activity, and plays a role in TKI resistance in leukemia stem cells (LSC) in CML cells co-cultured with bone marrow stromal cells[182]. The loss of the desmosome complex could potentially be a by-product of the K562-IR cell line acquiring a phenotype similar to LSCs in the context of the bone marrow microenvironment, which helps to confer imatinib resistance.

The overexpression of AXL could potentially mediate the loss of the desmosome complex in K562-IR cells. EMT is the process by which cells metastasize, and the loss of the desmosome is necessary for EMT to occur. AXL has been shown to be integral to EMT progression and the disruption of cell-cell adhesion[183]. The overexpression of AXL is also highly correlated with increased Wnt and TGFβR signaling, which are also upregulated during EMT, and associated with the loss of the desmosome complex and the EN-switch[184]. The association between AXL upregulation and the loss of the desmosome complex would mark a yet-uncharacterized link between the receptor tyrosine kinase and the desmosome complex. Leukemia stem cells are intrinsically resistant to imatinib, and the upregulation of AXL could potentially be an important step

in the disruption of the cell-cell adhesion anchoring LSCs in the bone marrow, allowing TKI-resistant CML cells to enter the bloodstream. Future studies should profile the expression of EMT-related genes such as *vimentin* and *snail* in response to AXL upregulation to determine in AXL upregulation corresponds with the induction of EMT-related gene expression. Additionally, it would be interesting to determine the molecular time-line of AXL upregulation and the loss of the desmosome complex, which could be easily determined using standard western blotting. The association of AXL upregulation and the induction of Wnt/TGFβ signaling has not been described in CML models, and it would be very interesting to explore the possibility that AXL upregulation serves as a conduit to drive cell proliferation by activating the Wnt/TGFβ pathway, and the loss of the desmosome is a consequence. Leukemia stem cells are intrinsically resistant to imatinib, so it raises the possibility that resistance to imatinib *in vivo* progresses through upregulation of AXL and the corresponding loss of the desmosome complex and the release of differentiated TKI-resistant CML cells from the bone marrow. AXL activates the MAPK pathway, which enhances WNT signaling[185]. It would be interesting to dissect the interaction between AXL and WNT, and if it proceeds through MAPK signaling, resulting in the loss of the desmosome and acquisition of an EMT-like phenotype.

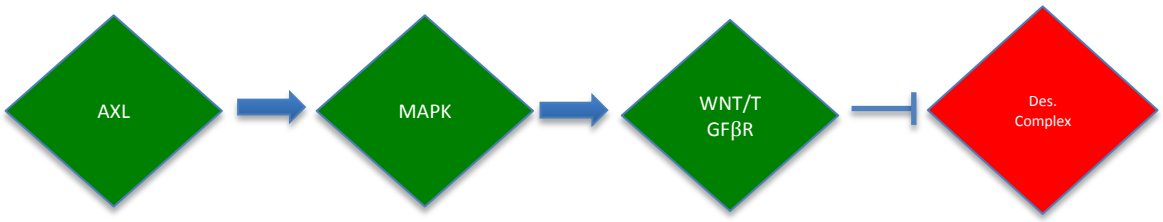

Figure 8. Proposed Model of K562-IR Resistance. AXL activates the MAP Kinase pathway, activating WNT/TGFβR, which leads to the downregulation of the proteins composing the desmosome complex, allowing for cell motility to increase.

Table 8. K562-NR proteins found to have significant (p<0.05) differential expression with respect to parental K562 cells determined by SWATH-MS. Differential expression was analyzed using Skyline, and statistics were compiled using MSStats.

Protein	log2 Fold Change	p-value
SLC25A3	4.308	1.93E-04
KRT14	3.798	4.80E-03
RPN1	3.603	3.40E-02
PHB2	3.546	1.53E-02
TUBA4A	3.478	1.31E-03
LRRC59	3.146	1.37E-04
VDAC2	3.084	1.93E-03
COMT	2.938	2.55E-02
VDAC1	2.647	3.27E-05
RHOA	2.549	4.35E-03
HBE1	2.218	1.66E-06
VAPA	2.202	1.08E-04
RAB33B	2.062	3.09E-04
RAB35	2.053	4.83E-05
PHGDH	2.006	5.59E-04
PEX19	-2.041	1.50E-03
PTMS	-2.045	2.06E-05
PAGE5	-2.089	2.04E-05
UQCRH	-2.125	2.77E-05
MFAP1	-2.138	8.63E-03

Table 8 Continued.

BTF3	-2.148	5.86E-05
NOLC1	-2.149	5.43E-04
TMSB10	-2.159	2.36E-04
NACA2	-2.237	1.70E-06
RPS21	-2.238	1.13E-03
DNAAF2	-2.251	1.00E-01
AHNAK2	-2.284	4.13E-03
POLR2I	-2.322	5.79E-02
SH3BGRL3	-2.338	1.05E-02
TIMM8A	-2.34	5.16E-03
ATOX1	-2.42	4.63E-04
ZC3H18	-2.483	2.36E-02
GRN	-2.502	5.39E-03
TPM4	-2.533	1.51E-04
ZPR1	-2.576	4.28E-02
C14orf142	-2.591	3.82E-05
SH3BGRL	-2.645	4.31E-05
AHNAK	-2.747	3.25E-01
MYL6	-2.833	3.17E-04
COX6B1	-2.875	4.47E-04
CCDC86	-2.876	5.17E-02
TRIP6	-3.16	1.80E-01
KIAA0101	-3.368	2.63E-04
RBM3	-3.475	2.80E-02
ZNF428	-3.563	1.09E-03
NUCKS1	-3.704	7.28E-04
CAST	-3.727	9.19E-03
BTF3L4	-4.169	1.78E-04

The K562-NR cells followed suit with the results from our RNA-seq analysis and showed the most differential expression of proteins of the three conditions as compared to the

parental K562 cell line. However, the results also demonstrated a majority of proteins downregulated, as 33 of 48 significantly differentially expressed proteins bearing a log2 fold change of ± 2 were downregulated. One of the most significantly upregulated genes was PHB2, a trait shared with the K562-IR cells. However, in the K562-NR cell line there was not a common trend of desmosome-related proteins in the downregulated group, with the only member of that group being KRT14, which was highly upregulated. A common feature of several upregulated proteins is their involvement in mitochondrial membrane transport, as VDAC1 and VDAC2 are upregulated in many cancers and their inhibition promotes apoptosis[186], and SLC25A3, a protein integral to phosphate transport from the cytosol to the mitochondrial matrix, has been reported to be significantly overexpressed in blast phase CML as compared to chronic phase CML[187]. Similarly, NUCKS1 upregulation is a biomarker of recurrence-free survival in cervical squamous carcinoma, and its downregulation is a predictor of vincristine-resistance in childhood acute lymphoblastic leukemia[188, 189].

CAST, also known as calpain-10, is significantly downregulated in K562-NR cells as compared to K562 cells. CAST has been shown to be a negative regulator of apoptosis in pancreatic cells, and may serve a similar purpose in K562-NR cells[190]. Although there has been no established role of these proteins in CML, it is worth noting there has been some role established on other cancer models that may translate to nilotinib resistance in K562 cells.

Table 9. K562-DR proteins found to have significant ($p<0.05$) differential expression with respect to parental K562 cells determined by SWATH-MS. Differential expression was analyzed using Skyline, and statistics were compiled using MSStats.

Protein	log2 Fold Change	p-value
SLC25A3	3.26	2.43E-04
COMT	3.242	3.35E-02
ASNS	3.184	3.27E-03
RPN1	2.887	6.42E-02
PHB2	2.846	3.10E-02
RAB10	2.727	9.17E-05
SLC25A5	2.721	1.13E-03
LRRC59	2.486	3.45E-04
NOP16	2.287	5.08E-02
VDAC1	2.222	6.55E-05
TK1	2.169	1.08E-02
CBS	2.155	3.82E-02
VDAC2	2.154	7.19E-03
VAPA	2.093	1.32E-04
GCN1L1	2.06	3.03E-01
KRT2	-2.018	3.58E-02
C14orf142	-2.147	8.04E-05
SH3BGRL3	-2.209	1.27E-02
CCDC86	-2.22	1.02E-01
PAGE5	-2.242	1.54E-05
ZC3H18	-2.243	3.25E-02
SH3BGRL	-2.254	8.12E-05
PTMS	-2.27	1.36E-05
TIMM8A	-2.385	4.82E-03
TRIP6	-2.391	2.04E-01
MYL6	-2.417	5.88E-04
PNISR	-2.461	1.03E-01

Table 9 Continued.

ZPR1	-2.639	3.99E-02
AHNAK	-2.772	3.22E-01
CALML5	-2.899	3.27E-03
DSG1	-3.295	1.86E-02
NUCKS1	-3.384	1.03E-03
CAST	-3.46	1.19E-02
BTF3L4	-3.932	2.24E-04

The K562-DR cells profile of significantly differentially expressed proteins shared many common features with the K562-NR cells, namely, the significant downregulation of BTF3L4, CAST, NUCKS1, AHNAK, ZPR1, TIMM8A, SH3BGRL, ZC3H18 and MYL6, along with the upregulation of SLC25A3, VDAC1 and VDAC2, LRRC59, and VAPA. The upregulated proteins in both conditions are involved in metabolic reprogramming, and might be indicative of the evolution of a more general cancer-survival reprogramming. Of note is the high level of TK1 in K562-DR cells, a thymidine kinase, and biomarker of poor prognosis in chronic lymphocytic leukemia, breast, gastric, rectal, colorectal, lung, brain cancer, and hepatoma[191, 192].

