

MOLECULAR ADAPTATION TO ANTI-CANCER CHEMOTHERAPY IN LEUKEMIA

Eric Iredell Zimmerman

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ABSTRACT

Eric I. Zimmerman

Molecular Adaptation to Anti-cancer Chemotherapy in Leukemia
(Under the direction of Dr. Lee M. Graves)

Drug resistance to anti-cancer chemotherapy is a significant barrier to the treatment of leukemia patients. Many times, resistance results from molecular adaptation to drug exposure, such as genetic mutation of key enzymes, up-regulation of pro-survival compensatory signaling pathways, and altered drug transport. In this dissertation, we describe two examples of molecular drug resistance in cell models of 1) Ara-C-resistant acute lymphoblastic leukemia (ALL) and 2) imatinib-resistant chronic myelogenous leukemia (CML). First, we determined that nucleoside transport is deficient in the Ara-C-resistant T-cell ALL CCRF-CEM cell line (Ara-C/8C) in comparison to drug-sensitive parental CCRF-CEM cells. Further study found a single point mutation in glycine residue 24 (G24) within equilibrative nucleoside transporter 1 (ENT1), a protein responsible for nucleoside uptake in these cells. Therefore, we tested the ability of G24A, G24R, and G24E ENT1 mutants to transport uridine and Ara-C and localize to the plasma membrane. Our data suggest that mutation of G24 disrupts ENT1 transport activity without altering localization; thus, expression of mutant ENT1 may confer Ara-C resistance in CCRF-CEM AraC/8C cells. In the second portion of the dissertation, we studied the role of Lyn tyrosine kinase (Lyn) in imatinib-resistant CML MYL-R cells. In

comparison to drug-sensitive parental MYL cells, Lyn was hyper-active, and loss of Lyn activity sensitized cells to imatinib treatment. We determined that Lyn inhibited miR181 microRNA (miRNA) expression in MYL-R cells at the transcriptional level. In addition, we determined that miR181b targeted the 3' UTR of Mcl-1, a pro-survival protein associated with drug-resistance, resulting in Mcl-1 degradation. Thus, we defined a molecular signaling axis by which Lyn may confer drug resistance in imatinib-resistant CML. We attempted to elucidate the mechanism of Lyn-dependent miR181 expression and found that 1) the transcription factors CREB and STAT5 do not regulate miR181a/b and 2) Lyn may regulate miR181c/d expression through modification of histone acetylation. These studies contribute to our knowledge of drug resistance mechanisms in leukemia and provide novel biomarkers for the identification of drug-resistant cancer.

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LIST OF ABBREVIATIONS

5-aza-C	5-aza cytidine
Abl	Abelson tyrosine kinase
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
Ara-C	Cytarabine
Bcl-2	B-cell CLL/lymphoma-2
BCR	Breakpoint cluster region
CLL	Chronic lymphoblastic leukemia
CML	Chronic myelogenous leukemia
CNT	Concentrative nucleoside transporter
CR	Complete remission
CREB	cAMP response element-binding protein
DNMT	DNA methyltransferase
ENT	Equilibrative nucleoside transporter
FLT3	Fms-like tyrosine kinase
HCT	Hematopoietic cell transplant
HDAC	Histone deacetylase
HSP	Heat shock protein
ITD	Internal tandem duplication
JAK	Janus kinase
KIB	Kinase inhibitor bead
Lyn	Lyn tyrosine kinase

MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia-1
miRNA	microRNA
NBMPR	Nitrobenzylmercaptapurine riboside
Ph	Philadelphia chromosome
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
RISC	RNA-induced silencing complex
SFK	Src family kinase
STAT	Signal transducer and activator of transcription
TCL-1	T-cell leukemia/lymphoma-1
TF	Transcription factor
TM	Transmembrane
TSA	Trichostatin A
TSS	Transcriptional start site
UTR	Untranslated region
VPA	Valproic acid
WBC	White blood cell
WT	Wild-type

CHAPTER I

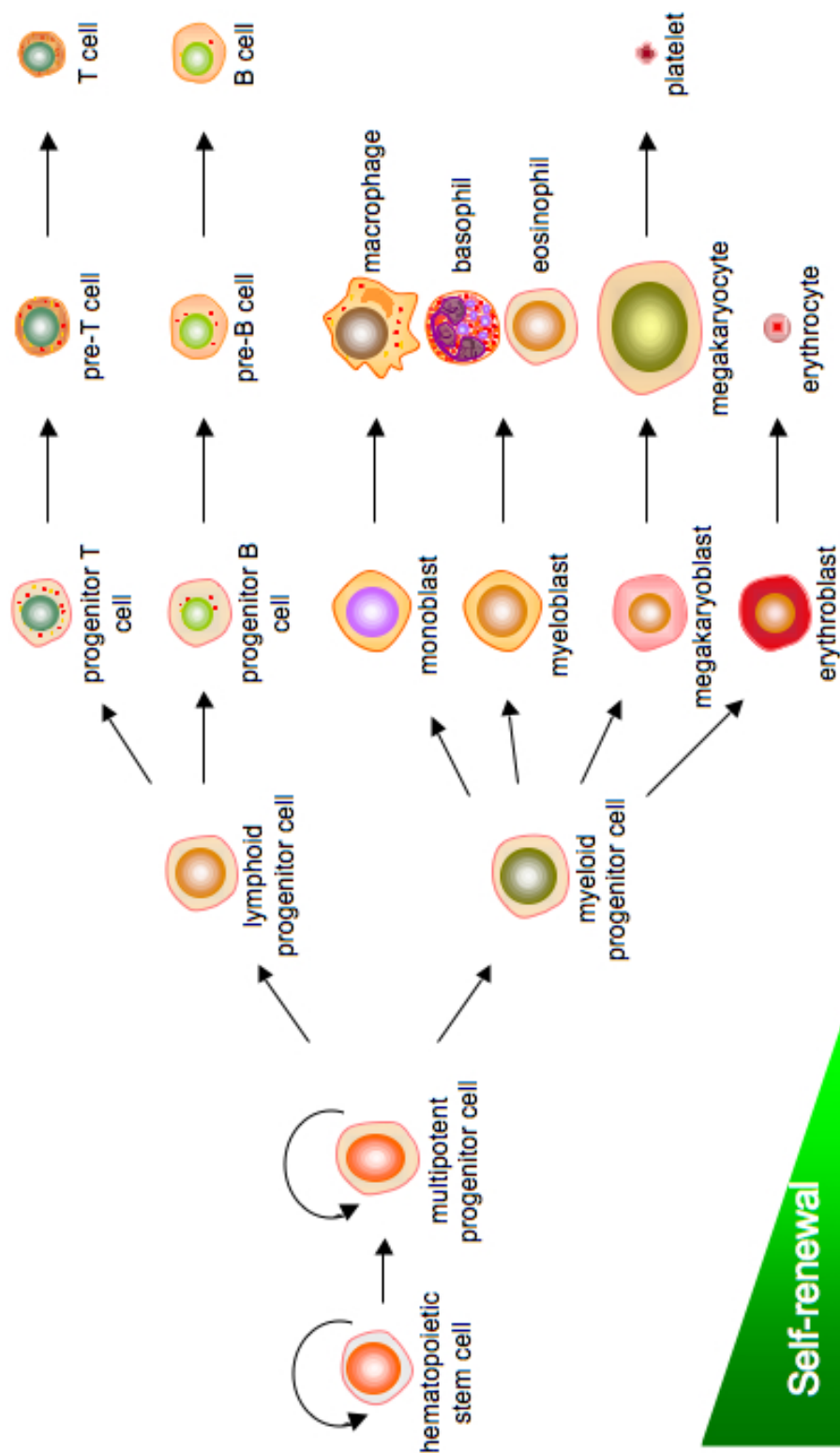
Introduction

All figures contributed by Eric I. Zimmerman.

1.1. Introduction to leukemia

1.1.1. Overview of leukemia

Leukemia is characterized by the unregulated growth of hematopoietic cells. In 2010, there were estimated to be approximately 43,000 new cases of leukemia with nearly 22,000 deaths expected to result from this disease [1]. The four major types of leukemia include, from the myeloid cell lineage, acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) and, derived from the lymphoid cell lineage, acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL) (Fig. 1.1). Classification of leukemia as acute or chronic depends on the overall proliferation rate of the disease as well as the level of cellular differentiation. Acute leukemia is fast growing and requires immediate treatment; cells are typically immature and undifferentiated. In contrast, cells in chronic leukemia are more differentiated and slower growing and have enhanced survival in comparison to normal cells. Leukemia patients are less able to combat infection due to compromised immune function and typically succumb to secondary infections.



Differentiation

Self-renewal

Figure 1.1. Hematopoiesis of blood-derived cells. Schematic depiction of normal human blood cell differentiation.

1.1.2. Lymphoblastic leukemia

1.1.2.1. CLL overview and standard of care

Hematopoietic cells of the lymphoid cell lineage mature into B cells or T cells, depending on the site of maturation (bone marrow and thymus, respectively), and serve as integral components of the innate immune system. CLL is characterized by lymphocytosis, including the following criteria: 1) expression of B cell-associated antigens (CD19, CD20, and CD23); expression of the T cell-associated CD5 antigen; and 3) low levels of surface membrane immunoglobulin (Ig) [2]. This disease predominates in older individuals with a median age of diagnosis of 70 years [3]. In addition, CLL is the most common leukemia in Western society where it accounts for approximately 30% of all diagnosed leukemias [4]. Cytogenetic abnormalities have not been consistently associated with either the cause or pathogenesis of CLL, though some have been associated with prognosis. In particular, 11q and 17p deletions are associated with progressive disease and shorter survival [5, 6]. In addition, up-regulation of the Bcl-2 pro-survival protein often occurs in CLL [7].

Due to the heterogeneity of the disease, the prognosis of CLL patients is extremely variable. Correlation studies of variable region immunoglobulin heavy chains (vHC) have determined that 30-40% of CLL patients express mutations or rearrangements of these genes [8]. Though patients with vHC mutations tend to have less malignant cancers, the appearance of mutation is independent of CLL clinical stage. Recently, research has focused on CD38 and Zap-70 as prognostic factors; patients who express both markers tend to have a poor prognosis [9].

Whether the expression of these proteins is predictive of response to therapy is unknown.

Traditionally, CLL has been treated with chlorambucil (Leukeran®), a nitrogen mustard-alkylating agent; however low complete remission (CR) responses in patients have given way to treatment with antimetabolite agents or monoclonal antibodies. Treatment with purine analogs, such as fludarabine (Fludara®), results in higher CR rates (20-40% vs. 10%) and longer disease-free survival [10]. Furthermore, the efficacy of fludarabine is improved upon combination with other agents. For example, the combination of fludarabine, cyclophosphamide (Cytoxan®), and the monoclonal CD20 antibody rituximab (Rituxan®) results in a superior response rate than fludarabine alone and longer patient survival [11].

1.1.2.2. ALL overview and standard of care

The majority of ALL is diagnosed in children, comprising approximately 30% of all childhood malignancies [12]. This disease is sub-divided into B-cell ALL (B-ALL) and T-cell ALL (T-ALL) depending on the cell lineage affected, which is determined by the expression of lineage-specific CD antigens. Genetic underpinnings for these diseases are often stratified; however, common to both B-ALL and T-ALL is rearrangement of the Ig and T-cell receptor (TCR) genes [13]. The presence of these and other genetic mutations, as well as the age of the patient, leukocyte count, and presence or absence of central nervous system or testicular leukemia involvement has a large impact on the treatment regimen. Of note, the presence of the Philadelphia chromosome (Ph+) (discussed in detail in Section 1.1.3.1 *CML Overview and Standard of Care*) is found in approximately 15-20% of

all ALL cases; the incidence increases with age with approximately 3% of children and 25% of adult ALL cases Ph+ [14, 15]. This mutation gives rise to the BCR-Abl oncogene; thus, treatment with BCR-Abl-targeted inhibitors, such as imatinib (Gleevec®), is appropriate.

The overall survival is high (75-80%) for childhood ALL, whereas an overall survival rate of only 40-50% is typically obtained in adults with ALL [16, 17]. Ph+ patients have a poor prognosis and experience a low CR rate; however, the introduction of imatinib as a front-line treatment has dramatically improved the 5-year CR rate (80-90%) [14]. Ph- ALL is first treated with short-term intensive chemotherapy consisting of antimetabolites (cytarabine (Ara-C; Cytosar-U®), methotrexate (Trexall®)), alkylating agents (cyclophosphamide), immunosuppressants (dexamethasone (Decadron®), prednisone (Deltasone®)), and/or cytotoxic agents (vincristine (Oncovin®), anthracyclines) [18]. Specifically, treatment of adult ALL patients with the hyperfractionated cyclophosphamide, vincristine, doxorubicin (Adriamycin®), and dexamethasone (Hyper-CVAD) regimen can obtain a CR rate >80% [19].

1.1.3 Myelogenous leukemia

1.1.3.1. CML overview and standard of care

In contrast to ALL, CML is relatively rare in children (<5% of cases) and has a median age of onset between 55 and 60 years of age with nearly half of CML patients over the age of 60 [20]. The hallmark of CML is the presence of a translocation between chromosomes 9 and 22 (t(9;22)(q34;q11), which creates what

is known as the Philadelphia (Ph) chromosome. This mutation was first described by Peter Nowell in the mid-1960s and is found in approximately 95% of CML patients [21, 22]. This specific translocation gives rise to the BCR-Abl kinase, a fusion of part of the breakpoint cluster region (BCR) with the Abelson (Abl) tyrosine kinase. BCR-Abl is the causative oncogene in Ph+ CML (reviewed in Section 1.3.2 *BCR-Abl kinase*).