K562-DR cells also shared common downregulated proteins with K562-IR cells, such as DSG1, and CALML5, the ubiquitination of which has been linked to breast cancer carcinogenesis in premenopausal women[193]. Compared to the transcriptomic data, which contained twenty-four commonly differentially expressed genes, the proteomic

analysis of TKI-resistant K562 cells yielded only eighteen commonly significantly differentially expressed proteins across all three conditions.

Table 10. Common proteins in TKI-resistant K562 cells found to have significant ($p<0.05$) differential expression with respect to parental K562 cells determined by SWATH-MS. Differential expression was analyzed using Skyline, and statistics were compiled using MSStats.

Protein	IR $\log_2$ Fold Change	NR $\log_2$ Fold Change	DR $\log_2$ Fold Change
ABCE1	1.062	1.649	1.405
ASNS	1.954	1.91	3.184
CA1	-3.017	-1.528	-1.632
CAST	-2.441	-3.727	-3.46
DDX6	1.149	1.331	1.005
DDX21	1.19	1.227	1.985
EIF4B	1.116	-1.191	-1.295
FKBP3	-1.322	-1.865	-1.74
FRA10AC1	-1.439	-1.097	-1.1
IGF2BP1	1.097	1.153	1.159
MYL6	-1.084	-2.833	-2.417
NFYA	-1.222	-1.618	-1.589
NUCKS1	-2.787	-3.704	-3.384
PHB2	2.463	3.546	2.846
SLC25A3	1.526	4.308	3.26
SLC3A2	1.229	1.303	1.118
TPM4	-1.231	-2.533	-1.77
VTN	-1.792	-1.773	-1.562

The commonly expressed proteins are involved in cell proliferation, apoptotic resistance, or are biomarkers of chemoresistance, but it would be premature to speculate about any potential role any play in TKI resistance. It may be worthwhile to probe PHB2's role in ERK1/2 signaling in TKI-resistant cells. Interestingly, we observed the upregulation of ABCE1, an ATP-binding cassette sub-family member that is known to inhibit endoribonuclease activity and influences mRNA turnover. ABCE1 upregulation is found in several other cancers, and siRNA-mediated depletion of ABCE1 induces inhibits cellular proliferation, sensitizes cancer cells to chemotherapy, and induces apoptosis in breast, lung, and esophageal cancers[194-196]. ASNS is also upregulated in all three TKI-resistant cell lines. ASNS catalyzes the conversion of arginine to asparagine, and is known to mediate cisplatin-resistance in nasopharyngeal carcinoma, doxorubicin resistance in uterine cancer, and is highly upregulated in melanoma and lymphoma[197-200]. Further study of ASNS in TKI-resistance may be warranted. Also noteworthy is the upregulation of DDX6 and DDX21, which are ATP-dependent RNA helicases that are integral components of the mRNA degradation machinery. Ablation of DDX6 in colorectal cancer cells inhibits cell proliferation and promotes apoptosis[201, 202]. DDX21 is another ATP-dependent RNA helicase that was recently uncovered to be a crucial regulator of ribosome biogenesis, and interacts with a wide variety of RNA, including non-coding RNAs involved in the formation of ribonucleoprotein complexes, including ribosomal RNA, small nucleolar RNAs (snoRNAs) and 7SK RNA[203]. DDX21 is also highly upregulated in lymphomas, breast, and colon cancer, and is known to drive AP-1 transcription and promote rRNA processing, driving tumorigenesis in multiple

fashions[204-206]. The upregulation of DDX6 and DDX21 may play key roles in the imatinib, nilotinib, and dasatinib resistance in our K562-TKI resistant cell lines by inhibiting the synthesis of proteins involved in TKI-mediated apoptosis, and partially explain the dramatic differences observed between the transcriptome and proteome in our K562-TKI resistant cell lines.

A comparison of the significantly differentially expressed genes between the transcriptome and proteome of TKI-resistant K562 cells with respect to the parental K562 cells requires less stringent differential expression requirements, as no proteins with a $\log_2$ fold change of ±2 were detected in both our SWATH analysis and RNA-seq analysis. We only detected one protein that displayed a significant fold change at the mRNA level and protein level across all TKI-resistant samples as compared to the parental K562 cell line, TPM4.

Table 11. Common genes and proteins in K562-TKI resistant cells found to have significant ($p<0.05$) differential expression with respect to parental K562 cells determined by RNA-seq and SWATH-MS. Differential expression of proteins was analyzed using Skyline, and statistics were compiled using MSStats. Differential expression of mRNA trancripts was analyzed using Cufflinks on the Galaxy platform. Fold change is expressed in terms of $\log_2$.

Cell Line	TPM4 Transcript log_2 Fold Change	TPM2 Protein log_2 Fold Change
IR	0.808	-1.231
NR	0.467	-2.533
DR	-0.698	-1.77

In both the K562-IR and K562-NR cells the transcription of TPM4 was upregulated, but the protein levels were downregulated, and in the K562-DR cells there was significantly more change in protein levels than mRNA levels, which simply demonstrates the transient nature of protein stability. It is possible that the protein was never translated from the mRNA, or the rates of protein degradation far surpassed the rates of protein synthesis in the K562-IR and K562-NR samples. Without further experimentation, it will be impossible to accurately explain the nature of this seemingly contradictory observation. However, it is not particularly surprising that the levels of mRNA and protein do not support each other, and illustrates the need to develop biomarkers of disease progression and response to chemotherapy at the protein level, the level at which most therapies exert their targeted action.

Transcriptomic data and proteomic data does not necessarily correlate, as there are many levels of post-transcriptional and post-translational modifications that contribute to protein levels at any given time. Previous studies have demonstrated a significant lack of correlation between the transcriptome and the proteome: *Ghazalpour et al* found a correlation coefficient of only 0.27 for transcript/protein expression similarity[169], *Foss et al* found a mRNA-protein correlation coefficient of 0.24[168], and *de Sousa Abreu R et*

al reported a mRNA-protein correlation coefficient of 0.22[207]. All of these studies support the concept that much of the level of protein expression is post-transcriptional and post-translational regulation. Given the significant genetic and metabolic reprogramming necessary to evolve resistance to imatinib, nilotinib, or dasatinib, it is unsurprising that our data reflects a low correlation between the transcriptome and proteome.

One explanation for the low correlation between our RNA-seq and SWATH-MS data could also be the significant difference in sensitivity between the measurement techniques. RNA-seq is extremely sensitive, and generally detects all transcripts regardless of their abundance. When using SWATH-MS to analyze the proteome of a sample set, it is imperative to develop a spectral reference library with maximum coverage of the proteome, which is typically acheived by using reverse-phase chromatography to fractionate the samples into many fractions of the same sample, which are then sequentially analyzed by the mass spectrometer. Without fractionation, many peptides are eluted at the same time, and the results are heavily biased towards only the most abundant peptides, as the instrument selects the most abundant peptides in a given MS1 window to fractionate for MS2 detection. Fractionation allows for the total sample to be spread out over many analytical runs, allowing detection of less abundant peptides. Due to instrument limitations we were unable to perform

fractionation up-front of the mass spectrometric analysis, which severely limited the depth of coverage we were able to attain in our proteomic experiments.

3.5 Identification of Unreported Peptides in SWATH Data

Proteogenomics is an emerging field combining transcriptomics and proteomics to expand the identification of spectra collected in a proteomics experiment to the genome. To do this, peptides obtained from RNA-seq experiments that are not contained in the reference database are appended to freely available protein reference databases to create a custom database unique to the sample set that is being analyzed. This approach is particularly useful for the study of cancer pathologies, as genetic mutations such as the emergence of fusion genes, can yield novel peptides that are not contained in common protein reference databases. We used a proteogenomic approach to translate unreported sequences identified in our RNA-seq experiments into peptides we might detect in our SWATH experiments. We first used GalaxyP to convert the format of our raw RNA-seq data from FASTQ to FASTA, and then concatenated the mapped reads from our Tophat2 runs to the human GRCh37 assembly reference FASTA file, and filtered all of the repeated genomic sequences[92]. Next, we used a 3-frame translation to convert the concatenated BED files from the previous step, into a peptide FASTA output file containing the GRCh37 reference peptides, and the peptides unique to our samples. Finally, we compared the 3-frame translated GRCh37 reference FASTA

file with the RNA-seq-appended reference file to filter out all shared peptides using BLAST-P[208]. The results are shown in Table 12 below.

Table 12. Unreported peptides identified in K562-TKI resistant cells using GalaxyP. RNA-seq reads were appended to the GRCh37 human reference database and converted to a peptide FASTA file, and novel peptides were filtered from the original reference database to yield peptides present in our RNA-seq samples hat were not reported in the GRCh37 human reference database.