If untreated, CML progresses through three phases; in order of severity these are chronic, accelerated, and blast crisis. Individuals in chronic phase may be asymptomatic but have an elevated white blood cell (WBC) count (<15% myeloblasts); most patients first present in this stage. However, eventually the accelerated production of WBCs, including the increased presence of undifferentiated myeloblasts (>30%), occurs as patients transition through the accelerated phase into blast crisis. At this point, the production of normal blood cells and blood-clotting platelets is compromised, typically resulting in mortality due to anemia, infection, uncontrolled bleeding, or other complications. Treatment regimens depend on the phase of disease. A younger patient of reasonable health in the relatively indolent chronic phase typically undergoes allogeneic hematopoietic cell transplant (HCT) within the first year of diagnosis [23]. HCT may be an option for patients with advanced CML; however, the success of HCT treatment decreases with increasing disease severity.

Traditionally, CML has been treated with antimetabolites (cytarabine), interferon, or hydroxyurea (Hydrea®). These regimens result in a complete cytogenetic response (0% Ph+ cells in bone marrow) in approximately 30-35% of

patients; nearly 80% of these patients experienced disease-free survival 10 years after treatment [24, 25]. However, in 2001, the approval of imatinib, a BCR-Abl inhibitor, revolutionized the treatment of CML. Treatment with imatinib produces a major cytogenetic response in approximately 60% of patients and a complete hematologic response (blood platelet cell counts return to normal) in 95% of patients [26]. A study of 553 patients with chronic phase CML treated with continuous imatinib therapy found that the 5-year survival rate was approximately 89% [27]. These superior results have made imatinib the front-line treatment used to treat CML. More recently, second generation BCR-Abl inhibitors, such as nilotinib (Tasinga®) and dasatinib (Sprycel®), have been approved for treatment of newly diagnosed CML and CML patients resistant to imatinib [28-30].

1.1.3.2. AML overview and standard of care

AML is the most common type of leukemia in adults with a median age of diagnosis between 65 and 75 years [12]. This disease has the lowest survival rate of any leukemia and many patients are non-responsive to treatment or relapse after initial therapy [31]. In contrast to CML, AML is a heterogeneous disease with a variety of cytogenetic and molecular alterations. Cytogenetic abnormalities occur in approximately 50-60% of newly diagnosed patients and some genetic rearrangements are indicative of poor prognosis [32]. A common molecular marker is the expression of mutant forms of FMS-like kinase 3 (FLT3). Constitutive activation of the FLT3 tyrosine kinase occurs after point mutation (D835) or gene translocation (FLT3-ITD); these mutations occur in approximately 20-30% of AML patients and are associated with a poor prognosis [33, 34].

FLT3 plays a critical role in governing hematopoiesis and cellular growth. Expression of constitutively active FLT3 has transformative capacity in Ba/F3 B-cells [35]. In addition, FLT3-ITD expression in lymphoid and myeloid progenitor cells in a bone marrow transplant mouse model produced myeloproliferative disease [36]. Ligand-independent cell hyper-proliferation induced by FLT3-ITD is primarily mediated through RAS/MAPK and STAT5 activation [37].

The standard of care for treatment of AML includes a combination of anthracycline and cytarabine, sometimes with the addition of etoposide (Eposin®), cladribine (Leustatin®), and fludarabine. Inhibitors of FLT3, such as PKC412 (midostaurin), are currently in clinical trials and have produced a favorable response when given alone or in combination with the standard of care [38, 39]. Though with only 20-40% of patients responding to initial therapy and nearly 50-70% of those patients experiencing relapse within 3 years, novel therapies for the treatment of AML are needed [40].

1.2. ENTs and drug resistance

1.2.1. ENT overview and functional aspects

Nucleosides are transported across cellular membranes by the equilibrative and concentrative nucleoside transporters (ENT and CNT, respectively). ENTs transport nucleoside substrates bidirectionally across membrane barriers down their concentration gradient, whereas CNTs concentrate substrates in a sodium-dependent manner [41]. The studies herein focus on the regulation of ENT-

dependent transport; therefore, the remainder of this introduction highlights our knowledge of ENT function.

In humans, the ENT family is composed of 4 members (ENT1-4), of which the function of ENT1 and ENT2 is best understood [42]. ENT1 has nearly ubiquitous tissue distribution and transports various purine and pyrimidine nucleosides, with the exception of uracil, in a K_m range of 50 μM (adenosine) to 680 μM (cytidine) [43]. ENT2 is 49% identical (69% similar) to ENT1 and is found in a variety of tissues, though it is primarily expressed in skeletal muscle [44]. Immunohistochemical studies have shown ENT2 expression on the plasma membrane [42]. In addition, other studies suggest that ENT2 and/or ENT2 splice variants function as nuclear transporters [45, 46]. ENT2 transports a broad range of purine and pyrimidine nucleosides with the exception of cytidine and displays a lower affinity to these substrates in comparison to ENT1. In contrast to ENT1, ENT2 can also transport nucleobases, such as hypoxanthine [43, 47]. Of note, uridine is a permeant of both ENT1 and ENT2 with a K_m of 260 μM and 250 μM , respectively [43].

Selective inhibitors can be used to delineate ENT1- and ENT2-mediated transport, as demonstrated by Ward et al. [43]. Nitrobenzylmercaptapurine riboside (NBMPR) is a purine analog and a potent inhibitor of ENT1 activity; its IC_{50} to inhibition of ENT1-mediated uridine transport is 0.4 \pm 0.1 nM, whereas inhibition of ENT2-mediated transport occurs at an IC_{50} of 2.8 \pm 0.3 μM , a nearly 10,000-fold difference in potency. Dipyridamole (Persantine®) is a less selective inhibitor with a difference in IC_{50} for ENT1 and ENT2 of approximately 100-fold (5.0 \pm 0.9 nM vs. 356 \pm 13 nM, respectively).

ENT1 contains 11 transmembrane (TM) α -helices with a cytosolic N-terminus and an extracellular C-terminus [48]. Based on glycosylation scanning mutagenesis, ENT1 is glycosylated on N48, which is located between TM1 and TM2; however, glycosylation is not essential for activity [49]. Using chimeric recombinant proteins of NBMPR-sensitive rat ENT1 and NBMPR-insensitive rat ENT2, it was determined that the binding domains for NBMPR reside within amino acids 100-231 encompassing TM3-6 [50]. In addition, site-directed mutational analysis of ENT1 has uncovered additional residues outside this region that are important for NBMPR binding, including F334, N338, and L92 [51, 52].

Because NBMPR is a direct competitor of nucleoside transport, many regions that are important for NBMPR binding are also contact points for nucleoside binding. For example, site-directed mutagenesis of human ENT1 expressed in *S. cerevisiae* demonstrated that G179 in TM5 was required for uridine transport and sensitivity to NBMPR [53]. In addition, this study suggested that G184 may partially determine targeting of the transporter to the plasma membrane [53]. G154 was also reported to be important for nucleoside transport and sensitivity to the inhibitors NBMPR, dipyridamole, and dilazep [54].

Similar to NBMPR binding, studies suggest that amino acids in TM domains other than 3-6 may also be critical for the transport of nucleoside substrates. Specifically, M33 in TM1 and I429 in the TM11 of human ENT1 and ENT2 and *C. elegans* ENT1 were reported to be required for nucleoside transport and may contribute to the binding of dipyridamole [55]. A report by Paproski et al.

demonstrated that W29 within TM1 was important for inhibitor binding, and ENT1s containing mutations of W29 have altered nucleoside transport kinetics [56].

1.2.2. Regulation of ENT1

ENT expression and activity are regulated at both the transcriptional and post-translational levels. In addition, ENT1 transcription in cancer cells is coordinated with the cell cycle, with an approximate doubling of ENT1 content between the G1 and G2/M phases [57]. In response to lack of oxygen, ENT1 and ENT2 mRNA expression is decreased in a hypoxia inducible factor 1 (HIF1)-dependent manner; this may provide a molecular mechanism to elevate extracellular adenosine, subsequently dilating blood vessels [58]. In addition, functional analysis of the mouse ENT1 promoter revealed regulation of gene expression by the MAZ and Sp-1 transcription factors [59]. Furthermore, research in our lab has uncovered a mechanism by which N-terminal c-Jun kinase (JNK) can regulate ENT expression through the activation of signaling transducer and activator of transcription 3/5 (STAT3/5) proteins [60].

Of particular note, a recent study by Jin et al. demonstrated that ENT1 is regulated at the transcriptional level by FLT3 [61]. Cell lines that express constitutively activated FLT3-ITD down-regulated ENT1 expression and promoter activity and were resistant to cytarabine treatment. This regulation was dependent on HIF-1 and reversal of this phenotype was observed after treatment with the FLT3 inhibitor PKC412 [61].

Additionally, ENT activity may be regulated at the post-translational level. Previous studies have documented protein kinase C (PKC)-dependent enhancement

of ENT1, but not ENT2, activity in various cell lines [62-64]. Though PKC-dependent ENT1 phosphorylation has not been clearly demonstrated, PKC membrane localization is required for ENT1 modulation and bioinformatics analysis reveals potential consensus PKC phosphorylation sites within ENT1 [62, 65] (PROSITE Database, <http://www.expasy.ch/prosite/>). Furthermore, Coe et al. demonstrated that administration of PMA, an activator of PKC, increased the binding affinity between ENT1 and NBMPR without altering the number of NBMPR binding sites, suggesting a PKC-dependent protein modification that may induce a conformation change [63]. In addition to PKC, ENT1 may be positively regulated by casein kinase 2 (CK2) [66]. Treatment with the CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) or deletion of the CK2 consensus phosphorylation site in mouse ENT1 (S254) reduced nucleoside transport activity [66].

1.2.3. Resistance to nucleoside analog treatment

1.2.3.1. Introduction to nucleoside analogs

In addition to natural nucleosides, ENTs transport nucleoside and nucleobase analogs, which are commonly used for the treatment of cancer, particularly leukemia. Once inside the cell, these compounds exert their cytotoxic effects primarily by incorporating into DNA during replication or DNA repair. For example, cytarabine, and gemcitabine (Gemzar®) accumulate in cells in their triphosphate forms, Ara-CTP and dFdCTP, respectively, and compete with CTP for DNA incorporation [67, 68]. In addition, analogs can disrupt specific metabolic pathways to induce cell death. For instance, 5-fluorouracil (5-FU, Aducril®), a nucleobase

antimetabolite, competes with dUMP for thymidylate synthase, resulting in depletion of dTTP and inhibition of DNA replication [69]. Furthermore, the purine nucleoside analog fludarabine directly inhibits ribonucleotide reductase, depleting dNTP pools [70].

Numerous nucleoside analogs are FDA-approved for the treatment of hematological malignancies. Cytarabine is given for induction and maintenance therapy of AML, from which a CR rate of 60-80% can be achieved [40]. Gemcitabine is an analog of cytarabine that is more commonly used against solid tumors, such as pancreatic cancer and metastatic bladder cancer [71, 72]. However, in combination with other agents, gemcitabine effectively treats lymphoid malignancies [73]. Fludarabine and the closely related purine nucleoside prodrug cladribine are used to treat low-grade lymphomas and CLL [74, 75]. The standard of care for leukemia and application of nucleoside analogs is reviewed in Section 1.1 *Introduction to Leukemia*.

1.2.3.2. Nucleoside analog transport and resistance

Studies suggest that the transport of nucleoside analogs is necessary for, or at least greatly increases, the cytotoxic effects of these agents. Mackey et al. demonstrated that the IC_{50} value of gemcitabine-dependent inhibition of cell proliferation was 100-3000-fold higher in cells that lack nucleoside transport in comparison to cells that actively transport gemcitabine [76]. In a study of childhood AML, Hubeek et al. found an inverse correlation between ENT1 mRNA expression and sensitivity to cytarabine, such that low ENT1 levels were associated with cytarabine resistance [77]. ENT1 mRNA expression was also determined to be a

predictor of 5-FU resistance in pancreatic cancer and pre-treatment with NBMPPR increases drug sensitivity [78]. Numerous other clinical and pre-clinical studies have determined a relationship between ENT1 abundance and chemosensitivity [79-87].

In addition, ENT dysfunction has been associated with resistance to nucleoside analogs [88]. Genetic mutation may result in non-functional ENTs or ENTs that have reduced activity. In a cytarabine-resistant cell line, Cai et al. described two independent mutations in ENT1 that resulted in a non-functional protein due to a shortened transcript and a transcript lacking exon 13, both of which were associated with increased drug resistance [89]. In addition, profiling of an ethnically diverse population uncovered ENT1 proteins with variations in the coding region; however, kinetic analysis of these proteins in yeast found no difference in transporter function [90]. Furthermore, single nucleotide polymorphisms (SNPs) in ENT genes may determine individual responsiveness to nucleoside analog treatment [91].

1.3. Kinase signaling in leukemia

1.3.1. Overview of kinase function

Reversible phosphorylation was first uncovered by Ed Krebs and Ed Fischer during the mid-1950s [92]. Their discovery of cAMP-dependent protein kinase (PKA) created a new field of research for the discovery and characterization of kinases. These proteins catalyze the transfer of a negatively charged phosphate (PO_4) group from ATP to specific serine, threonine, and tyrosine residues on protein, oftentimes resulting in an alteration in protein conformation and catalytic activity. Currently, there are 518 known kinases in the human genome with approximately 90 kinases

that phosphorylate tyrosine residues, whereas the large majority of kinases (385) phosphorylate serine and threonine residues [93]. These events are integral to molecular signaling and kinases are involved in every aspect of cellular regulation, including cell growth, proliferation, and apoptosis.