Peptides Identified Using GalaxyP	Gene Associated with Peptide
ARFPQQKK	TRAF2
DFVSSRTVTFIQTR	N/A
LTVAPPLAEPGAGK	ODO2
MLLYANMIR	N/A
NQVESEVRR	N/A
QGARGLVER	N/A
RPLSANSGIDLSK	N/A
TSSSLLGTGWAV	N/A

Six of the eight peptides identified in our RNA-seq data did not map to a specific gene, and may be false positives, or map to miRNAs or genes that have yet to be characterized. However, we were able to match two peptides from the RNA-seq data with their corresponding genes. The first peptide, ARFPQQKK, mapped to the gene TRAF2, which regulates the activity of JNK and NFκB and plays a central role in cell proliferation and apoptosis, and is an essential component of several E3-ubiquitin ligase complexes.

Unfortunately, ARFPQQKK could not be identified in the SWATH-MS data. Conversely, LTVAPPLAEPGAGK, a peptide corresponding to the ODO2 gene was identified in both the RNA-seq data and the SWATH-MS data. However, the predicted lysine residue (**L**TVAPPLAEPGAGK) identified in the transcript detected in the RNA-seq data actually corresponds to a proline residue (**P**TVAPPLAEPGAGK)in the peptide detected in the SWATH-MS data, which is the correct peptide according to published FASTA databases. RNA-seq is a highly sensitive technique, and it is likely that a very small fraction of transcripts contained this mutation, but were filtered as novel peptides despite very low transcript levels. Despite the L-P mutation, the **P**TVAPPLAEPGAGK peptide was still only identified in our database searches using the custom FASTA file derived from our RNA-seq data. The identification of point mutations is an important goal of proteogenomics, as transcriptomic data does not inform about translational errors that could potentially be drivers of oncogenesis. By appending a data from our RNA-seq analysis with an existing protein database we were able to identify a mutation that would have been overlooked without the implementation of a proteogenomic approach. Future experiments will optimize our SWATH-MS methods to increase the proteomic coverage of our samples, and we expect that optimization of our methods will allow for identification of novel peptides that we were unable to detect in the data presented here.

3.6 Conclusions

In this chapter we report the results of a proteomic analysis of the K562-IR, K562-NR, and K562-DR cell lines with respect to the parental K562 cell line. Across all three TKI-resistant cell lines we identified the upregulation of PHB2, a protein involved in cell proliferation, inhibition of apoptosis, and ERK1/2 signaling. In the K562-IR cells we observed a significant downregulation of eleven proteins involved in the formation of the desmosome complex. The K562-NR cell line and K562-DR cell line shared many common proteins differentially expressed at similar levels, namely, the significant downregulation of BTF3L4, CAST, NUCKS1, AHNAK, ZPR1, TIMM8A, SH3BGRL, ZC3H18 and MYL6, along with the upregulation of SLC25A3, VDAC1 and VDAC2, LRRC59, and VAPA. Finally, we used a proteogenomic approach to add novel peptides identified in our RNA-seq experiment to generate a custom FASTA file for which to search our SWATH-MS data against. We were able to identify a peptide corresponding to ODO2 which was not identified using the GRCh37 human reference database, demonstrating the utility of proteogenomics in cancer research. Without utilizing a proteogenomic approach, the finding of an unreported peptide would have been lost between the transcriptomic and proteomic data, overlooking a potentially significant finding. The ability to append protein databases to include peptides predicted to be present in a sample based on RNA-seq data is a crucial crossover between transcriptomics and proteomics, and provides a deeper understanding of potential drivers of cancer pathology by adding a dimension of information to previously existing modes of analysis.

CHAPTER 4. SUMMARY AND FUTURE DIRECTIONS

The work in this thesis provides many avenues to explore potentially novel mechanisms of imatinib, nilotinib, and dasatinib resistance in a cell-culture model of evolved resistance using K562 cells. In chapter 2, we describe the use of RNA-seq to identify differences in transcript expression in K562-IR, K562-NR, and K562-DR cells with respect to the parental K562 cell line, and then distinguish transcripts that are differentially expressed across all three TKI-resistant cell lines as compared to the parental K562 cell line, which suggest potential avenues for future research into general mechanisms of TKI-resistance in a CML cell-culture model. We also describe the differences in transcriptexpression within each individual cell line, which serves to highlight the different evolutionary pressures that an individual TKI can exert on a cell line, and how an individual TKI can affect the acquisition of a resistant phenotype through the alteration of differential expression of a wide variety of genes, ultimately leading to resistance.

We identified several genes we suspected might be involved in general TKI-resistance, AXL, ACP5, and CYP2S1. AXL had been previously demonstrated to be involved in

imatinib and nilotinib resistance, presumably through a scaffolding function that allowed for compensatory activation of LYN kinase[42], and the ablation of the AXL transcript restored sensitivity to imatinib and nilotinib in resistant cell lines to a level comparable to the parental K562 cell line, which served as an internal control to validate our cell culture model as a clinically-relevant model of acquired TKI-resistance. However, the K562-DR cell line did not sharply respond to AXL ablation in the same manner as the K562-IR and K562-NR cell lines did, suggesting that the mechanisms underlying dasatinib resistance are not AXL-dependent. It is interesting to note that AXL upregulation at the transcript level correlates with the downregulation of many proteins involved in the formation of the desmosome complex. The desmosome complex is integral in cell-cell adhesion and the loss of the proteins involved in the formation of the desmosome complex could serve as a biomarker of imatinib resistance.

The ablation of ACP5 had a similar effect on the proliferation of K562-IR and K562-NR cells, restoring sensitivity to imatinib and nilotinib, respectively. However, the K562-DR cells again did not have a significant response to ACP5 ablation, signifying that dasatinib resistance is mediated through an alternative mechanism, and ACP5 upregulation may be an artifact of another mechanism conferring resistance to dasatinib.

The ablation of CYP2S1 in K562-IR cells dramatically increased sensitivity to imatinib, but increased resistance to imatinib in K562 cells. This seemingly illogical result underscores the complexity of drug-metabolism, and eliminates CYP2S1 as a serious candidate for further study. Ablation of CYP2S1 in K562-NR cells had a similar effect, though less pronounced. In K562-DR cells, the ablation of CYP2S1 slightly sensitized the cells to dasatinib, although the results mirror the effects of ablating AXL and ACP5 from previous experiments. The results of this RNA-seq study did not elucidate any promising mechanisms of resistance to dasatinib.

One of the advantages of RNA-seq is the ability to detect minor changes in the transcription profile between samples. We were able to detect the transcription of genes that were not present in the parental K562 sample, or the total loss of transcription in the TKI-resistant cell line. We identified a massive upregulation in transcription of miRNA3940 and miRNA637, and SNORD88A and SNORD88B in K562-IR cells, and massive upregulation of SNORD33 and SNORD35A in K562-NR cells, yet a massive downregulation of SNORD88C in K562-NR and K562-DR cells. At this time, there is little known about the function of any of these transcripts, but there has been several reports that indicate miRNA's and snORD's play a role in translational regulation, which may partially explain the lack of correlation between the transcriptome and proteome, and the fact that the majority of differentially expressed proteins are downregulated.

miRNA and snORDS may be useful information for future studies to follow up on the emergence or disappearance of these transcripts with regards to TKI-resistance.

Finally, we described the detection of novel fusion transcripts emergent in our TKI-resistant cells. Fusion genes are responsible for many disease pathologies, CML being one of them. Detection of novel fusion genes is integral to the characterization of disease pathology, especially in drug resistance, as the detection of these transcripts could help predict future resistance to chemotherapy and guide therapeutic regimens for patients.

In the third chapter, we used SWATH-MS to profile the proteomic differences between K562 parental cells and the TKI-resistant cells we have generated in-house. The significantly differentially expressed proteins in our K562-IR cell line alluded to the loss of the desmosome as a biomarker of TKI-resistance, as we observed the loss of eleven proteins composing the desmosome, a structure that mediated cell-cell adhesion, and the loss of which is a hallmark of CML progression. K562-NR cells showed a loss of proteins involved with the regulation of cellular metabolism, suggesting that chronic nilotinib exposure has a role in rewiring the metabolic functions of K562 cells, conferring resistance to nilotinib. Dasatinib-resistant K562 cells showed a very similar protein expression profile to K562-NR cells, as many of the proteins differentially expressed play

some role in metabolism. Overall, eighteen proteins were commonly differentially expressed at a $\log_2$ fold change of ±1 between the three TKI-resistant cells, including PHB2, CAST, NUCKS1, DDX6, and DDX21. PHBS was significantly unregulated, and has been shown to be involved in apoptosis resistance, cell-cycle checkpoint release, and ERK1/2 signaling. CAST and NUCKS1 are known biomarkers of oncogenesis in many other cancers, so it may be worth tracking their protein expression in chemoresistant cells as biomarkers of TKI-resistance in CML. We also identified the upregulation of DDX6 and DDX21, proteins involved in translation of mRNA, which could partially explain the lack of correlation between the transcriptomic and proteomic data.

Lastly, we used a proteogenomic approach to identify novel peptides in TKI-resistant cells. We were able to identify one novel mutant peptide, corresponding to the ODO2 gene that was not represented in the canonical GRCh37 FASTA database, and contained a lysine-proline mutation from the mRNA to protein level. The ability to add a layer of information blending transcriptomic and proteomic data is critical to understanding complex pathological phenotypes, and this data demonstrates the power of the proteogenomic approach in cancer research.

Optimization of SWATH-MS methods will certainly increase the coverage of protein identification, and up-front fractionation of the samples will improve base protein

identification. The results of the data reported in this thesis suggest several promising therapeutic avenues to explore, but there is room to improve. First and foremost, the SWATH-MS methodology needs to be optimized, specifically the need for up-front fractionation and a longer HPLC gradient. These factors will certainly improve the identification of proteins, and allow a more accurate comparison of our results to our RNA-seq data. It is clear that there is little correlation between the transcriptome and proteome of our K562-TKI resistant cells, and protein levels in a cell do not yield any information about the activity of those proteins. Moving forward, a major effort will be made to understand the post-translational modifications that dictate the activity of the signaling proteins in our K562-TKI resistant cells. By enriching for phospho-peptides we will be able to construct a map of the signaling events underlying chemoresistance in our model of acquired TKI-resistance, which will be more informative than the transcriptome or proteome alone.

The goal of this research is to identify biomarkers of resistance and propose novel resistance mechanisms, and the best way to understand the dynamics of how a cell responds to a stimulus is by integrating multiple layers of information to determine which genes are transcribed, which proteins are translated, and how post-translational modification of proteins effect the molecular signaling pathways underlying TKI-resistance. In this thesis we have described the transcriptional and translational levels; the next step in elucidating the mechanisms of chemoresistance must be a

comprehensive characterization of the phospho-proteome in our model cell lines. The findings of our research must then be compared to human patient samples to validate our findings. The findings of this thesis have shined a light on many paths leading to the acquisition of chemoresistance. As Rosalia de Castro once said, “We can see the path, but we don’t know where it leads. Not knowing where it leads is what inspires us to travel it.”

LIST OF REFERENCES

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1. Cassio, A., et al., *Current loss-of-function mutations in the thyrotropin receptor gene: when to investigate, clinical effects, and treatment.* J Clin Res Pediatr Endocrinol, 2013. **5 Suppl 1**: p. 29-39.
2. Arjunaraja, S. and A.L. Snow, *Gain-of-function mutations and immunodeficiency: at a loss for proper tuning of lymphocyte signaling.* Curr Opin Allergy Clin Immunol, 2015. **15**(6): p. 533-8.
3. Silva, J.L., et al., *Expanding the prion concept to cancer biology: dominant-negative effect of aggregates of mutant p53 tumour suppressor.* Biosci Rep, 2013. **33**(4).
4. Olivier, M., M. Hollstein, and P. Hainaut, *TP53 mutations in human cancers: origins, consequences, and clinical use.* Cold Spring Harb Perspect Biol, 2010. **2**(1): p. a001008.
5. Davies, H., et al., *Mutations of the BRAF gene in human cancer.* Nature, 2002. **417**(6892): p. 949-54.
6. Drooger, J.C., et al., *Diagnostic and therapeutic ionizing radiation and the risk of a first and second primary breast cancer, with special attention for BRCA1 and BRCA2 mutation carriers: a critical review of the literature.* Cancer Treat Rev, 2015. **41**(2): p. 187-96.
7. Goh, A.M., C.R. Coffill, and D.P. Lane, *The role of mutant p53 in human cancer.* The Journal of pathology, 2011. **223**(2): p. 116-26.
8. Kozusko, K., et al., *Clinical and molecular characterization of a novel PLIN1 frameshift mutation identified in patients with familial partial lipodystrophy.* Diabetes, 2015. **64**(1): p. 299-310.
9. Guerreiro, R., et al., *Nonsense mutation in PRNP associated with clinical Alzheimer's disease.* Neurobiol Aging, 2014. **35**(11): p. 2656.e13-6.
10. Lajus, T.B. and R.M. Sales, *CDH1 germ-line missense mutation identified by multigene sequencing in a family with no history of diffuse gastric cancer.* Gene, 2015. **568**(2): p. 215-9.
11. Kishimoto, T., et al., *Molecular Clock of Neutral Mutations in a Fitness-Increasing Evolutionary Process.* PLoS Genet, 2015. **11**(7): p. e1005392.
12. Telwatte, S., et al., *Silent mutations at codons 65 and 66 in reverse transcriptase alleviate indel formation and restore fitness in subtype B HIV-1 containing D67N and K70R drug resistance mutations.* Nucleic Acids Res, 2015. **43**(6): p. 3256-71.
13. Cortes, J., et al., *Dynamics of BCR-ABL kinase domain mutations in chronic myeloid leukemia after sequential treatment with multiple tyrosine kinase inhibitors.* Blood, 2007. **110**(12): p. 4005-11.

14. Deangelo, D.J., *Managing chronic myeloid leukemia patients intolerant to tyrosine kinase inhibitor therapy.* Blood Cancer J, 2012. **2**: p. e95.
15. Donato, N.J., et al., *BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571.* Blood, 2003. **101**(2): p. 690-8.
16. Hughes, T., et al., *Impact of baseline BCR-ABL mutations on response to nilotinib in patients with chronic myeloid leukemia in chronic phase.* J Clin Oncol, 2009. **27**(25): p. 4204-10.
17. Hughes, T.P., et al., *BCR-ABL1 mutation development during first-line treatment with dasatinib or imatinib for chronic myeloid leukemia in chronic phase.* Leukemia, 2015. **29**(9): p. 1832-8.
18. Mahon, F.X., et al., *Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance.* Blood, 2000. **96**(3): p. 1070-9.
19. Pendergast, A.M., et al., *BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein.* Cell, 1993. **75**(1): p. 175-85.
20. Hiljadnikova Bajro, M., et al., *A new case with 10q23 interstitial deletion encompassing both PTEN and BMPR1A narrows the genetic region deleted in juvenile polyposis syndrome.* J Appl Genet, 2013. **54**(1): p. 43-7.
21. Brianti, M.T., G. Ananina, and L.B. Klaczko, *Differential occurrence of chromosome inversion polymorphisms among Muller's elements in three species of the tripunctata group of Drosophila, including a species with fast chromosomal evolution.* Genome, 2013. **56**(1): p. 17-26.
22. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
23. Peti, W. and R. Page, *Molecular basis of MAP kinase regulation.* Protein Sci, 2013. **22**(12): p. 1698-710.
24. Solus, J.F. and S. Kraft, *Ras, Raf, and MAP kinase in melanoma.* Adv Anat Pathol, 2013. **20**(4): p. 217-26.
25. Sullivan, R.J. and K. Flaherty, *MAP kinase signaling and inhibition in melanoma.* Oncogene, 2013. **32**(19): p. 2373-9.
26. Goodman, L.S., et al., *Landmark article Sept. 21, 1946: Nitrogen mustard therapy. Use of methyl-bis(beta-chloroethyl)amine hydrochloride and tris(beta-chloroethyl)amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. By Louis S. Goodman, Maxwell M. Wintrobe, William Dameshek, Morton J. Goodman, Alfred Gilman and Margaret T. McLennan.* Jama, 1984. **251**(17): p. 2255-61.
27. Farber, S. and L.K. Diamond, *Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid.* N Engl J Med, 1948. **238**(23): p. 787-93.