Importantly, kinase dysregulation is a hallmark of many cancers. The non-receptor tyrosine kinase Src was the first cancer oncogene to be characterized, discovered by Bishop and Varmus and colleagues [94]. Since this discovery, many other kinases have been demonstrated, primarily when mutated, to be oncogenes [95]. Furthermore, 244 kinases have been mapped to disease loci or cancer amplicons, providing further evidence for the importance of these enzymes in disease [93].

1.3.2. BCR-Abl kinase

1.3.2.1. Overview and significance to CML

CML is a hematopoietic cancer characterized by the expression of the constitutively active tyrosine kinase, BCR-Abl [96, 97]. BCR-Abl is the protein product of the translocation of the c-Abl gene on chromosome 9 to a specific 5.8-kD region on chromosome 22 termed the breakpoint cluster region (BCR). This abnormality is a hallmark of CML and is present in approximately 95% of CML patients [98]. Expression of BCR-Abl enhances molecular signaling that controls cell division and proliferation, such as Ras [99] and PI3K/Akt [100, 101]. Compelling evidence from mouse models has demonstrated the importance of BCR-Abl in CML. Transduction of mouse bone marrow cells with BCR-Abl is sufficient to induce leukemogenesis, and this technique is employed to model CML [102]. Using an

inducible mouse system, Huettner et al. demonstrated that expression of BCR-Abl developed lethal leukemia, whereas repression of BCR-Abl resulted in complete remission [103]. Thus, BCR-Abl is required for both the induction and maintenance of leukemia.

1.3.2.2. BCR-Abl kinase signaling

BCR-Abl kinase has enhanced activity in comparison to c-Abl [104]. Whereas c-Abl is primarily located in the nucleus, BCR-Abl is distributed throughout the cytoplasm, suggesting that it signals to different substrates [105]. Expression of BCR-Abl has a transformative capacity in fibroblasts and hematopoietic cells in vitro [106] and can induce CML in mice [107]. In addition, BCR-Abl expression activates multiple molecular signaling cascades to enhance cell survival and proliferation, including PI3K/Akt, Ras/MAPK, and JAK/STAT.

A major substrate for BCR-Abl is the adaptor protein CRKL. The phosphorylation of CRKL correlates with BCR-Abl activity in CML patients and is used to measure the status of leukemia development [108]. CRKL forms complexes with BCR-Abl and important cell signaling molecules, including c-Cbl and STAT5 [109, 110]. Disruption of BCR-Abl-CRKL complex formation strongly reduces the proliferation of primary CML cells and BCR-Abl-positive cell lines [111]. Furthermore, over-expression of CRKL in BCR-Abl transgenic mice leads to more rapid leukemia development [112].

An additional consistent regulator of BCR-Abl-dependent signaling is the transcription factor STAT5. RNAi-mediated silencing of STAT5 inhibited cell proliferation and induced apoptosis in leukocytes from CML patients [113].

Expression of dominant-negative STAT5 reduced the induction and maintenance of CML in mice [114]. STAT proteins are a substrate of BCR-Abl kinase; however, studies disagree as to the importance of Janus kinases (JAKs) in the promotion of STAT activity and BCR-Abl-dependent leukogenesis [115, 116].

1.3.2.3. Imatinib and mechanisms of imatinib resistance

Developed initially as a platelet-derived growth factor receptor (PDGFR) inhibitor, imatinib (STI-571) is a selective and potent inhibitor of BCR-Abl and Abl kinase as well as the c-Kit receptor kinase [98]. The therapeutic relevance of imatinib was uncovered when it was observed that imatinib dramatically inhibited the proliferation and tumor formation of BCR-Abl-positive cells [117]. Subsequent in vitro and in vivo testing of this compound [117, 118] resulted in clinical trials to determine imatinib efficacy against CML [119] and the eventual approval of imatinib for the treatment of CML in 2001. Currently, imatinib is the front-line treatment for BCR-Abl-positive CML and ALL.

Imatinib competes for ATP binding to Abl kinase [120]. Specific mutations in the contact residues between imatinib and Abl and residues in the P loop and the kinase activation loop confer resistance to imatinib by preventing drug-induced inhibition [121]. Of note, the T315I mutant form of BCR-Abl is commonly found in imatinib-resistant patients and can confer resistance by both the prevention of inhibitor binding and by increasing kinase activity [122]. Other mechanisms of imatinib resistance include the increased expression of BCR-Abl [123] and drug export proteins (i.e. p-glycoprotein) [124].

An alternative BCR-Abl-independent mechanism of resistance is the up-regulation of compensatory kinase signaling pathways. Burchert et al. demonstrated that activation of the PI3K/Akt/mTor pathway could mediate resistance in cell lines and primary CML cells. In addition, treatment with the mTor inhibitor rapamycin reversed the resistant phenotype [125]. BCR-Abl promotes cell survival through the activation of JAK and subsequent phosphorylation and activation of the STATs [126]. Therefore, it is not surprising that up-regulation of JAK2 expression can confer resistance to imatinib treatment [127].

Of specific importance to this dissertation is the mediation of imatinib resistance by the up-regulation of the Src family kinase (SFK) Lyn. Previous studies demonstrated that up-regulation and hyper-activation of Lyn kinase (Lyn) confers imatinib resistance in imatinib-resistant CML cell lines [128-130] and primary cells from patients that were non-responsive to imatinib [131]. The mechanism by which Lyn confers drug resistance has not been fully elucidated; however, this may involve the activation of GRB2-associated binder 2 (Gab2) and de-stabilization of c-Cbl [132]. The Lyn-dependent increase in the expression of the Bcl-2 family of proteins has also been attributed to a drug-resistant phenotype [128].

1.3.2.4. Second generation BCR-Abl kinase inhibitors

A minority of chronic phase CML patients is refractory to imatinib treatment or display secondary resistance to imatinib after therapy. However, as much as 75% of patients with advanced disease (accelerated phase, blast crisis) are refractory to treatment or relapse after initial imatinib therapy [133]. Therefore, second-line BCR-Abl inhibitors (i.e. nilotinib, dasatinib) have been developed and are currently

approved for treatment of imatinib non-responsive CML patients [134]. These inhibitors have affinity to the BCR-Abl kinase in addition to SFKs. The hematopoietic SFKs Hck and Lyn are important mediators of cell survival and are often up-regulated after imatinib treatment [135]. Moreover, SFK molecular signaling overlaps with that of BCR-Abl (i.e. STAT, CRKL). Thus, the efficacy of second line inhibitors is believed to be due to inhibition of BCR-Abl compensatory signaling.

1.3.3. Lyn kinase in CML/AML

1.3.3.1. Overview of Lyn kinase function

Lyn is a SFK member and a non-receptor tyrosine kinase. Two splice isoforms of this kinase exist, which differ by the excision of 21 amino acids in the N-terminal SH4 domain (Lyn A – p56, Lyn B – p53; Yi et al., 1991). Currently, no functional significance has been attributed to Lyn splice variation; however, the N-terminal region is important for kinase localization [136] and this splice site is in proximity to sites of lipid post-translational modification, implying splice variant-specific localization [137]. In addition, regions within the Lyn kinase domain and N-terminal SH4 domain are necessary for localization to Golgi membranes [138], and inhibition of kinase activity increases Lyn nuclear localization [139].

Similar to Src kinase, Lyn activity is regulated in a phosphorylation-dependent manner. Lyn activity is inhibited by phosphorylation of a conserved tyrosine in the extreme C-terminal tail (Y508 – Lyn; Y527 – Src). Phosphorylation of this residue by C-terminal Src kinase (CSK) induces an inactive conformation, during which the SH2 domain interacts with the phosphorylated tyrosine residue [140, 141]. Upon dephosphorylation of Y508, Lyn is trans-autophosphorylated on residue Y397 (Y416

– Src) within the kinase domain, inducing an activating conformational change. Kinase activity is attributed to phosphorylation of this tyrosine residue. However, in contrast to similar deletions in Src, loss of the SH2 and SH3 domains decreased Lyn activity, implying individual differences in the autoregulation of Lyn in comparison to Src [142].

Lyn is exclusively expressed in hematopoietic cells, particularly in cells of the myeloid lineage and B-cell lymphocytes [143, 144]. Lyn functions as a catalytic transducer of the B-cell receptor complex (BCR) and erythropoietin receptor (EpoR) to promote B-cell development [145] and erythrocyte differentiation [146, 147], respectively. In addition, stimulation of c-Kit, a cell surface receptor similar to PDGFR, leads to activation of Lyn, a result necessary for granulocyte colony stimulating factor (G-CSF)-induced cell proliferation in hematopoietic cells [148, 149]. Thus, Lyn plays an active role in the promotion of hematopoietic cell survival.

1.3.3.2. Lyn and drug resistance

In myeloid cells, Lyn can both positively and negatively affect cell signaling. Studies using Lyn^{-/-} mice suggest that Lyn is an inhibitor of normal myeloid cell development; these mice have enhanced myeloproliferation and a large number of cells with a blast-like phenotype [150]. However, the contribution of secondary inflammatory responses to the hyper-proliferation of these cells cannot be discounted.

In contrast, numerous studies have suggested that myeloid leukemias depend on Lyn activity. AML cell lines and primary progenitor cells display a high level of active Lyn [151]. Lyn associates with FLT3, a kinase that is commonly

mutated in AML, to mediate constitutive activation of STAT5 [152]. Treatment with PP2, a SFK inhibitor, prevented tumor formation and tumor growth in mice transplanted with AML cells expressing constitutively active FLT3 [152]. Moreover, Lyn is involved in many aspects of CML cell survival [131, 153].

Lyn over-expression and hyper-activation has been implicated in drug resistance in CML. Over-expression of Lyn in hematopoietic cells contributes to cell proliferation and drug resistance through increased STAT5 phosphorylation, Bcl-2 expression, and other pro-survival responses [128, 129, 154]. Inhibition or knockdown of Lyn reduced pro-survival signaling and increased imatinib sensitivity in CML cells [130, 154]. Of note, therapeutic targeting of Lyn using dual SFK and BCR-Abl inhibitors, such as dasatinib, leads to remission in patients that are non-responsive to imatinib [155]. Currently, dasatinib and other dual BCR-Abl/SFK inhibitors are FDA-approved for the treatment of imatinib-resistant CML.

1.4. MicroRNAs and leukemia

1.4.1. MicroRNA biogenesis

MicroRNAs (miRNAs) are small (19-24 nt) non-coding RNA molecules that are key regulators of protein expression through their targeted binding to specific mRNAs. Since the first description of this RNA class in *C. elegans* [156], identification of numerous miRNAs has been made in both plant and animal phyla. There are estimated to be as many as 1000 miRNAs encoded by the human genome and approximately half of these have been cloned and confirmed.

The majority of miRNAs are transcribed by RNA polymerase II as a pri-miRNA precursor molecule (~1000-3000 bp) (Fig. 1.2). These transcripts are

primarily located within introns of either protein-coding or non-protein-coding transcripts; however, some are found within the exons of non-protein-coding transcripts, the 3' UTR of mRNA, or isolated regions of the genome. In the nucleus, the RNAase III-like enzyme Drosha processes the pri-miRNA into one or more pre-miRNAs (~70 bp). These pre-miRNA molecules take on a stem-loop-stem structure and are exported out of the nucleus by a mechanism involving exportin-5. The pre-miRNAs are subsequently processed by Dicer RNAse III. The resulting mature miRNA product (~22 nt) forms a RNA duplex with target mRNAs in the RNA-induced silencing complex (RISC), triggering the degradation of the mRNA transcript, which requires complete complementarity between the miRNA and mRNA sequence, or the direct inhibition of protein translation [157]. It has been suggested that a single miRNA targets multiple mRNA transcripts and that a given mRNA has binding sites for multiple miRNAs. Thus, miRNAs may regulate protein expression in a complex manner.