28. Hall, B.E., et al., *Evaluation of 6-mercaptopurine in the treatment of leukemia; a preliminary report.* Stanford Med Bull, 1953. **11**(4): p. 237-40.
29. van Vuuren, R.J., et al., *Antimitotic drugs in the treatment of cancer.* Cancer Chemother Pharmacol, 2015. **76**(6): p. 1101-12.
30. Decker, D.G., et al., *Cyclophosphamide in the treatment of ovarian cancer.* Clin Obstet Gynecol, 1968. **11**(2): p. 382-400.
31. Xu, Y. and C. Her, *Inhibition of Topoisomerase (DNA) I (TOP1): DNA Damage Repair and Anticancer Therapy.* Biomolecules, 2015. **5**(3): p. 1652-70.
32. Sleijfer, D.T., S. Meijer, and N.H. Mulder, *Cisplatin: a review of clinical applications and renal toxicity.* Pharm Weekbl Sci, 1985. **7**(6): p. 237-44.
33. Giannatempo, P., et al., *The Impact of Adding Taxanes to Gemcitabine and Platinum Chemotherapy for the First-Line Therapy of Advanced or Metastatic Urothelial Cancer: A Systematic Review and Meta-analysis.* Eur Urol, 2015.
34. Ribeiro, M.P., A.E. Santos, and J.B. Custodio, *Rethinking tamoxifen in the management of melanoma: New answers for an old question.* Eur J Pharmacol, 2015. **764**: p. 372-8.
35. Alam, H., B. Gu, and M.G. Lee, *Histone methylation modifiers in cellular signaling pathways.* Cell Mol Life Sci, 2015. **72**(23): p. 4577-92.
36. Li, P., et al., *Function and mechanism of tumor suppressor gene LRRC4/NGL-2.* Mol Cancer, 2014. **13**: p. 266.
37. Toulany, M. and H.P. Rodemann, *Phosphatidylinositol 3-kinase/Akt signaling as a key mediator of tumor cell responsiveness to radiation.* Semin Cancer Biol, 2015. **35**: p. 180-90.
38. Zhao, Y. and X. Yang, *The Hippo pathway in chemotherapeutic drug resistance.* Int J Cancer, 2015. **137**(12): p. 2767-73.
39. Burnett, G. and E.P. Kennedy, *The enzymatic phosphorylation of proteins.* J Biol Chem, 1954. **211**(2): p. 969-80.
40. Roskoski, R., Jr., *A historical overview of protein kinases and their targeted small molecule inhibitors.* Pharmacol Res, 2015. **100**: p. 1-23.
41. Dengjel, J., I. Kratchmarova, and B. Blagoev, *Receptor tyrosine kinase signaling: a view from quantitative proteomics.* Mol Biosyst, 2009. **5**(10): p. 1112-21.
42. Gioia, R., et al., *Quantitative phosphoproteomics revealed interplay between Syk and Lyn in the resistance to nilotinib in chronic myeloid leukemia cells.* Blood, 2011. **118**(8): p. 2211-21.
43. Wiley, H.S. and P.M. Burke, *Regulation of receptor tyrosine kinase signaling by endocytic trafficking.* Traffic, 2001. **2**(1): p. 12-8.
44. Belfiore, A., et al., *Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease.* Endocr Rev, 2009. **30**(6): p. 586-623.
45. Hojlund, K., *Metabolism and insulin signaling in common metabolic disorders and inherited insulin resistance.* Dan Med J, 2014. **61**(7): p. B4890.

46. Kurzrock, R., et al., *Philadelphia chromosome-positive leukemias: from basic mechanisms to molecular therapeutics.* Ann Intern Med, 2003. **138**(10): p. 819-30.
47. Gu, L., et al., *Pharmacologic inhibition of Jak2-Stat5 signaling By Jak2 inhibitor AZD1480 potently suppresses growth of both primary and castrate-resistant prostate cancer.* Clin Cancer Res, 2013. **19**(20): p. 5658-74.
48. Ohanian, M., et al., *Acute myeloid leukemia with MYC rearrangement and JAK2 V617F mutation.* Cancer Genet, 2015.
49. Bixby, D. and M. Talpaz, *Mechanisms of resistance to tyrosine kinase inhibitors in chronic myeloid leukemia and recent therapeutic strategies to overcome resistance.* Hematology Am Soc Hematol Educ Program, 2009: p. 461-76.
50. Chai, S.K., G.L. Nichols, and P. Rothman, *Constitutive activation of JAKs and STATs in BCR-Abl-expressing cell lines and peripheral blood cells derived from leukemic patients.* J Immunol, 1997. **159**(10): p. 4720-8.
51. Weisberg, E. and J.D. Griffin, *Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines.* Blood, 2000. **95**(11): p. 3498-505.
52. Shah, N.P., *Medical management of CML.* Hematology Am Soc Hematol Educ Program, 2007: p. 371-5.
53. Witte, O., *The role of Bcr-Abl in chronic myeloid leukemia and stem cell biology.* Semin Hematol, 2001. **38**(3 Suppl 8): p. 3-8.
54. Burchert, A., et al., *Compensatory PI3-kinase/Akt/mTor activation regulates imatinib resistance development.* Leukemia, 2005. **19**(10): p. 1774-82.
55. Croucher, D.R., et al., *Involvement of Lyn and the atypical kinase SgK269/PEAK1 in a basal breast cancer signaling pathway.* Cancer Res, 2013. **73**(6): p. 1969-80.
56. Danhauser-Riedl, S., et al., *Activation of Src kinases p53/56lyn and p59hck by p210bcr/abl in myeloid cells.* Cancer Res, 1996. **56**(15): p. 3589-96.
57. Klejman, A., et al., *The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells.* EMBO J, 2002. **21**(21): p. 5766-74.
58. Ptasznik, A., et al., *Short interfering RNA (siRNA) targeting the Lyn kinase induces apoptosis in primary, and drug-resistant, BCR-ABL1(+) leukemia cells.* Nat Med, 2004. **10**(11): p. 1187-9.
59. Keeshan, K., T.G. Cotter, and S.L. McKenna, *High Bcr-Abl expression prevents the translocation of Bax and Bad to the mitochondrion.* Leukemia, 2002. **16**(9): p. 1725-34.
60. Li, Q., et al., *BCR/ABL oncogene-induced PI3K signaling pathway leads to chronic myeloid leukemia pathogenesis by impairing immuno-modulatory function of hemangioblasts.* Cancer Gene Ther, 2015. **22**(5): p. 227-37.
61. Gorska-Flipot, I., et al., *Molecular pathology of chronic myelogenous leukemia.* Tumour Biol, 1990. **11 Suppl 1**: p. 25-43.
62. Granatowicz, A., et al., *An Overview and Update of Chronic Myeloid Leukemia for Primary Care Physicians.* Korean J Fam Med, 2015. **36**(5): p. 197-202.

63. Chereda, B. and J.V. Melo, *Natural course and biology of CML.* Ann Hematol, 2015. **94 Suppl 2**: p. S107-21.
64. Kantarjian, H., et al., *Improved survival in chronic myeloid leukemia since the introduction of imatinib therapy: a single-institution historical experience.* Blood, 2012. **119**(9): p. 1981-7.
65. Kantarjian, H.M., et al., *Significance of increasing levels of minimal residual disease in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in complete cytogenetic response.* J Clin Oncol, 2009. **27**(22): p. 3659-63.
66. Gruber, F.X., et al., *Dynamics of the emergence of dasatinib and nilotinib resistance in imatinib-resistant CML patients.* Leukemia, 2012. **26**(1): p. 172-7.
67. Mauro, M.J. and B.J. Druker, *STI571: targeting BCR-ABL as therapy for CML.* Oncologist, 2001. **6**(3): p. 233-8.
68. O'Brien, S.G., et al., *Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia.* N Engl J Med, 2003. **348**(11): p. 994-1004.
69. Yang, K. and L.W. Fu, *Mechanisms of resistance to BCR-ABL TKIs and the therapeutic strategies: A review.* Crit Rev Oncol Hematol, 2015. **93**(3): p. 277-92.
70. Kantarjian, H., et al., *Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL.* N Engl J Med, 2006. **354**(24): p. 2542-51.
71. le Coutre, P., et al., *Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is active in patients with imatinib-resistant or -intolerant accelerated-phase chronic myelogenous leukemia.* Blood, 2008. **111**(4): p. 1834-9.
72. Jabbour, E., H. Kantarjian, and J. Cortes, *Use of second- and third-generation tyrosine kinase inhibitors in the treatment of chronic myeloid leukemia: an evolving treatment paradigm.* Clin Lymphoma Myeloma Leuk, 2015. **15**(6): p. 323-34.
73. Camgoz, A., et al., *Mechanisms responsible for nilotinib resistance in human chronic myeloid leukemia cells and reversal of resistance.* Leuk Lymphoma, 2013. **54**(6): p. 1279-87.
74. Apperley, J.F., et al., *Dasatinib in the treatment of chronic myeloid leukemia in accelerated phase after imatinib failure: the START a trial.* J Clin Oncol, 2009. **27**(21): p. 3472-9.
75. Muller, M.C., et al., *Dasatinib treatment of chronic-phase chronic myeloid leukemia: analysis of responses according to preexisting BCR-ABL mutations.* Blood, 2009. **114**(24): p. 4944-53.
76. Talpaz, M., et al., *Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias.* N Engl J Med, 2006. **354**(24): p. 2531-41.
77. Kantarjian, H., et al., *Dasatinib or high-dose imatinib for chronic-phase chronic myeloid leukemia resistant to imatinib at a dose of 400 to 600 milligrams daily: two-year follow-up of a randomized phase 2 study (START-R).* Cancer, 2009. **115**(18): p. 4136-47.