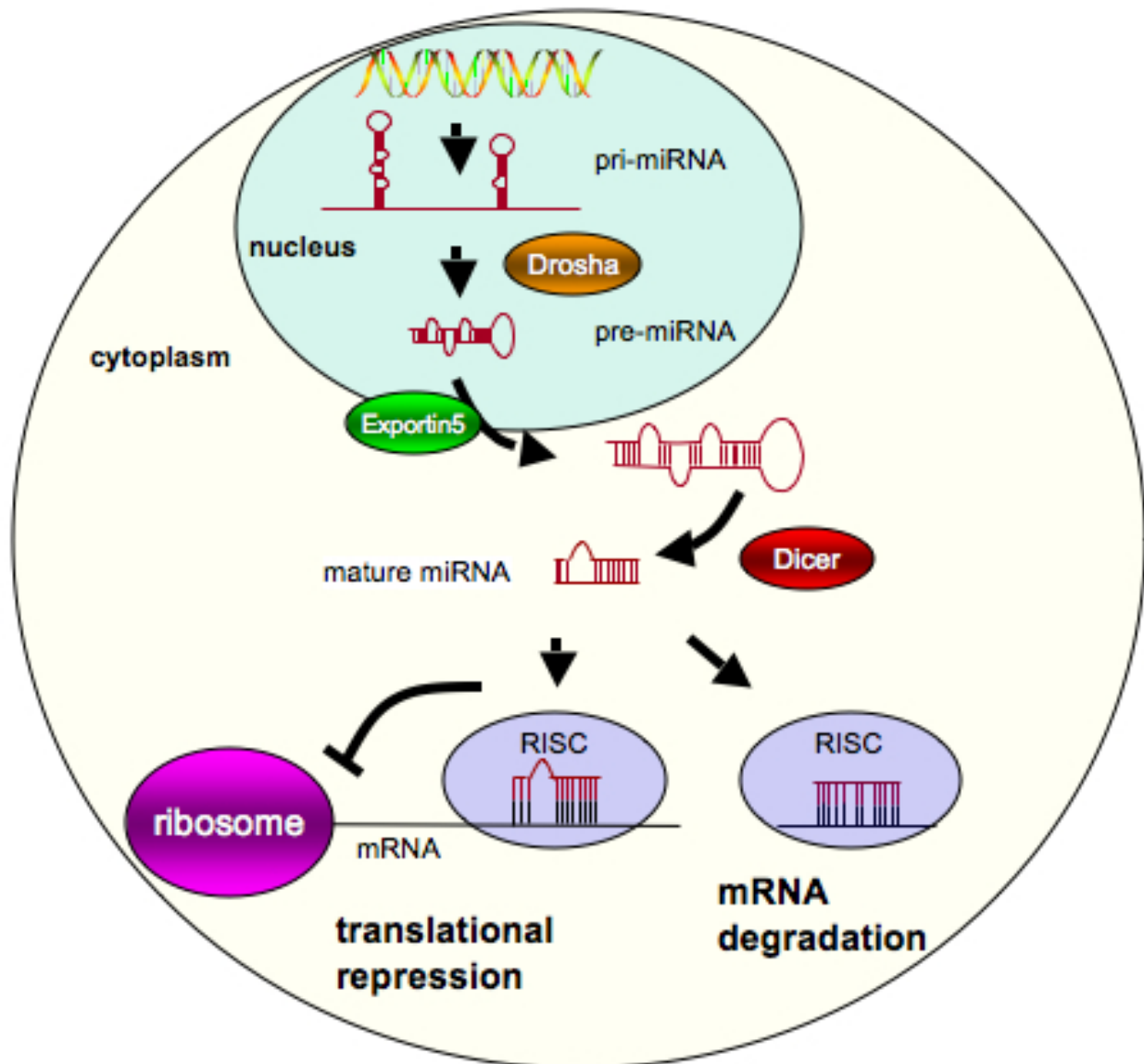


Figure 1.2. MicroRNA biogenesis. MicroRNAs are initially transcribed as pri-miRNA transcripts and are subsequently processed into pre-miRNA and mature miRNA by Drosha and Dicer, respectively. Mature miRNA associate with the RISC complex and target and bind mRNA to induce translational repression or mRNA degradation.

1.4.2. MicroRNA regulation

MiRNAs may be transcribed singly or in clusters from both intragenic (within the exon or intron of a transcribed RNA) and intergenic (between transcribed RNAs) regions of the genome. Transcriptional regulation can be imposed by epigenetic (methylation, acetylation, etc.) factors. For example, Agirre et al. recently demonstrated that hyper-methylation of the promoter region of miR124a increased the expression of CDK6 and contributed to enhanced cell proliferation in ALL [158].

Transcription factors, such as CEBP α , are also important regulators of miRNA expression. CEBP α is a basic region leucine zipper transcription factor that is essential for myeloid cell differentiation and growth control and is commonly inactivated in AML by mutation or post-transcriptional modification [159-161]. CEBP α directly regulates the expression of several miRNAs, such as miR661 [162] and miR223 [163]. Furthermore, distinct miRNA signatures in AML have been attributed to the status of CEBP α activity; in particular, the up-regulation of miR181 family is associated with CEBP α mutation [164]. These studies of transcription factor and miRNA expression have become increasingly important for the diagnosis of leukemias.

On the post-transcriptional level, miRNAs are regulated by proteins that affect miRNA processing. Seminal work by the laboratories of Richard Gregory and Scott Hammond has elucidated the role of the RNA-binding protein Lin28 in the regulation of the let-7 miRNA family. Lin28 specifically interacts with the precursor let-7 molecule to inhibit further processing by Dicer into the mature miRNA species [165, 166]. This regulation is observed in a developmentally timed manner, and increased

Lin28 expression and loss of let-7 is a marker of cellular differentiation. Furthermore, Lin28 is a Src kinase-regulated protein [167, 168] and aberrant expression of Lin28 has been attributed to cancer progression and Hmga2 and Ras oncogene expression.

1.4.3. MicroRNAs and leukemia

The consequences of dysregulated miRNA expression can be severe, resulting in developmental defects or cancer. MiRNAs may act as oncogenes to promote cancer progression. For instance, the miR17-92 locus encodes a cluster of 7 microRNAs that can enhance c-Myc activity and accelerate tumor development [158]. Elevated expression of this cluster is associated with an aggressive cancer phenotype in hepatocellular carcinoma [169] and lung cancer [170].

In addition, the loss of tumor suppressor miRNAs can enhance cell proliferation and cancer progression. For example, miR29a suppresses the expression of pro-survival genes including Myeloid Cell Leukemia-1 (Mcl-1) and inhibition of this miRNA using antisense antagomirs sensitizes cells to drug-induced cell death [171]. In addition, expression of miR29b in AML cell lines and primary AML cells reduces cell growth and promotes apoptosis [172]. The regulation of Mcl-1 expression by miRNAs is an important determinant of drug sensitivity and cell survival. Other tumor suppressor miRNAs that target Mcl-1 include miR320 and miR133b [173, 174].

Another putative tumor suppressor miRNA that has been implicated in AML is miR181. This miRNA family (miR181a-d) is preferentially expressed in hematopoietic cells and its expression can modulate cell differentiation [175].

Studies of AML patients have determined that the loss of miR181 is an indicator of poor disease prognosis and is associated with a reduced survival rate compared to patients with high miR181 expression [164, 176, 177]. This microRNA family is suggested to target Bcl-2 [178, 179], and over-expression of miR181a sensitizes glioma cells to ionizing radiation [178].

1.5. Bcl-2 family proteins

1.5.1. Overview of the Bcl-2 family

The B cell CLL/lymphoma-2 (Bcl-2) family of proteins is composed of both pro-survival and pro-death members that dictate the integrity of the outer mitochondrial membrane (OMM) (Fig. 1.3). The anti-apoptotic Bcl-2 proteins contain four Bcl-2 homology (BH1-4) domains and the major members are Bcl-2, Mcl-1, A1, Bcl-x, and Bcl-w. These proteins directly bind to Bcl-2 antagonist killer 1 (Bak) and Bcl-2 associated x protein (Bax). Bax/Bak comprise one branch of pro-apoptotic Bcl-2 proteins and, upon homo-oligomerization, form pores within the OMM to promote permeabilization. After permeabilization, soluble proteins, such as cytochrome c, diffuse into the cytosol and activate apoptotic protease activating factor-1 (APAF-1) and subsequent caspase activation. Thus, blockade of Bax/Bak oligomerization prevents cellular apoptosis.

The second branch of pro-apoptotic Bcl-2 proteins are termed “BH3-only” proteins and consist of Bcl-2 antagonist of cell death (Bad), Noxa, Bcl-2-interacting domain death agonist (Bid), and Bcl-2-interacting mediator of cell death (Bim). As the name suggests, these proteins do not contain any of the domains found in other family members, with the exception of the BH3 domain. BH3-only proteins bind to

and inhibit anti-apoptotic Bcl-2 family members, thereby promoting apoptosis. In addition, Bid and Bim can directly affect Bax and Bak oligomerization to induce cell death [180].

Dysregulation of Bcl-2 protein expression can influence cell survival and is often a barrier to cancer therapy. Bcl-2^{-/-} mice are embryotically viable yet undergo massive apoptosis in the thymus and spleen subsequent to birth [181]. Upon over-expression, Bcl-2 suppresses apoptosis induced by a variety of agents both in vitro and in vivo [182, 183]. Furthermore, over-expression of pro-survival Bcl-2 proteins in transgenic mice results in malignancies [184, 185].

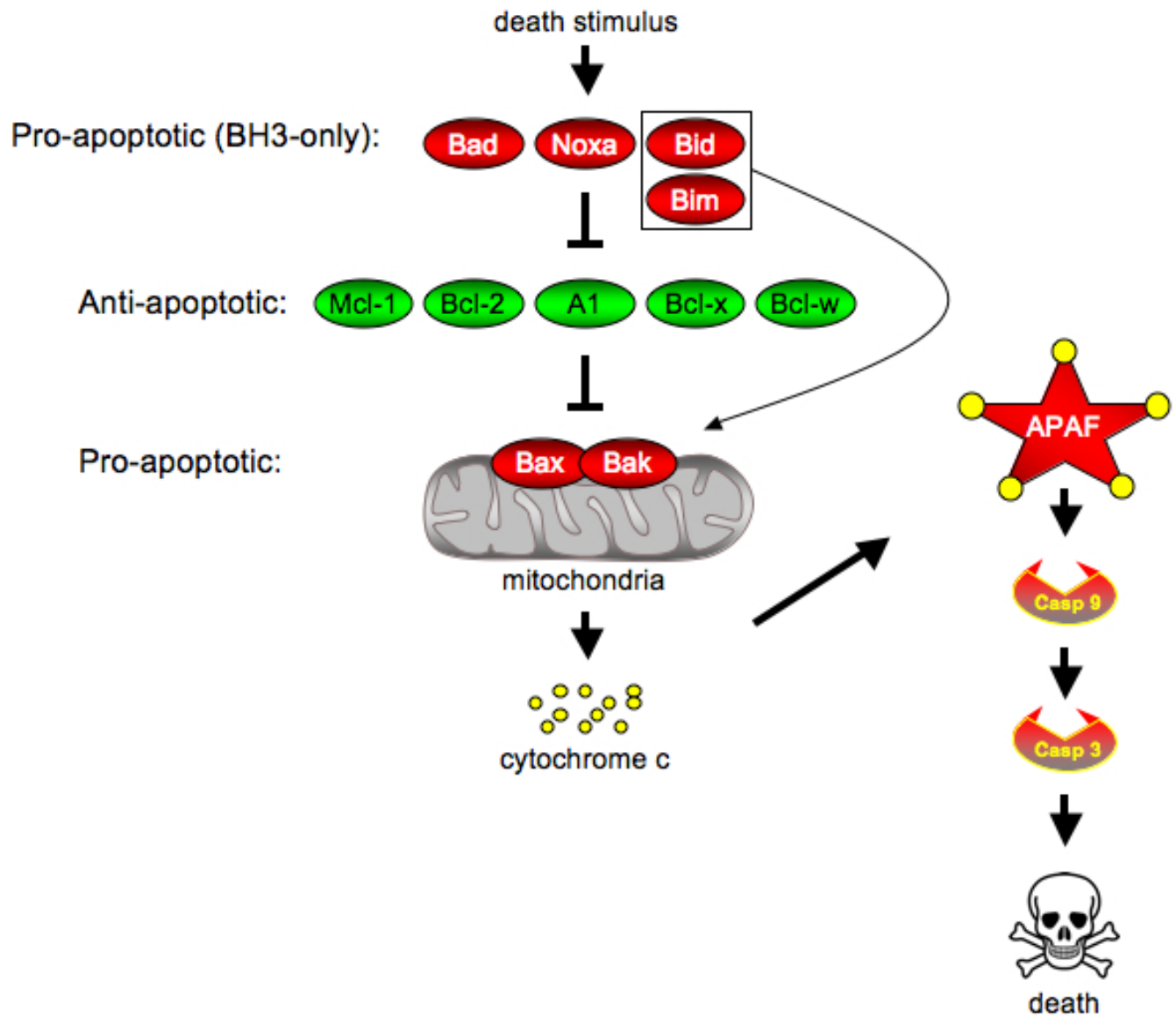


Figure 1.3. Regulation of mitochondria-mediated apoptosis by the Bcl-2 family of proteins. Schematic depiction of mitochondria-mediated apoptosis and the components affected by pro- and anti-apoptotic Bcl-2 proteins (APAF, apoptotic protease activating factor; casp, caspase).

1.5.2. Unique aspects of Mcl-1

Mcl-1 is a pro-survival Bcl-2 protein that is necessary for embryonic development as well as the survival of hematopoietic stem cells [186, 187]. In contrast to other pro-survival Bcl-2 family members, Mcl-1 does not contain a BH4 domain in its N-terminus [188]. Rather, Mcl-1 contains two PEST sequences that have been shown to be important for mitochondrial localization [189]. In addition, this region contains JNK phosphorylation sites (S121, T163) that, upon oxidative stress-induced phosphorylation, inhibit the anti-apoptotic function of Mcl-1 [190]. Phosphorylation of T163 is dynamic in that ERK-dependent phosphorylation in response to differentiation factor stimulation slows Mcl-1 protein turnover [191].

Mcl-1 is also regulated at the post-transcriptional level by microRNAs (see Section 1.4.3. *MicroRNAs and leukemia*) and alternative splicing. Splicing of exon 2 produces Mcl-1s, which lacks the BH1, BH2, and BH4 domains. This variant is unable to sequester Bax and Bak, and binds to and inhibits full-length Mcl-1. Thus, splicing of Mcl-1 generates a pro-apoptotic protein [192]. This natural variation may be exploited by the use of splice-switching oligonucleotides, similar to that demonstrated with Bcl-x [193].

Mcl-1 is a high-turnover protein with a constitutive half-life of approximately 40 minutes [194]. Mcl-1 is ubiquitinated and directed for degradation by the MULE/LASU1 E3 ligase [195]. Interestingly, this ligase contains a BH3 domain that is similar to the Bak BH3 domain, which allows for specific targeting of Mcl-1 [196]. As stated above, phosphorylation can affect the E3 ligase-dependent degradation of Mcl-1.

1.5.3. Bcl-2 family proteins and drug resistance

Pro-survival Bcl-2 family members are important regulators of mitochondrial membrane integrity and cytochrome c release and are often modulated by oncogenic proteins, such as BCR-Abl and FLT3-ITD [197, 198]. These proteins have been implicated in the promotion of drug resistance to a variety of chemotherapeutic drugs, including alkylating agents [199], topoisomerase inhibitors [200], antimetabolite drugs [201, 202], anticancer monoclonal antibody therapy [203], and signal transduction inhibitors [204], as well as ionizing radiation [205]. Of note, up-regulation of Bcl-2 and Mcl-1 expression has been observed in cell models of imatinib-resistant CML [128, 206], and compounds that effectively inhibit imatinib-resistant CML cell survival down-regulate Mcl-1 expression [207-209]. Furthermore, co-treatment of cells with siRNA against Bcl-2 members sensitizes cells to drug treatment [210-212].

These observations provide rationale for the development of Bcl-2 inhibitors for the treatment of cancer, such as ABT-737, which has received significant attention due to its dramatic effects on cancer cell apoptosis. This small molecule is a BH-3 mimetic and targets the hydrophobic groove in pro-survival Bcl-2 proteins produced by BH domains 1-4 [213]. ABT-737 treatment induces cell death and sensitizes cells to drug treatment [213, 214]. However, due to the lack of BH4 domain, Mcl-1 is not targeted by ABT-737; expression of Mcl-1 confers resistance to drug treatment [215], and down-regulation of Mcl-1 increases drug-induced cytotoxic effects [216]. Thus, novel Mcl-1 targeted therapies would be beneficial as an adjuvant to ABT-737 treatment.

1.6. Dissertation Summary

As stated above, drug resistance to anti-cancer chemotherapy is a significant barrier to the treatment of leukemia patients. Prolonged exposure to drug may induce alterations in drug transport proteins or adaptations in molecular signaling through alteration of kinase activity or miRNA expression. The overall goal of my dissertation was to identify the molecular mechanisms of drug resistance in order to provide substrates for the second-line chemotherapy as well as biomarkers for the identification of drug-resistant cancer.

The remainder of this dissertation is divided into 5 chapters. In chapter III, “Identification of a Novel Point Mutation in ENT1 that Confers Resistance to Ara-C in Human T-cell Leukemia CCRF-CEM Cells”, I describe that loss of ENT1 function due to a point mutation at G24 may explain Ara-C resistance in a cell model of ALL. Chapter IV, “Lyn Kinase-dependent Regulation of miR181 and Mcl-1 Expression: Implications for Drug Resistance in Myelogenous Leukemia”, focuses on a cell model of imatinib-resistant CML. In this chapter, I elucidate a novel signaling axis by which Lyn hyper-activation increases Mcl-1 expression through the repression of miR181 miRNAs. In the next chapter, “Elucidating the Mechanism(s) of miR181 Transcriptional Regulation”, I extend these findings in an attempt to determine the mechanism by which Lyn regulates miR181 expression. Finally, in Chapter VI, “Conclusions and Future Directions”, I discuss the broad impact of these findings and propose future experiments for the study of Lyn and miR181 in myelogenous leukemia.

CHAPTER II

Materials and Methods

2.1. Chapter 3 materials and methods

2.1.1. Cell culture and reagents

The nucleoside transport-deficient swine epithelial cell line PK15-NTD (PK15) was generously provided by Dr. Chung-Ming Tse (School of Medicine, Johns Hopkins University) and was maintained as described previously [43]. The CCRF-CEM cell line was obtained from the ATCC (Rockville, MD.) and the CCRF-CEM Ara-C/8C cell line, an Ara-C resistant cell line derived from the CCRF-CEM cell line, was kindly provided by Dr. Buddy Ullman (Dept. of Biochemistry and Molecular Biology, Oregon Health Sciences University). CCRF-CEM and Ara-C/8C cells were maintained as described previously [217]. NBMPR (6-[(4-nitrobenzyl) thio]-9-(β -d-ribofuranosyl) purine) was purchased from Sigma-Aldrich (St. Louis, MO). [^3H]-NBMPR (specific activity: 22.0 Ci/mmol), [5, 6- ^3H]-Uridine (specific activity: 35-50 Ci/mmol), and [5- ^3H]-Ara-C (cytosine- β -d-arabinofuranoside, specific activity: 15-30 Ci/mmol) were obtained from Moravsek Biochemicals (Brea, CA).

2.1.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from CCRF-CEM and Ara-C/8C cells using Trizol® reagent (Invitrogen; Carlsbad, CA) following the manufacturer's instruction. Two micrograms of total RNA were used for first-strand cDNA synthesis using oligo (dT) as primers (Invitrogen) and SuperScript II RNase H⁻ Reverse Transcriptase (SuperScript™ First-Strand Synthesis System for RT-PCR; Invitrogen); the manufacturer's instructions were followed.

2.1.3. Non-quantitative RT-PCR and DNA sequencing

One-tenth of the resulting first-strand cDNA was then used for PCR amplification with High Fidelity pfu DNA polymerase (Invitrogen) following the manufacturer's instructions. The primers for amplifying the entire coding sequence of hENT1 were 5'-CCGCTCGAGATGACAACCAGTCACCTCAG-3' (sense primer) and 5'-AGACTCGAGTCACACAATTGCCCGGAACAGG-3' (antisense primer). Products were visualized on an ethidium bromide-stained agarose gel. PCR products were cloned into the TOPO blunt cloning vector (Invitrogen) and expressed in DH5α *E. Coli* (Invitrogen). Plasmids were submitted for automatic sequencing (UNC-CH Genome Analysis Facility, <http://152.19.68.152/gafsite/Main.asp>).

2.1.4. Construction of expression vectors and cell transfection

Human ENT1 cDNA was subcloned into the pcDNA 3.1 HisC expression vector (Invitrogen). Construction of the G24 ENT1 mutant expression vectors was performed using the Quickchange Site-directed Mutagenesis kit (Stratagene; Cedar Creek, TX) with primers specific to ENT1; the G24R sense primer (5'-CTTCATGCTGGGTCTGAGAACGCTGCTCCCGTGG-3') and antisense primer (5'-CCACGGGAGCAGCGTTCTCAGACCCAGCATGAAG-3'), G24A sense primer (5'-CTTCATGCTGGGTCTGGCAACGCTGCTCCCGTGG-3') and antisense primer (5'-CCACGGGAGCAGCGTTGCCAGACCCAGCATGAAG-3'), and G24E sense primer (5'-CTTCATGCTGGGTCTGGAAACGCTGCTCCCGTGG-3') and antisense primer (5'-CCACGGGAGCAGCGTTTCCAGACCCAGCATGAAG-3') each confer a missense mutation at base pair 72. Wild-type (wt) and G24R ENT1 constructs were subcloned into the EGFP-C3 expression vector (Clontech; Mountain View, CA) for

confocal microscopy. Expression vectors were introduced into PK15 or HeLa cells by Lipofectamine™ transfection (Invitrogen) according to the manufacturer's instructions.

2.1.5. [³H]-Uridine and [³H]-Ara-C uptake assays

The uptake of [³H]-uridine was measured in CEM cell lines or transfected PK15 cells exactly as described previously [218]. The same method was used to measure [³H]-Ara-C uptake with the substitution of cold cytidine for uridine used to stop the reaction. The protein concentration of each sample was quantified using Bradford reagent (Pierce; Rockford, IL).

2.1.6. Confocal microscopy

pEGFP-C3 constructs were transfected into HeLa cells using Lipofectamine™ reagent (Invitrogen) according to the manufacturer's instructions. This cell type was used to obtain high transfection efficiency and ease for microscopy. Twenty-four hr after transfection, images were captured using a 63X oil immersion objective on a Zeiss LSM 510 Meta confocal microscope (Thornwood, NY). Image capture was resolved using a 488 nm argon laser.

2.1.7. Isolation of membranes and [³H]-NBMPR binding assay

Crude cell membranes from CCRF-CEM cells were prepared as described previously with minor modification [219]. Briefly, approximately 5×10^7 cells were washed 3 times with room temperature (r.t.) phosphate-buffered saline (PBS), suspended in 500 μ l of ice-cold 5 mM Na₂HPO₄ buffer (pH 8.0), and sonicated to completely lyse the cells. After a 30-min incubation on ice, the cell membranes were

washed twice (40 000xg centrifugation for 40 min) with ice-cold 5 mM Na₂HPO₄ buffer and suspended in 200 µl of the ice-cold 5 mM Na₂HPO₄ buffer. Binding assays were performed at r.t. in 10 mM Tris (pH 7.1) containing 0.01% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (w/v) to prevent non-specific binding of the radioligand. Incubations were initiated by adding an aliquot of 500 µg cell membrane to r.t. 5 mM Na₂HPO₄ (final volume = 1 ml) containing 10 nM [³H]-NBMPR and were terminated after 45 min by dilution with 5 ml of ice-cold 10 mM Tris (pH 7.1) followed by rapid filtration through Whatman GF/B filters (Piscataway, NJ), which were then washed once with 5 ml of ice-cold 10 mM Tris (pH 7.1). Radioactivity was measured by liquid scintillation using Scintisafe™ Econo-2 scintillation fluid (Fisher Scientific; Pittsburgh, PA) on a LS6500 Multi-purpose Scintillation Counter (Beckman Coulter; Fullerton, CA). Non-specific binding of [³H]-NBMPR was determined in the presence of 20 µM unlabelled NBMPR.

2.1.8. Statistics

Data were analyzed using ANOVA and t-tests where appropriate using Prism 4 software (Graphpad Software, La Jolla, CA).

2.2. Chapter 3 addendum materials and methods

2.2.1. Cell culture and reagents

MYL and MYL-R human CML cells were a generous gift from Dr. Hideo Tanaka (Dept. of Haematology and Oncology, Hiroshima University, Hiroshima, Japan). Cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals; Norcross, GA) and 1%

antibiotic/antimycotic (Invitrogen). The imatinib-resistant cells (MYL-R, K562R) were not continuously cultured in the presence of imatinib; however, imatinib resistance and Lyn kinase over-expression were routinely checked and were found to be stably maintained. Cell cultures were maintained and incubated with drug at 37°C in a 5% CO₂ humidified atmosphere. Imatinib was purchased from LC Laboratories (Woburn, MA). [³H]-NBMPR (specific activity: 22.0 Ci/mmol), [5, 6-³H]-Uridine (specific activity: 35–50 Ci/mmol), and [5-³H]-Ara-C (specific activity: 15–30 Ci/mmol) were obtained from Moravsek Biochemicals. All other reagents were purchased from Sigma-Aldrich unless otherwise indicated.

2.2.2. Cell viability assay

MYL and MYL-R cells were plated at 1.2×10^4 cells/well in 96-well plates. Vehicle or drug was added to wells in triplicate at the indicated concentrations and cells were incubated for 48 hr. After incubation, the MTS assay was performed according to the manufacturer's protocol (CellTiter 96®AQ_{ueous} One Solution Reagent, Promega; Madison, WI). Absorbance was read at 490 nm on a SpectraMAX plate reader (Molecular Devices; Sunnyvale, CA).

2.2.3. Caspase 3 activity assay

To determine caspase 3 activity, cells (6×10^4 /well) were incubated with drug or vehicle at the indicated concentration in a 96-well plate. Plates were then centrifuged for 5 min to pellet the cells. Cells were lysed on ice with buffer containing 250 mM HEPES (pH 7.4), 25 mM CHAPS, and 25 mM dithiothreitol. The non-ionic detergent disrupts the plasma membrane and releases the cytosolic proteins without

denaturing the caspase protein. The activity of caspase 3 in these samples was determined using the Caspase 3 Fluorimetric Assay Kit (CASP3F-1KT, Sigma-Aldrich) according to the manufacturer's instructions. Fluorescence was measured using a FLUOstar Galaxy plate reader (BMG Labtech; Durham, NC) with a 360 nm excitation filter and a 460 nm emission filter.

2.2.4. [³H]-Uridine and [³H]-Ara-C uptake assays

Transport assays were performed as described in Section 2.1.5. *[³H]-Uridine and [³H]-Ara-C uptake assays*. 3×10^5 cells were used for each sample.

2.2.5. Isolation of membranes and [³H]-NBMPR binding assay

[³H]-NBMPR binding assays were performed as described in Section 2.1.7. *Isolation of membranes and [³H]-NBMPR binding assay*. 6×10^5 cells were used for each sample.

2.2.6. Quantitative RT-PCR

ENT mRNA was quantified by qRT-PCR performed on a 7500 Fast Real-Time PCR System (Applied Biosystems; Foster City, CA) according to the manufacturer's instructions. The PCR reaction mixture contained 900 nM each of the sense primer (5'-CTGGCTTTCTCTGTCTGCTTCA-3') and antisense primer (5'-CTCAACAGTCACGGCTGGAA-3') for ENT1 or the sense primer (5'-ATGAGAACGGGATTCCCAGTAG-3') and the antisense primer (5'-TCCTTCTCCAGGTCAAGATCCA-3') for ENT2 or the sense primer (5'-TTGTTACAGGAAGTCCCTTGCC-3') and antisense primer (5'-ATGCTATCACCTCCCCTGTGTG-3') for β actin. One hundred micrograms of cDNA,

as well as the SYBR Green PCR master mix (Applied Biosystems), were also added to the reaction mixture. After an initial 10-min incubation at 95.0°C, cDNA was amplified by 40 cycles of 95.0°C for 15 seconds and 60°C for 1 minute. Transcript levels and expression fold changes between treatments were determined as previously described [220].

2.2.7. Statistics

Data were analyzed using ANOVA and t-tests where appropriate using Prism 4 software (Graphpad Software).