78. Guilhot, F., et al., *Dasatinib induces significant hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in accelerated phase.* Blood, 2007. **109**(10): p. 4143-50.
79. Hochhaus, A., et al., *Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy.* Blood, 2007. **109**(6): p. 2303-9.
80. Soverini, S., et al., *Presence or the emergence of a F317L BCR-ABL mutation may be associated with resistance to dasatinib in Philadelphia chromosome-positive leukemia.* J Clin Oncol, 2006. **24**(33): p. e51-2.
81. Hoy, S.M., *Ponatinib: a review of its use in adults with chronic myeloid leukaemia or Philadelphia chromosome-positive acute lymphoblastic leukaemia.* Drugs, 2014. **74**(7): p. 793-806.
82. Wu, J., et al., *Association between imatinib-resistant BCR-ABL mutation-negative leukemia and persistent activation of LYN kinase.* J Natl Cancer Inst, 2008. **100**(13): p. 926-39.
83. Soverini, S., et al., *Resistance to dasatinib in Philadelphia-positive leukemia patients and the presence or the selection of mutations at residues 315 and 317 in the BCR-ABL kinase domain.* Haematologica, 2007. **92**(3): p. 401-4.
84. Aceves-Luquero, C.I., et al., *ERK2, but not ERK1, mediates acquired and "de novo" resistance to imatinib mesylate: implication for CML therapy.* PLoS One, 2009. **4**(7): p. e6124.
85. Widmer, N., et al., *Resistance reversal by RNAi silencing of MDR1 in CML cells associated with increase in imatinib intracellular levels.* Leukemia, 2007. **21**(7): p. 1561-2; author reply 1562-4.
86. Rumpold, H., et al., *RNAi-mediated knockdown of P-glycoprotein using a transposon-based vector system durably restores imatinib sensitivity in imatinib-resistant CML cell lines.* Exp Hematol, 2005. **33**(7): p. 767-75.
87. White, D.L., et al., *OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib.* Blood, 2006. **108**(2): p. 697-704.
88. Crossman, L.C., et al., *hOCT 1 and resistance to imatinib.* Blood, 2005. **106**(3): p. 1133-4; author reply 1134.
89. Sattler, M., et al., *Critical role for Gab2 in transformation by BCR/ABL.* Cancer Cell, 2002. **1**(5): p. 479-92.
90. Thomas, J., et al., *Active transport of imatinib into and out of cells: implications for drug resistance.* Blood, 2004. **104**(12): p. 3739-45.
91. White, D., et al., *Measurement of in vivo BCR-ABL kinase inhibition to monitor imatinib-induced target blockade and predict response in chronic myeloid leukemia.* J Clin Oncol, 2007. **25**(28): p. 4445-51.
92. Blankenberg, D., et al., *Manipulation of FASTQ data with Galaxy.* Bioinformatics, 2010. **26**(14): p. 1783-5.

93. Kim, D., et al., *TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions.* Genome Biol, 2013. **14**(4): p. R36.
94. Trapnell, C., et al., *Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation.* Nat Biotechnol, 2010. **28**(5): p. 511-5.
95. McPherson, A., et al., *deFuse: an algorithm for gene fusion discovery in tumor RNA-Seq data.* PLoS Comput Biol, 2011. **7**(5): p. e1001138.
96. Xiao, W.H., et al., *Identification of commonly dysregulated genes in colorectal cancer by integrating analysis of RNA-Seq data and qRT-PCR validation.* Cancer Gene Ther, 2015. **22**(5): p. 278-84.
97. Cloonan, N., et al., *Stem cell transcriptome profiling via massive-scale mRNA sequencing.* Nat Methods, 2008. **5**(7): p. 613-9.
98. Wang, Z., M. Gerstein, and M. Snyder, *RNA-Seq: a revolutionary tool for transcriptomics.* Nat Rev Genet, 2009. **10**(1): p. 57-63.
99. Chng, W.J., et al., *Gene expression profiling of pulmonary mucosa-associated lymphoid tissue lymphoma identifies new biologic insights with potential diagnostic and therapeutic applications.* Blood, 2009. **113**(3): p. 635-45.
100. Rumer, K.K., et al., *Siglec-6 is expressed in gestational trophoblastic disease and affects proliferation, apoptosis and invasion.* Endocr Relat Cancer, 2012. **19**(6): p. 827-40.
101. Wang, H., et al., *Decreased expression and prognostic role of cytoplasmic BRSK1 in human breast carcinoma: correlation with Jab1 stability and PI3K/Akt pathway.* Exp Mol Pathol, 2014. **97**(2): p. 191-201.
102. Khan, J., et al., *Overexpression of active Aurora-C kinase results in cell transformation and tumour formation.* PLoS One, 2011. **6**(10): p. e26512.
103. Tsou, J.H., et al., *Aberrantly expressed AURKC enhances the transformation and tumourigenicity of epithelial cells.* J Pathol, 2011. **225**(2): p. 243-54.
104. Oguri, T., et al., *The Kruppel-type zinc finger family gene, HKR1, is induced in lung cancer by exposure to platinum drugs.* Gene, 1998. **222**(1): p. 61-7.
105. Dufies, M., et al., *Mechanisms of AXL overexpression and function in Imatinib-resistant chronic myeloid leukemia cells.* Oncotarget, 2011. **2**(11): p. 874-85.
106. Grosso, S., et al., *Gene expression profiling of imatinib and PD166326-resistant CML cell lines identifies Fyn as a gene associated with resistance to BCR-ABL inhibitors.* Mol Cancer Ther, 2009. **8**(7): p. 1924-33.
107. Hochhaus, A., et al., *Nilotinib is associated with a reduced incidence of BCR-ABL mutations vs imatinib in patients with newly diagnosed chronic myeloid leukemia in chronic phase.* Blood, 2013. **121**(18): p. 3703-8.
108. O'Connor, C., et al., *Nfix expression critically modulates early B lymphopoiesis and myelopoiesis.* PLoS One, 2015. **10**(3): p. e0120102.
109. Ravichandran, V., et al., *Interactions between c-Jun, nuclear factor 1, and JC virus promoter sequences: implications for viral tropism.* J Virol, 2006. **80**(21): p. 10506-13.

110. Villaruz, L.C., et al., *MicroRNA expression profiling predicts clinical outcome of carboplatin/paclitaxel-based therapy in metastatic melanoma treated on the ECOG-ACRIN trial E2603.* Clin Epigenetics, 2015. **7**(1): p. 58.
111. Hsu, S.I., et al., *TRIP-Br: a novel family of PHD zinc finger- and bromodomain-interacting proteins that regulate the transcriptional activity of E2F-1/DP-1.* EMBO J, 2001. **20**(9): p. 2273-85.
112. Li, J., et al., *Dissection of CDK4-binding and transactivation activities of p34(SEI-1) and comparison between functions of p34(SEI-1) and p16(INK4A).* Biochemistry, 2005. **44**(40): p. 13246-56.
113. Jung, S., et al., *Oncogenic function of p34SEI-1 via NEDD41mediated PTEN ubiquitination/degradation and activation of the PI3K/AKT pathway.* Int J Oncol, 2013. **43**(5): p. 1587-95.
114. Takano, S., et al., *Apolipoprotein C-1 maintains cell survival by preventing from apoptosis in pancreatic cancer cells.* Oncogene, 2008. **27**(20): p. 2810-22.
115. Braoudaki, M., et al., *Protein biomarkers distinguish between high- and low-risk pediatric acute lymphoblastic leukemia in a tissue specific manner.* J Hematol Oncol, 2013. **6**: p. 52.
116. Martin, P., et al., *Predicting response to vascular endothelial growth factor inhibitor and chemotherapy in metastatic colorectal cancer.* BMC Cancer, 2014. **14**: p. 887.
117. Claerhout, S., et al., *Gene expression signature analysis identifies vorinostat as a candidate therapy for gastric cancer.* PLoS One, 2011. **6**(9): p. e24662.
118. Morrow, K., et al., *Anti-leukemic mechanisms of pegylated arginase I in acute lymphoblastic T-cell leukemia.* Leukemia, 2013. **27**(3): p. 569-77.
119. Stone, J.C., *Regulation and Function of the RasGRP Family of Ras Activators in Blood Cells.* Genes Cancer, 2011. **2**(3): p. 320-34.
120. Reuther, G.W., et al., *RasGRP4 is a novel Ras activator isolated from acute myeloid leukemia.* J Biol Chem, 2002. **277**(34): p. 30508-14.
121. Watanabe-Okochi, N., et al., *Possible involvement of RasGRP4 in leukemogenesis.* Int J Hematol, 2009. **89**(4): p. 470-81.
122. Liu, J., et al., *High expression of beta3GnT8 is associated with the metastatic potential of human glioma.* Int J Mol Med, 2014. **33**(6): p. 1459-68.
123. Hua, D., et al., *beta3GnT8 regulates laryngeal carcinoma cell proliferation via targeting MMPs/TIMPs and TGF-beta1.* Asian Pac J Cancer Prev, 2012. **13**(5): p. 2087-93.
124. Zhang, Z., et al., *Glycomic alterations are associated with multidrug resistance in human leukemia.* Int J Biochem Cell Biol, 2012. **44**(8): p. 1244-53.
125. Tang, H., et al., *LRRC4 inhibits glioma cell growth and invasion through a miR-185-dependent pathway.* Curr Cancer Drug Targets, 2012. **12**(8): p. 1032-42.
126. Kelly, K.R., et al., *The novel Aurora A kinase inhibitor MLN8237 is active in resistant chronic myeloid leukaemia and significantly increases the efficacy of nilotinib.* J Cell Mol Med, 2011. **15**(10): p. 2057-70.