2.3. Chapter 4 materials and methods

2.3.1. Cell culture and reagents

MYL and MYL-R human CML cells were obtained and cultured as described in Section 2.2.1. *Cell culture and reagents*. K562-R cells were obtained from Dr. Steven Grant (Massey Cancer Center, Virginia Commonwealth University; K562-R(1)). A separate isolate of imatinib-resistant K562 cells were kindly provided by Dr. Nicholas Donato (Dept. of Internal Medicine, University of Michigan; K562-R(2)). HL-60 cells were obtained from the UNC Tissue Culture Facility. Cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% antibiotic/antimycotic (Invitrogen). The imatinib-resistant cells (MYL-R, K562R) were not continuously cultured in the presence of imatinib; however, imatinib resistance and Lyn kinase over-expression were routinely checked and were found to be stably maintained. HEK293T cells were a generous gift from Dr. Channing Der (Dept. of Pharmacology, UNC) and were maintained in

DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. Cell cultures were maintained and incubated with drug at 37°C in a 5% CO₂ humidified atmosphere. Imatinib and dasatinib were purchased from LC Laboratories (Woburn, MA). SMARTpool siRNA was purchased from Thermo Scientific (Lafayette, CO). 1,25-dihydroxyvitamin D₃ (1,25-D₃) was purchased from Enzo Life Sciences International (Plymouth Meeting, PA).

2.3.2. Nucleofection

Plasmids and siRNA were incorporated into cells using the Amaxa nucleofection system (Walkersville, MD; >90% transfection efficiency with >90% cell viability, data not shown). Briefly, 1.0-1.5x10⁶ cells were washed with PBS and re-suspended in nucleofection solution (Mirus Bio LLC; Madison, WI). Cells were nucleofected using the T-16 nucleofector program and added to 4.5 ml of nutrient-rich media. Cells were cultured in a 5% CO₂ humidified atmosphere at 37°C for 24 hr before experimentation.

2.3.3. Plasmid constructs and site-directed mutagenesis

The mature human miR181b sequence was cloned into the SDSA 3.0 expression plasmid using the BglII and XhoI restriction sites. After incorporation into cells, this plasmid expresses miR181b concomitantly with GFP. The Mcl-1 3' UTR luciferase expression construct was a generous gift from Dr. Serge Nana-Sinkam (Div. of Pulmonary, Allergy, Critical Care and Sleep Medicine, Ohio State University). This is a psicheck-2 plasmid vector that incorporates the 3' UTR of Mcl-1 down-stream of the luciferase gene [174]. Site-directed mutagenesis of the miR181b

binding site was performed using sense (5'-CCATTTAAAAATAGGTATGAATAAGATGACTAAGATACTAATGGGGAAGAACTGCCCTG-3') and antisense (5'-CAGGGCAGTTCTTCCCCATTAGTATCTTAGTCATCTTATTCATACCTATTTTTAAATGG-3') primers. The reaction mix included 1-ng template DNA, dNTPs, and forward and reverse primers. Touchdown PCR was performed on a thermocycler (Eppendorf; Hauppauge, NY); after an initial incubation at 95°C for 2 min, the following steps were cycled 18 times: 95°C for 50 sec, 70°C->55°C (each cycle was performed with a temperature 1 degree lower) for 50 sec, and 72°C for 14 min. The PCR product was treated with Dpn1, and DH5 α *E. Coli* (Invitrogen) were transformed according to the manufacturer's instructions. Mutation was confirmed by sequencing (UNC Genome Analysis Facility).

2.3.4. Caspase 3 activity assay

Caspase 3 activity was determined as described in Section 2.2.3. *Caspase 3 activity assay.*

2.3.5. Quantitative RT-PCR-based miRNA expression profiling

Total RNA was extracted from 40.0x10⁶ MYL and MYL-R cells using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. MiRNA enrichment was then performed using the Small RNA Isolation Kit (SABiosciences; Frederick, MD), and the miRNA purity was assessed using an Agilent Bioanalyzer Chip (UNC Genomics and Bioinformatics Core). Finally, 100 ng of miRNA was 3'

polyadenylated and converted to cDNA using the First Stand Synthesis Kit (SABiosciences) according to the manufacturer's instructions.

Approximately 100 ng of cDNA was added to the Human Cancer RT² miRNA qPCR Array (#MAH102C; SABiosciences; ~1.0 ng/well), and qRT-PCR was performed on an Applied Biosystems 7500 Fast Real-Time Thermocycler according to the manufacturer's instructions. Melting curve analysis of the resulting transcript was determined. Analysis of the qRT-PCR data was performed using the RT² miRNA PCR Array Data Analysis program (<http://www.sabiosciences.com/pcr/arrayanalysis.php>).

2.3.6. Quantitative RT-PCR analysis of miR181 expression

MiRNA 181 expression was measured using the Taqman MicroRNA Assay (Applied Biosystems; miR181b ID#: 001098; miR181d ID#: 001099) according to the manufacturer's instructions with slight modification. Briefly, total RNA was extracted from cells using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. One microgram of RNA was added to the following reaction mix: RT buffer, 1 mM dNTPs, U6 small nuclear RNA (snRNA) RT primers (Applied Biosystems; ID#: 001173), and RT primers against miR181. Twenty-five units of the Multiscribe RT enzyme (Applied Biosystems) were added, and the reverse transcription reaction was performed on a thermocycler (Eppendorf). Twenty-five nanograms of the resulting cDNA were added to a qPCR mix containing the Taqman Fast Universal PCR Master Mix (Applied Biosystems) and the PCR primer/probe reaction mix against either miR181 or U6 snRNA. Quantitative PCR was performed on an Applied Biosystems 7500 Fast Real-Time System, and data were analyzed

using the 7500 Fast Real-Time software (Applied Biosystems). Primer specificity was determined by the inclusion of a no-RT sample and melting curve analysis. MiRNA expression was normalized to the expression of U6 snRNA.

2.3.7. Western blot analysis

Cells were collected, washed twice with PBS, and lysed using RIPA buffer (without SDS; 150 mM NaCl, 9.1 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, 1% NP-40, and 0.5% deoxycholic acid; pH 7.4) supplemented with protease and phosphatase inhibitors (150 µM Na₃VO₄, 0.25 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, and 10 nM microcystin). The lysate was centrifuged and the protein concentration was determined using Bradford reagent (Pierce). Sample buffer (2X: 0.5 M Tris, 20% glycerol, 10% β-mercaptoethanol, 0.002 µg/ml Bromphenol blue; pH 6.8) was added to an equal volume of total protein (30-50 µg) and the samples were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore; Billerica, MA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline supplemented with Tween-20 (TBST; 9.9 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 8.0) for 1 hr at r.t. Primary antibodies against Lyn (#sc-7274; Santa Cruz Biotechnology; Santa Cruz, CA), Mcl-1 (#sc-819; Santa Cruz), GFP (#G1544; Sigma), SFK pY416 (#2101; Cell Signaling Technology; Beverly, MA), and SFK pY527 (#2105, Cell Signaling) were diluted in 1% bovine serum albumin/TBST and applied to a membrane overnight at 4°C or for 1 hr at r.t. Following the primary antibody incubation, membranes were washed 3 times for 5 min in TBST. Secondary antibodies (horseradish peroxidase (HRP)-conjugated anti-mouse (#sc-2008) or anti-rabbit (#sc-2004) immunoglobulin;

Santa Cruz) diluted in 1% gelatin/TBST were then applied to the membrane for 1 hr at r.t. β -actin (#sc-47778; Santa Cruz) or α -tubulin (#T9026; Sigma) expression was measured as a loading control. Membranes were developed using enhanced chemiluminescence (ECL; GE Healthcare, Piscataway, NJ) with exposure to autoradiography film. Densitometry was performed using a FluorChem FC2 imager (Cell Biosciences, Santa Clara, CA).

2.3.8. Transfection and luciferase activity assay

HEK293T cells (3×10^5 /well) were plated in poly-L-lysine-treated 6-well plates. The next day, cells were transfected using Lipofectamine™ reagent (Invitrogen) according to the manufacturer's instructions.

Twenty-four hr after transfection, cells were lysed at r.t. with RIPA buffer (without SDS). The lysate was centrifuged for 10 min at 10000 RPM. Ten-microliter aliquots of each sample were applied to a 96-well plate, and 150 μ l of the luciferase assay reaction mix (25 mM glycyl glycine, 15 mM MgSO_4 , 15 mM KPO_4 , 4 mM EGTA, pH 7.8) containing 0.1 mg/ml luciferin (Sigma) was added to each well. Luciferase activity was measured on a Pherastar luminometer (BMG Labtech). Western blot analysis of GFP was performed to ensure equivalent transfection, and β -actin expression was determined as a loading control.

2.3.9. Statistics

Data were analyzed using ANOVA and t-tests where appropriate using Prism 4 software (Graphpad Software).

2.4 Chapter 4 supplemental methods

2.4.1. Quantitative RT-PCR analysis of miR181b expression

Quantitative RT-PCR of individual miR181b expression was performed using the method of Thomson et al, 2006. Briefly, 10 µg of total RNA was DNase (Promega) treated and then polyadenylated using Poly(A) polymerase (Applied Biosystems) according to the manufacturer's instructions. Two micrograms of RNA were then reverse transcribed by the Superscript II reverse transcriptase (Invitrogen) using the oligo(dT) adapter primer (5'-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTTVN-3') according to the manufacturer's instructions. Quantitative RT-PCR was performed on a DNA Engine Opticon 2 thermocycler (MJ Research; Waltham, MA) using 25 ng of cDNA. An exact sequence-specific sense primer was used for miR181b in conjunction with a universal antisense primer (5'-GCGAGCACAGAATTAATACGACTCAC-3'). Sense (5'-CTCGCTTCGGCAGCACA-3') and antisense (5'-AACGCTTCACGAATTTGCGT-3') primers were used to amplify the U6 snRNA transcript. The PCR reaction cocktail was mixed with the SYBER green master mix (Applied Biosystems) and qPCR was performed using the following profile: 10 min incubation at 95.0°C followed by 40 cycles of 95.0°C for 15 sec and 60°C for 1 min. Primer specificity was determined by the inclusion of a no-RT sample and melting curve analysis. MiRNA expression was normalized to the expression of U6 snRNA.

2.4.2. Plasmid constructs and cell transfection

The pENTR™ Lyn kinase (A isoform) Gateway® expression construct (Invitrogen) was a generous gift from Dr. David Lawrence (Div. of Medicinal Chemistry and Natural Products, UNC School of Pharmacy). The Lyn kinase expression construct was subcloned into the pLenti6.2/V5-DEST vector (Invitrogen) according to the manufacturer's instructions. HEK293T cells were transfected using Lipofectamine™ reagent (Invitrogen) according to the manufacturer's instructions.

2.4.3. Lyn kinase peptide construction and peptide-based Lyn activity assay

A fluorochrome-conjugated peptide containing a Lyn consensus phosphorylation site was developed as described in Wang et al., 2010. Lyn activity was determined in MYL and MYL-R cells using a peptide-based assay as described in [221].

2.5. Chapter 5 materials and methods

2.5.1. Cell culture and reagents

MYL, MYL-R, and HEK293T cells were obtained and maintained as described in Section 2.2.1. *Cell Culture and Reagents*. Cell cultures were incubated with drug at 37°C in a 5% CO₂ humidified atmosphere. Trichostatin A and Valproic acid were a generous gift from Dr. Pilar Blancafort (Dept. of Pharmacology, UNC). 5-aza-C was obtained from Sigma-Aldrich. Go6983, H89, and U0126 were purchased from Enzo Life Sciences International. SNX5422-conjugated beads were a kind gift from Dr. Tim Haystead (Dept. of Pharmacology and Cancer Biology, Duke University).

2.5.2. Phospho-kinase antibody array

Kinase activity was profiled using the Proteome Profiler Human Phospho-kinase Array System (#ARY003; R and D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, cells were lysed in buffer supplied by the kit. After a blocking step, membranes were incubated with total cell lysate (500 µg) overnight at 4°C. This was followed by 3 washes in buffer supplied by the kit for 10 min each. Membranes were then incubated with a secondary antibody solution for 2 hr at r.t. After a 3 x 10 min wash, membranes were incubated with a streptavidin-HRP solution for 30 min. A final 3 x 10 min wash was followed by incubation with ECL reagent (Thermo Scientific) and exposure to autoradiography film. Densitometry was performed using a FluorChem FC2 imager (Cell Biosciences).

2.5.3. Western blot analysis

Western blot analysis of protein expression was performed as described in Section 2.3.7. *Western blot analysis*. Antibodies against pLyn (Y397; #2101), pCREB (S133; #9191), CREB (#9192), pSTAT5 (Y694; #9351), and STAT5 (#9363) were purchased from Cell Signaling Technology. Antibodies against histone H3-Ac (K27 (#39135) and K18 (#39755)), histone H3K4me2 (#39913), and histone H4-tetra-Ac (#39177) were purchased from Active Motif (Carlsbad, CA). Antibodies against histone H3 were a generous gift from Dr. Brian Strahl (Dept. of Biochemistry, UNC). Antibodies against HSP90 (#13119) were purchased from Santa Cruz Biotechnology.

2.5.4. Total RNA extraction and cDNA synthesis

Total RNA was extracted from cells and cDNA was synthesized as described in Section 2.1.2. *Total RNA extraction and cDNA synthesis.*

2.5.5. Non-quantitative RT-PCR and DNA sequencing

Twenty-five nanograms of the resulting first-strand cDNA were then used for PCR amplification with High Fidelity pfu DNA polymerase (Invitrogen). The primers for amplifying pri-181b-1 were 5'-CGGCCATGTTTTGCTTAATT-3' (sense primer) and 5'-TCCACCTTTGGTTTCCTGTC-3' (antisense primer). The primers for amplifying pri-181b-2 were 5'-CCCCACCAACTGAAAACACT-3' (sense primer) and 5'-CAGTAGCTGCTGCACTCAGG-3' (antisense primer). The primers for amplifying β 2M were 5'-GAATTGCTATGTGTCTGGT-3' (sense primer) and 5'-CATCTTCAAACCTCCATGATG-3' (antisense primer). The PCR reaction was performed on a thermocycler (Eppendorf) as follows: 94°C for 30 sec followed by 30 cycles of 94°C for 20 sec, 56.2°C for 30 sec, and 72°C for 30 sec. Products were visualized on an ethidium bromide-stained agarose gel. Densitometry was performed using a FluorChem FC2 imager (Cell Biosciences).