127. Baldini, E., et al., *Aurora kinases are expressed in medullary thyroid carcinoma (MTC) and their inhibition suppresses in vitro growth and tumorigenicity of the MTC derived cell line TT.* BMC Cancer, 2011. **11**: p. 411.
128. Hosseini, S., et al., *Expression Analysis of Aurora-C and Survivin, Two Testis-Specific Genes, in Patients with Colorectal Cancer.* Clin Lab, 2015. **61**(5-6): p. 475-80.
129. Goruppi, S., E. Ruaro, and C. Schneider, *Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH3T3 fibroblasts.* Oncogene, 1996. **12**(3): p. 471-80.
130. Goruppi, S., et al., *Requirement of phosphatidylinositol 3-kinase-dependent pathway and Src for Gas6-Axl mitogenic and survival activities in NIH 3T3 fibroblasts.* Mol Cell Biol, 1997. **17**(8): p. 4442-53.
131. Jacob, A.N., et al., *A receptor tyrosine kinase, UFO/Axl, and other genes isolated by a modified differential display PCR are overexpressed in metastatic prostatic carcinoma cell line DU145.* Cancer Detect Prev, 1999. **23**(4): p. 325-32.
132. Rankin, E.B., et al., *AXL is an essential factor and therapeutic target for metastatic ovarian cancer.* Cancer Res, 2010. **70**(19): p. 7570-9.
133. Shieh, Y.S., et al., *Expression of axl in lung adenocarcinoma and correlation with tumor progression.* Neoplasia, 2005. **7**(12): p. 1058-64.
134. Vuoriluoto, K., et al., *Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer.* Oncogene, 2011. **30**(12): p. 1436-48.
135. Xia, L., et al., *ACP5, a direct transcriptional target of FoxM1, promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma.* Oncogene, 2014. **33**(11): p. 1395-406.
136. Scott, K.L., et al., *Proinvasion metastasis drivers in early-stage melanoma are oncogenes.* Cancer Cell, 2011. **20**(1): p. 92-103.
137. Hung, M.J. and G.M. Oremek, *Value of TRAcP 5b as a diagnostic marker for detection of bone metastases in patients with breast cancer.* Eur J Gynaecol Oncol, 2011(0392-2936 (Print)).
138. Kawamura, M., et al., *Clinical significance of tartrate-resistant acid phosphatase type-5 expression in human gastric cancer.* Anticancer Res, 2014(1791-7530 (Electronic)).
139. Krumpel, M., et al., *The small chemical enzyme inhibitor 5-phenylnicotinic acid/CD13 inhibits cell migration and invasion of tartrate-resistant acid phosphatase/ACP5-overexpressing MDA-MB-231 breast cancer cells.* Exp Cell Res, 2015.
140. Wu, Y.Y., et al., *Serum tartrate-resistant acid phosphatase 5b activity as a prognostic marker of survival in breast cancer with bone metastasis.* BMC Cancer, 2010(1471-2407 (Electronic)).
141. Endo-Munoz, L., et al., *Loss of osteoclasts contributes to development of osteosarcoma pulmonary metastases.* Cancer Res, 2010. **70**(18): p. 7063-72.

142. Tan, B.S., et al., *CYP2S1 and CYP2W1 mediate 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole (GW-610, NSC 721648) sensitivity in breast and colorectal cancer cells.* Mol Cancer Ther, 2011. **10**(10): p. 1982-92.
143. Kumarakulasingham, M., et al., *Cytochrome p450 profile of colorectal cancer: identification of markers of prognosis.* Clin Cancer Res, 2005(1078-0432 (Print)).
144. Abedin, M.J., et al., *Potential roles of galectins in myeloid differentiation into three different lineages.* J Leukoc Biol, 2003. **73**(5): p. 650-6.
145. Williams, K.E., et al., *High-density array analysis of DNA methylation in Tamoxifen-resistant breast cancer cell lines.* Epigenetics, 2014(1559-2308 (Electronic)).
146. Booken, N., et al., *Sezary syndrome is a unique cutaneous T-cell lymphoma as identified by an expanded gene signature including diagnostic marker molecules CDO1 and DNM3.* Leukemia, 2008(1476-5551 (Electronic)).
147. Cho, H.K., et al., *HBx induces the proliferation of hepatocellular carcinoma cells via AP1 over-expressed as a result of ER stress.* Biochem J, 2015(1470-8728 (Electronic)).
148. Ghasemi, R., et al., *Multitargeting and antimetastatic potentials of silibinin in human HepG-2 and PLC/PRF/5 hepatoma cells.* Nutr Cancer, 2015(1532-7914 (Electronic)).
149. Sille, F.C., et al., *Post-GWAS functional characterization of susceptibility variants for chronic lymphocytic leukemia.* PLoS One, 2012. **7**(1): p. e29632.
150. Heidel, F.H., et al., *Genetic and pharmacologic inhibition of beta-catenin targets imatinib-resistant leukemia stem cells in CML.* Cell Stem Cell, 2012(1875-9777 (Electronic)).
151. Riether, C., et al., *Tyrosine kinase inhibitor-induced CD70 expression mediates drug resistance in leukemia stem cells by activating Wnt signaling.* Science Translational Medicine, 2015 (1946-6242 (Electronic)).
152. Sun, Y., et al., *Expression of miR-150 and miR-3940-5p is reduced in non-small cell lung carcinoma and correlates with clinicopathological features.* Oncol Rep, 2013. **29**(2): p. 704-12.
153. Zhang, J.F., et al., *Primate-specific microRNA-637 inhibits tumorigenesis in hepatocellular carcinoma by disrupting signal transducer and activator of transcription 3 signaling.* Hepatology, 2011. **54**(6): p. 2137-48.
154. Ono, M., et al., *Analysis of human small nucleolar RNAs (snoRNA) and the development of snoRNA modulator of gene expression vectors.* Mol Biol Cell, 2010. **21**(9): p. 1569-84.
155. Shugay, M., et al., *Oncofuse: a computational framework for the prediction of the oncogenic potential of gene fusions.* Bioinformatics, 2013. **29**(20): p. 2539-46.
156. Zheng, J., et al., *Structure of human MDM2 complexed with RPL11 reveals the molecular basis of p53 activation.* Genes Dev, 2015. **29**(14): p. 1524-34.
157. Edfeldt, K., et al., *TCEB3C a putative tumor suppressor gene of small intestinal neuroendocrine tumors.* Endocr Relat Cancer, 2014. **21**(2): p. 275-84.

158. Grosso, A.R., et al., *Pervasive transcription read-through promotes aberrant expression of oncogenes and RNA chimeras in renal carcinoma.* Elife, 2015. **4**.
159. Wang, L., et al., *Quantification of endogenous brassinosteroids in sub-gram plant tissues by in-line matrix solid-phase dispersion-tandem solid phase extraction coupled with high performance liquid chromatography-tandem mass spectrometry.* J Chromatogr A, 2014. **1359**: p. 44-51.
160. Tsou, C.C., et al., *DIA-Umpire: comprehensive computational framework for data-independent acquisition proteomics.* Nat Methods, 2015. **12**(3): p. 258-64, 7 p following 264.
161. Hausser, J., et al., *Analysis of CDS-located miRNA target sites suggests that they can effectively inhibit translation.* Genome Res, 2013. **23**(4): p. 604-15.
162. Li, S., et al., *MicroRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in Arabidopsis.* Cell, 2013. **153**(3): p. 562-74.
163. Plank, M., et al., *Targeting translational control as a novel way to treat inflammatory disease: the emerging role of microRNAs.* Clin Exp Allergy, 2013. **43**(9): p. 981-99.
164. Rogers, S., et al., *Investigating the correspondence between transcriptomic and proteomic expression profiles using coupled cluster models.* Bioinformatics, 2008. **24**(24): p. 2894-900.
165. Collins, B.C., et al., *Quantifying protein interaction dynamics by SWATH mass spectrometry: application to the 14-3-3 system.* Nat Methods, 2013. **10**(12): p. 1246-53.
166. Liu, Y., et al., *Glycoproteomic analysis of prostate cancer tissues by SWATH mass spectrometry discovers N-acylethanolamine acid amidase and protein tyrosine kinase 7 as signatures for tumor aggressiveness.* Mol Cell Proteomics, 2014. **13**(7): p. 1753-68.
167. Scheidweiler, K.B., M.J. Jarvis, and M.A. Huestis, *Nontargeted SWATH acquisition for identifying 47 synthetic cannabinoid metabolites in human urine by liquid chromatography-high-resolution tandem mass spectrometry.* Anal Bioanal Chem, 2015. **407**(3): p. 883-97.
168. Foss, E.J., et al., *Genetic variation shapes protein networks mainly through non-transcriptional mechanisms.* PLoS Biol, 2011. **9**(9): p. e1001144.
169. Ghazalpour, A., et al., *Comparative analysis of proteome and transcriptome variation in mouse.* PLoS Genet, 2011. **7**(6): p. e1001393.
170. Pedrioli, P.G., *Trans-proteomic pipeline: a pipeline for proteomic analysis.* Methods Mol Biol, 2010. **604**: p. 213-38.
171. Egertson, J.D., et al., *Multiplexed peptide analysis using data-independent acquisition and Skyline.* Nat Protoc, 2015. **10**(6): p. 887-903.
172. MacLean, B., et al., *Skyline: an open source document editor for creating and analyzing targeted proteomics experiments.* Bioinformatics, 2010. **26**(7): p. 966-8.
173. Equilibrina, I., et al., *ASURA (PHB2) interacts with Scc1 through chromatin.* Cytogenet Genome Res, 2013. **139**(4): p. 225-33.

174. Kasashima, K., et al., *Mitochondrial functions and estrogen receptor-dependent nuclear translocation of pleiotropic human prohibitin 2.* J Biol Chem, 2006. **281**(47): p. 36401-10.
175. Bavelloni, A., et al., *Prohibitin 2 represents a novel nuclear AKT substrate during all-trans retinoic acid-induced differentiation of acute promyelocytic leukemia cells.* Faseb j, 2014. **28**(5): p. 2009-19.
176. Kroger, C., et al., *Keratins control intercellular adhesion involving PKC-alpha-mediated desmoplakin phosphorylation.* J Cell Biol, 2013. **201**(5): p. 681-92.
177. Loschke, F., M. Homberg, and T.M. Magin, *Keratin Isotypes Control Desmosome Stability and Dynamics through PKCalpha.* J Invest Dermatol, 2015.

178. Arnemann, J., et al., *Chromosomal assignment of the human genes coding for the major proteins of the desmosome junction, desmoglein DGI (DSG), desmocollins DGII/III (DSC), desmoplakins DPI/II (DSP), and plakoglobin DPIII (JUP).* Genomics, 1991. **10**(3): p. 640-5.
179. Huber, O. and I. Petersen, *150th Anniversary Series: Desmosomes and the Hallmarks of Cancer.* Cell Commun Adhes, 2015. **22**(1): p. 15-28.
180. Melki, J.R., et al., *Hypermethylation of E-cadherin in leukemia.* Blood, 2000. **95**(10): p. 3208-13.
181. Gravdal, K., et al., *A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer.* Clin Cancer Res, 2007. **13**(23): p. 7003-11.
182. Zhang, B., et al., *Microenvironmental protection of CML stem and progenitor cells from tyrosine kinase inhibitors through N-cadherin and Wnt-beta-catenin signaling.* Blood, 2013. **121**(10): p. 1824-38.
183. Cichon, M.A., et al., *The receptor tyrosine kinase Axl regulates cell-cell adhesion and stemness in cutaneous squamous cell carcinoma.* Oncogene, 2014. **33**(32): p. 4185-92.
184. Asiedu, M.K., et al., *AXL induces epithelial-to-mesenchymal transition and regulates the function of breast cancer stem cells.* Oncogene, 2014. **33**(10): p. 1316-24.
185. Ehyai, S., et al., *A p38 MAPK regulated MEF2:beta-catenin interaction enhances canonical Wnt signalling.* Mol Cell Biol, 2015.
186. Shoshan-Barmatz, V., et al., *The mitochondrial voltage-dependent anion channel 1 in tumor cells.* Biochim Biophys Acta, 2015. **1848**(10 Pt B): p. 2547-75.
187. Oehler, V.G., et al., *The derivation of diagnostic markers of chronic myeloid leukemia progression from microarray data.* Blood, 2009. **114**(15): p. 3292-8.
188. Akbari Moqadam, F., et al., *MiR-125b, miR-100 and miR-99a co-regulate vincristine resistance in childhood acute lymphoblastic leukemia.* Leuk Res, 2013. **37**(10): p. 1315-21.

189. Gu, L., et al., *NUCKS1 overexpression is a novel biomarker for recurrence-free survival in cervical squamous cell carcinoma.* Tumour Biol, 2014. **35**(8): p. 7831-6.
190. Johnson, J.D., et al., *RyR2 and calpain-10 delineate a novel apoptosis pathway in pancreatic islets.* J Biol Chem, 2004. **279**(23): p. 24794-802.
191. Konoplev, S.N., et al., *High serum thymidine kinase 1 level predicts poorer survival in patients with chronic lymphocytic leukemia.* Am J Clin Pathol, 2010. **134**(3): p. 472-7.
192. He, Q., et al., *Concentration of thymidine kinase 1 in serum (S-TK1) is a more sensitive proliferation marker in human solid tumors than its activity.* Oncol Rep, 2005. **14**(4): p. 1013-9.
193. Debald, M., et al., *Specific expression of k63-linked ubiquitination of calmodulin-like protein 5 in breast cancer of premenopausal patients.* J Cancer Res Clin Oncol, 2013. **139**(12): p. 2125-32.
194. Huang, B., et al., *Depleting ABCE1 expression induces apoptosis and inhibits the ability of proliferation and migration of human esophageal carcinoma cells.* Int J Clin Exp Pathol, 2014. **7**(2): p. 584-92.
195. Huang, B., et al., *siRNAinduced ABCE1 silencing inhibits proliferation and invasion of breast cancer cells.* Mol Med Rep, 2014. **10**(4): p. 1685-90.
196. Kara, G., et al., *Downregulation of ABCE1 via siRNA affects the sensitivity of A549 cells against chemotherapeutic agents.* Med Oncol, 2015. **32**(4): p. 103.
197. Li, H., et al., *Knockdown of asparagine synthetase by RNAi suppresses cell growth in human melanoma cells and epidermoid carcinoma cells.* Biotechnol Appl Biochem, 2015.
198. Li, Y., et al., *Asparagine synthetase expression and its potential prognostic value in patients with NK/T cell lymphoma.* Oncol Rep, 2014. **32**(2): p. 853-9.
199. Liu, R.Y., et al., *Overexpression of asparagine synthetase and matrix metalloproteinase 19 confers cisplatin sensitivity in nasopharyngeal carcinoma cells.* Mol Cancer Ther, 2013. **12**(10): p. 2157-66.
200. Lin, S.T., et al., *Proteomic analysis of proteins responsible for the development of doxorubicin resistance in human uterine cancer cells.* J Proteomics, 2012. **75**(18): p. 5822-47.
201. Nishimura, T., et al., *The eIF4E-Binding Protein 4E-T Is a Component of the mRNA Decay Machinery that Bridges the 5' and 3' Termini of Target mRNAs.* Cell Rep, 2015. **11**(9): p. 1425-36.
202. Rouya, C., et al., *Human DDX6 effects miRNA-mediated gene silencing via direct binding to CNOT1.* Rna, 2014. **20**(9): p. 1398-409.
203. Calo, E., et al., *RNA helicase DDX21 coordinates transcription and ribosomal RNA processing.* Nature, 2015. **518**(7538): p. 249-53.
204. Bonzheim, I., et al., *Identification of C/EBPbeta target genes in ALK+ anaplastic large cell lymphoma (ALCL) by gene expression profiling and chromatin immunoprecipitation.* PLoS One, 2013. **8**(5): p. e64544.

205. Jung, Y., et al., *Clinical validation of colorectal cancer biomarkers identified from bioinformatics analysis of public expression data.* Clin Cancer Res, 2011. **17**(4): p. 700-9.
206. Zhang, Y., et al., *Elevated DDX21 regulates c-Jun activity and rRNA processing in human breast cancers.* Breast Cancer Res, 2014. **16**(5): p. 449.
207. de Sousa Abreu, R., et al., *Global signatures of protein and mRNA expression levels.* Mol Biosyst, 2009. **5**(12): p. 1512-26.
208. Altschul, S.F., et al., *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.* Nucleic Acids Res, 1997. **25**(17): p. 3389-402.

VITA

VITA

Brett Noel

Graduate School, Purdue University

Education

Purdue University

Doctor of Philosophy in Medicinal Chemistry and Molecular Pharmacology- December 2015

Major Focus- Molecular Signaling and Cancer Biology

Thesis: “Proteogenomic Insights Into The Nature Of Chemoresistance In Chronic Myelogenous Leukemia.”

University of Wisconsin-Madison

BS in Neurobiology, BS in Zoology 2009

Publications

Tantak MP, Kumar A, **Noel B**, Shah K, Kumar D. "Synthesis and biological evaluation of 2-arylamino-5-(3'-indolyl)-1,3,4- oxadiazoles as new potent cytotoxic agents." Chem Med Chem. 2013 Sep;8(9):1468-74.

Kumar D, Arun V, Kumar NM, Acosta G, **Noel B**, Shah K. "A facile synthesis of novel bis (indolyl)-1,3,4-oxadiazoles as potent cytotoxic agents." Chem Med Chem. 2012 Nov;7(11):1915-20.

Kumar D, Kumar NM, **Noel B**, Shah K. “A series of 2-arylamino-5-(indolyl)-1,3,4 thiadiazoles as potent cytotoxic agents." Eur J Med Chem. 2012 Sep;55:432-8.

Poster Presentation

Noel B, Ouellette S, Yang TY, Rochelle N, Parker L. *Using SWATH-MS to identify alterations in tyrosine kinase signaling pathways in chemoresistant Chronic Myelogenous Leukemia cells.* US-HUPO Annual Conference, Tempe, AZ. March 2015

Awards and Leadership

Teaching- Most Outstanding Graduate Teaching Assistant-Department of Chemistry, Purdue University (2012)

Writing- Purdue Center for Cancer Research SIRG Fellowship (awarded 2012-2013)

Purdue Lynn Fellow (2009-2010, Spring 2014)

Leadership- Purdue University Life Sciences Graduate Student Organization President (2011-2012)