2.5.6. Quantitative RT-PCR analysis of miR181 expression

MiRNA 181 expression was measured using the Taqman MicroRNA Assay (Applied Biosystems; miR181b ID#: 001098; miR181d ID#: 001099; miR181c ID#: 000482) as described in Section 2.3.6. *Quantitative RT-PCR analysis of miR181 expression.*

2.5.7. Nucleofection

Nucleofection was performed as described in Section 2.3.2. *Nucleofection*.

2.5.8. Statistics

Data were analyzed using ANOVA and t-tests where appropriate using Prism 4 software (Graphpad Software).

CHAPTER III

Identification of a Novel Point Mutation in ENT1 that Confers Resistance to Ara-C in Human T-cell Leukemia CCRF-CEM Cells

Elements of the work referenced in this chapter have been published in:

Zimmerman EI, Huang M, Leisewitz AV, Wang Y, Yang J, and Graves LM. Identification of a novel point mutation in ENT1 that confers resistance to Ara-C in human T cell leukemia CCRF-CEM cells. FEBS Lett. 2009 Jan 22;583(2):425-9.

All figures contributed by Eric I. Zimmerman.

3.1. Abstract

The genetic basis for the Ara-C resistance of CCRF-CEM Ara-C/8C leukemia cells was investigated. DNA sequencing revealed that these cells expressed an equilibrative nucleoside transporter 1 (ENT1) with a single missense mutation resulting in glycine to arginine replacement (G24R). To test the importance of this residue, additional G24 mutants were created and examined for [³H]-uridine and [³H]-Ara-C uptake. Both a G24E and G24A mutant showed reduced ENT1-dependent activity. An EGFP-tagged G24R ENT1 displayed plasma membrane localization even though it was unable to bind [³H]-NBMPR, an ENT1-specific inhibitor. These results define G24 as critical amino acid for ENT1 nucleoside uptake and suggest that mutations in TM1 may provide a mechanism for Ara-C resistance in CCRF-CEM Ara-C/8C cells.

3.2. Introduction

Cytarabine (Ara-C), a pyrimidine analog, is a conventional anti-cancer chemotherapeutic commonly used for the treatment of acute myeloid leukemia. Ara-C is a hydrophilic molecule that achieves intracellular penetration via nucleoside transporter proteins. Once inside the cell, this agent exerts cytotoxic effects on proliferating cancer cells by the direct inhibition of DNA polymerases and the arrest of DNA synthesis. Although Ara-C treatment often induces partial or complete remission of cancerous tissue, many patients eventually develop resistance [222]. Thus, understanding the mechanisms of resistance is important for the development of effective anti-cancer therapies.

CCRF-CEM Ara-C/8C (Ara-C/8C) cells, a nucleoside transport-deficient T-cell leukemia cell line, were isolated by single cell cloning and resistance to Ara-C; these cells demonstrate cross-resistance to gemcitabine and 2',3'-dideoxycytidine [217, 223]. Despite these observations, the molecular defect responsible for the loss of nucleoside transport in these cells was not elucidated.

The equilibrative nucleoside transporters (ENTs) are a sodium-independent class of nucleoside transporters (SLC29) responsible for the uptake of a large number of nucleosides and nucleoside analogs [42]. This includes purine and pyrimidine nucleosides and analogs such as gemcitabine, Ara-C, and fludarabine [224-226]. Four distinct members of this class of proteins have been identified (ENT1-4) [42]. ENT1 and ENT2 are the best-characterized members of this family; ENT1 is selectively inhibited by nitrobenzylthioinosine (NBMPR), whereas both ENT1 and ENT2 are inhibited by dipyrindimole and dilazep [219]. In comparison, ENT3 is distributed mainly within endomembranes [227] and studies suggest ENT4 may function as a monoamine/organic anion transporter [228, 229].

ENTs share a common 11-transmembrane (TM) α -helix topology [48] and structure-function studies suggest that amino acid residues within TM 3-6 may be involved in nucleoside binding [53, 54]. For example, site-directed mutagenesis of human ENT1 expressed in *Saccharomyces cerevisiae* demonstrated that glycine 179 in TM5 was required for uridine transport and sensitivity to NBMPR, whereas glycine 184 may partially determine targeting of the transporter to the plasma membrane [53]. Similarly, glycine 154 was reported to be important for nucleoside transport and sensitivity to the inhibitors NBMPR, dipyrindimole and dilazep [54].

However, recent studies suggest that amino acids in TM domains other than 3-6 may also be critical for the transport of nucleoside substrates and their analogs [55]. Specifically, methionine 33 in TM1 and isoleucine 429 in TM11 of human ENT1, human ENT2, and *Caenorhabditis elegans* ENT1 were reported to be required for nucleoside transport and may contribute to the binding of dipyridamole [55]. A report by Paproski et al. demonstrated that tryptophan 29 within TM1 was important for inhibitor binding and ENT1 proteins containing mutations of W29 have altered nucleoside transport kinetics [56].

In our study we identified a single point mutation at glycine 24 in TM1 of ENT1 in Ara-C/8C cells. To investigate the importance of G24, we developed ENT1 proteins with different point mutations of glycine 24 and expressed these mutants in PK15 cells, a nucleoside transport-deficient cell line. The mutant ENT1 proteins were defective in transport activity, as measured by [³H]-uridine and [³H]-Ara-C uptake and inhibitor binding. Thus, mutation of glycine 24 in ENT1 may explain the loss of nucleoside transport and resistance to nucleoside analogs in Ara-C/8C cells.

3.3. Results

3.3.1. Functional loss of ENT1 activity in Ara-C/8C cells is independent of mRNA expression

The nucleoside transport-deficient Ara-C/8C cells were isolated as a cell line highly resistant to Ara-C and gemcitabine [217, 223]. Comparison of [³H]-uridine uptake in parental and resistant CEM cells showed that the Ara-C/8C were completely deficient in uridine uptake (Fig. 3.5.1A). [³H]-Uridine uptake was abolished with the ENT1-specific inhibitor NBMPR in the parental CCRF-CEM cells,

demonstrating that ENT1 activity accounts for the majority of uridine transport in the CEM cell lines. In comparison, the binding of [³H]-NBMPR to crude membrane preparations of Ara-C/8C cells was almost completely abolished (Fig. 3.5.1B), suggesting that functional ENT1 protein was absent from Ara-C/8C cells.

ENT1 mRNA expression was determined using non-quantitative RT-PCR. As shown in Figure 3.5.1C, Ara-C/8C cells expressed similar levels of ENT1 mRNA as compared to the parental CCRF-CEM cells. This observation suggests that the defect in nucleoside uptake was independent of ENT1 expression.

3.3.2. Identification of a point mutation in ENT1 from Ara-C/8C cells that disrupts transport activity

We next determined whether ENT1 from the Ara-C/8C cell line contained mutations that would explain a loss of function. Full-length ENT1 was amplified by PCR and sequenced. Analysis of the ENT1 sequence revealed a single nucleotide missense mutation resulting in the substitution of arginine for glycine at amino acid position 24 (GGA to AGA). Complete sequence analysis of ENT1 from Ara-C/8C cells did not reveal any additional mutations. G24 is located in the TM1 domain of ENT1 and is conserved amongst species and ENT2 isoforms (Fig. 3.5.2A).

We created several point mutations of G24; substitution of alanine and glutamate were performed to determine the effect of amino acid size and charge on ENT1 function. Wt ENT1 and G24A, G24E, and G24R ENT1 mutants were transfected into nucleoside transport-deficient PK15 cells and [³H]-uridine uptake was measured as an index of ENT1 activity. As shown in Figure 3.5.2B, G24R ENT1 was deficient in [³H]-uridine transport compared to wt ENT1. ENT1 proteins with a

mutation of G24 to alanine or glutamate displayed only partial transport activity when compared to wt ENT1 (Fig. 3.5.2B). Similarly, the G24R ENT1 mutant displayed a deficiency in [³H]-Ara-C uptake; G24A ENT1 displayed partial uptake when compared to wt ENT1, whereas G24E ENT1 was uptake-deficient (Fig. 3.5.2C). These data further indicate that glycine 24 is important for ENT1 activity and mutation of G24 severely reduced transport activity.

3.3.3. The G24R ENT1 mutant maintains plasma membrane localization but is deficient in NBMPR binding

To determine if the G24R ENT1 mutant was localized to the plasma membrane as expected, wt and G24R ENT1 proteins were subcloned into the pEGFP-C3 vector. Transfection of HeLa cells showed that the G24R ENT1 mutant exhibited predominantly membranous expression, similar to that observed with wt ENT1 (Fig. 3.5.3A). These data demonstrated that the G24 mutation did not alter protein maturation or plasma membrane localization. To investigate whether the G24R ENT1 mutant was sensitive to inhibitor binding, [³H]-NBMPR binding assays were conducted with crude cell membrane preparations from PK15 cells expressing either wt or G24R ENT1. [³H]-NBMPR has selective affinity for ENT1 and inhibitor binding to cells expressing wt ENT1 was observed (Fig. 3.5.3B). In contrast, cells expressing the G24R ENT1 mutant did not display any detectable [³H]-NBMPR binding, suggesting that the G24R mutation affected both substrate and inhibitor recognition.

3.4. Discussion

In this study, we provide evidence for a novel missense mutation in ENT1 that conferred resistance to nucleoside uptake in an Ara-C-resistant cell line of CCRF-CEM cells. Our results demonstrate that a single point mutation (G24R) in TM1 results in the generation of an inactive ENT1. G24 is an amino acid that is highly conserved between ENT isoforms and amongst species, suggestive of its importance to hENT1 function. Substitution of G24 with amino acids that vary in size and charge confirmed the importance of G24 in ENT1 recognition of substrates. Moreover, G24 may be an important contact for inhibitor recognition since G24R mutants of ENT1 did not bind [³H]-NBMPR. Partial [³H]-Ara-C transport was observed with the G24A mutation, which may be due to the similarity of the amino acids. Importantly, our data suggest that differences in ENT1 G24 mutant activity were not due to differential expression but due to a functional difference since the wt and G24R ENT1 proteins had dissimilar [³H]-uridine transport/[³H]-NBMPR binding ratios (2.54 ± 0.10 vs. 0.67 ± 0.02). Thus these studies are the first to identify this conserved amino acid as an important determinant for nucleoside recognition and uptake.

The G24R point mutation in ENT1 may affect several processes related to the functional activity of ENT1, including substrate binding, protein folding or targeting of ENT1 to the plasma membrane. Topology predictions (SOSUI program; <http://bp.nuap.nagoya-u.ac.jp/sosui/>) suggest that G24R ENT1 is capable of targeting to plasma membrane similarly to the wt ENT1. This prediction is supported by our data showing similar membranous localization of EGFP-tagged wt and G24R

ENT1. Our studies also show that G24R ENT1 is incapable of binding the inhibitor NBMPR. This may result from a disruption of the substrate-binding site since NBMPR, a purine analog, is expected to bind at or near the substrate-binding pocket [53, 54]. Recent studies have shown that amino acids in close proximity (M33 and W29) are important to ENT1 nucleoside transport and inhibitor binding [55, 56]. Based on these observations, we speculate that the G24R substitution at this position similarly interferes with binding of nucleosides and nucleoside analogs to ENT1. Thus, TM1 may be a topologically-sensitive portion of ENT1, making the proper orientation of TM1 necessary for protein function.

ENT-mediated transport of nucleoside analogs is often the rate-limiting step for drug-induced cell cytotoxicity [225]. Thus, it is not surprising that studies have found a positive correlation between the expression of ENT1 and cancer cell sensitivity to the nucleoside analogs Ara-C [81] and gemcitabine [230]. The reduction in ENT1 expression is a common mechanism for resistance to antimetabolite treatment of cancer [226, 231]. In addition, a recent study using an Ara-C-resistant CCRF-CEM cell line reported that genetic mutations of ENT1 that alter mRNA splicing and protein translation provide mechanisms for resistance to drug treatment [89].

In conclusion, these studies provide new information on the specific role of G24 within the TM1 domain of ENT1 in nucleoside recognition and uptake. Expression of a G24R ENT1 mutant provides the first molecular explanation for the loss of ENT1-dependent nucleoside uptake in the Ara-C/8C cells and indicates that mutation of this amino acid could result in resistance to Ara-C treatment.

3.5. Figures

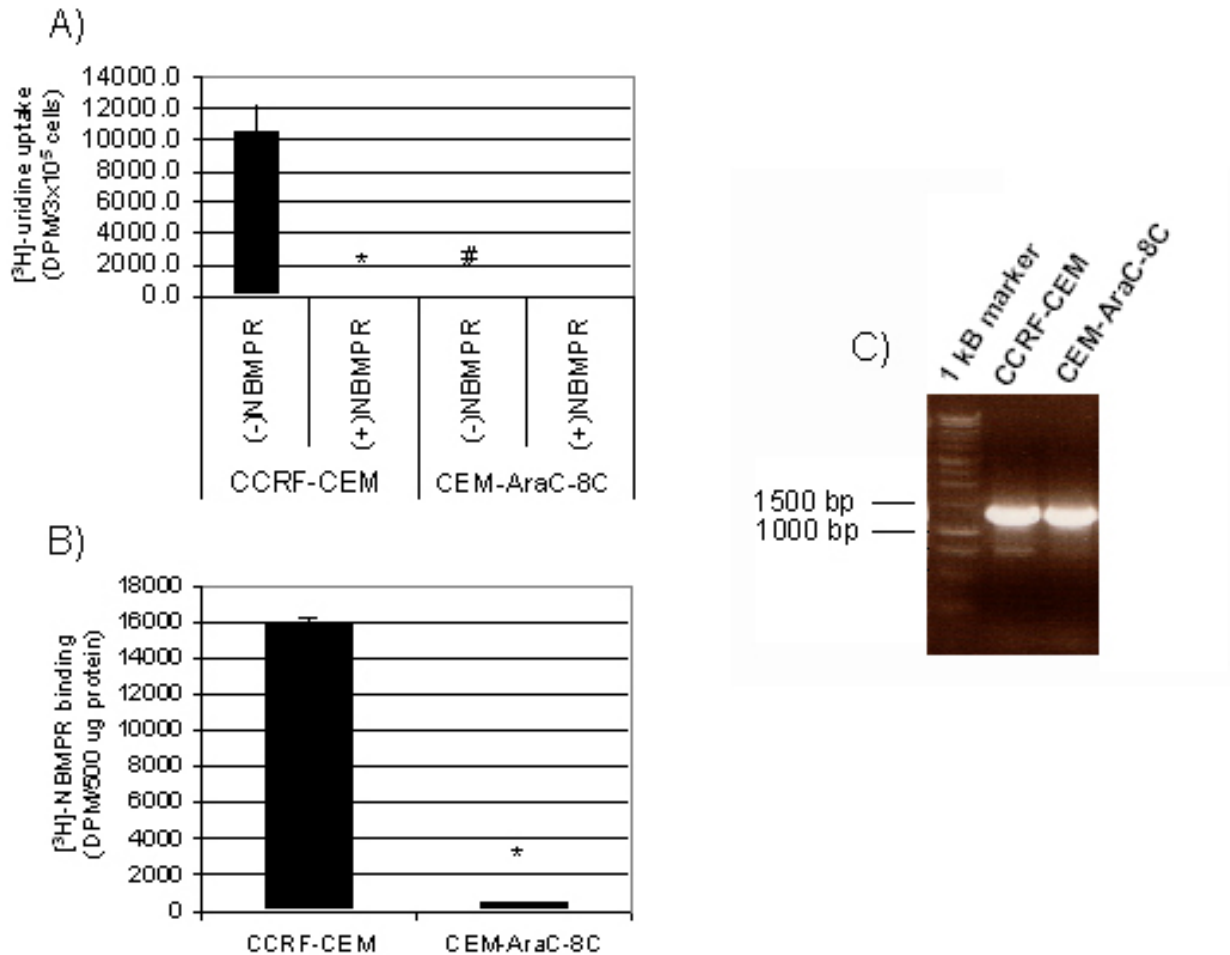


Figure 3.5.1. Comparison of [3H]-uridine uptake, [3H]-NBMPR binding, and ENT1 mRNA expression between CCRF-CEM cells and Ara-C/8C cells. A) 3×10^5 CCRF-CEM and Ara-C/8C cells (CEM-AraC-8C) cells were incubated with [3H]-uridine in sodium-free transport buffer for 5 min in the presence or absence of 1.0 μ M NBMPR, a selective inhibitor of ENT1. Data represent the mean \pm S.D. of four experiments performed with duplicate samples (*, $p < 0.01$ different from -NBMPR; #, $p < 0.01$ different from parental cell line, -NBMPR). B) [3H]-NBMPR binding was performed using isolated membranes from 3×10^5 CCRF-CEM and CEM-AraC-8C cells as described in Section 2.1.7. *Isolation of membranes and [3H]-NBMPR binding assay.* Data represent the mean \pm S.D. of two experiments performed with triplicate samples (*, $p < 0.01$). C) Total RNA was extracted from 3×10^5 CCRF-CEM and CEM-AraC-8C cells. ENT1 mRNA expression in CCRF-CEM and CEM-AraC-8C cells was determined by non-quantitative RT-PCR. A representative gel is shown.

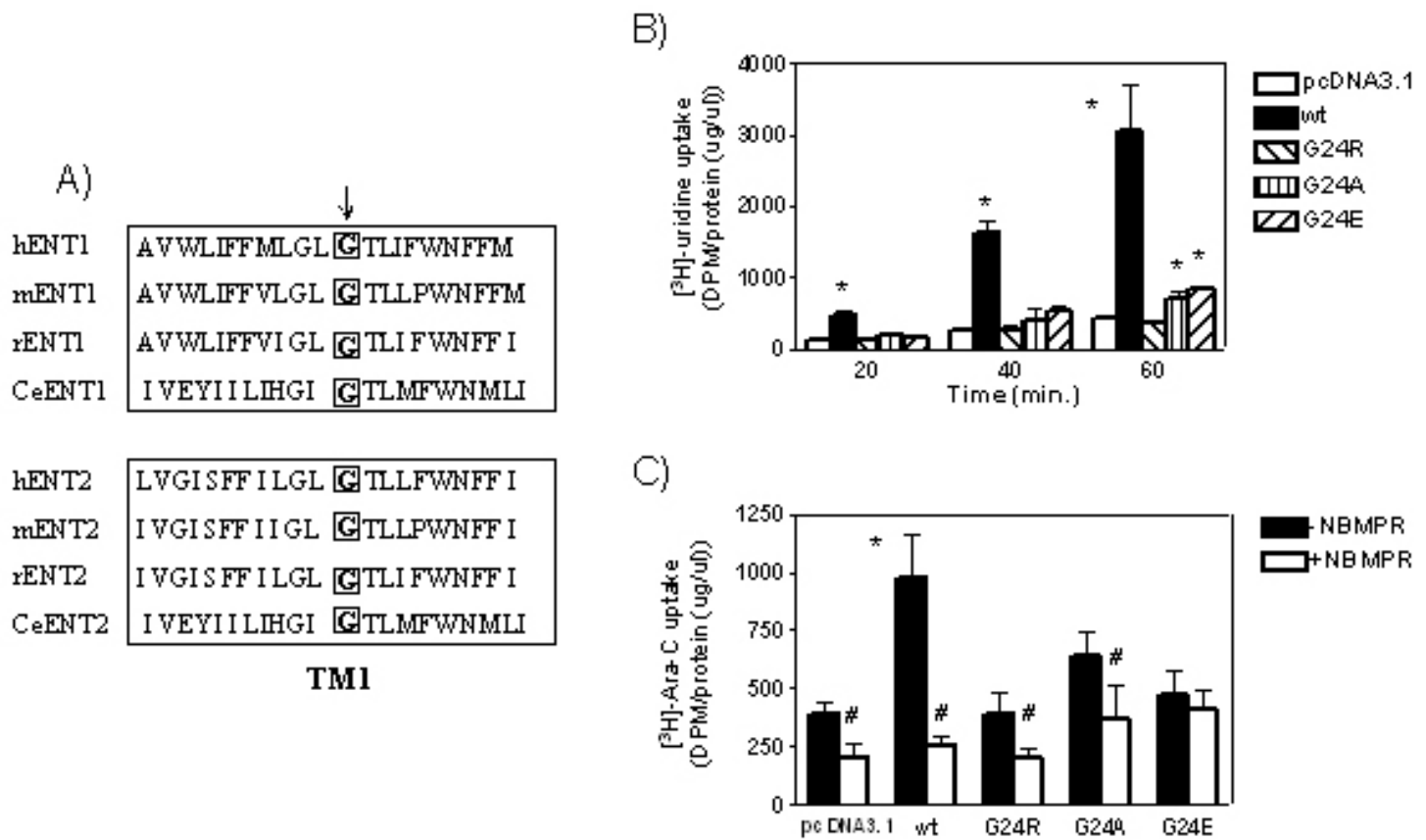


Figure 3.5.2. Substitution of G24 in ENT1 disrupts nucleoside and Ara-C transport activity. A) Alignment of amino acids 13–33 of ENT1 and ENT2 from different species using ClustalW software (<http://align.genome.jp/>). Arrow denotes residue G24 (TM1, transmembrane domain 1). B) Wt ENT1 and G24A, G24E, and G24R ENT1 mutants were expressed separately in PK15 cells (6×10^4) and [3 H]-uridine uptake was performed for 20, 40, and 60 min. The average transport by wt ENT1 was approximately 4.19 pmol/min/mg protein. Data represent the mean \pm S.D. of samples in quadruplicate (*, $p < 0.01$ different from 0 min). (C) Wt ENT1 and G24A, G24E, and G24R ENT1 mutants were expressed separately in PK15 cells (6×10^4) and [3 H]-Ara-C uptake was performed for 2 hr in the presence or absence of 1 μ M NBMPR. Average transport by wt ENT1 was approximately 2.08 pmol/min/mg protein. Data represent the mean \pm S.D. of samples in quadruplicate (*, $p < 0.01$ different from control; #, $p < 0.01$ different from -NBMPR).

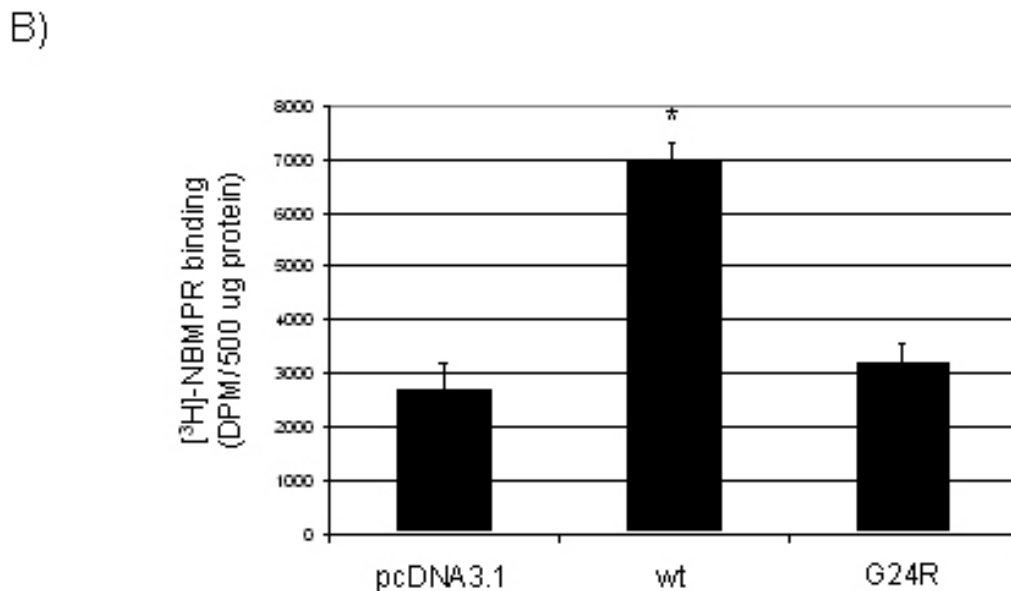
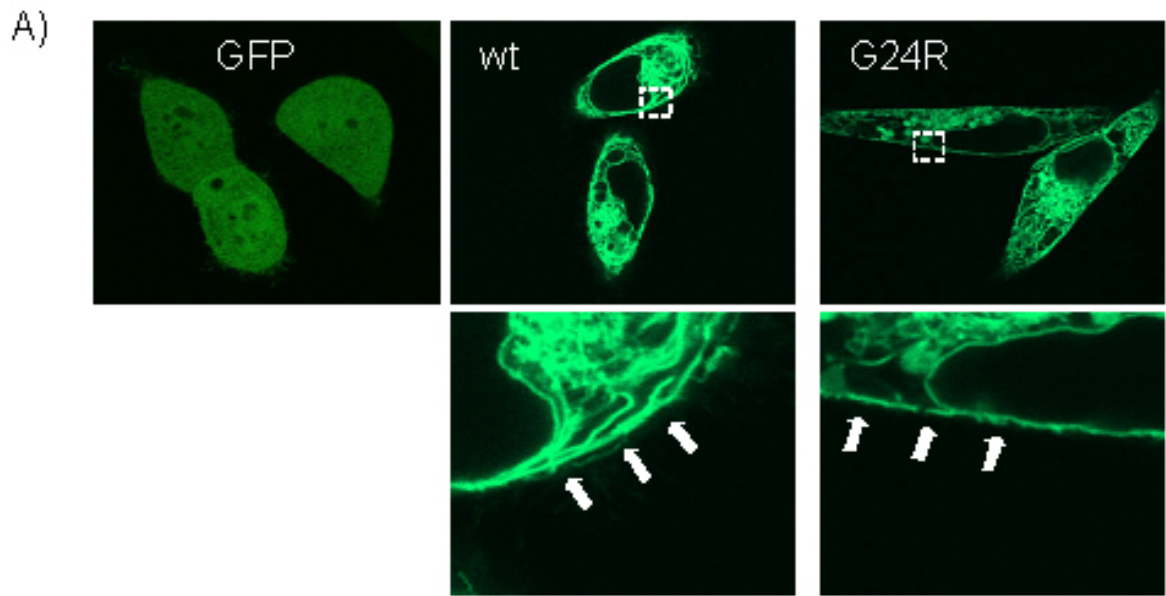


Figure 3.5.3. The G24R ENT1 mutant lacks [³H]-NBMPR binding even though plasma membrane expression is maintained. A) Wt and G24R ENT1-GFP proteins were expressed in HeLa cells and observed using confocal microscopy. Representative images are shown. Arrows denote approximate plasma membrane location. B) Wt ENT1 and G24R ENT1 were transfected separately into PK15 cells (6×10^4). Forty-eight hr post-transfection, cells were lysed, the membranes were isolated, and [³H]-NBMPR binding to the isolated membrane was determined as described in Section 2.1.7. *Isolation of membranes and [³H]-NBMPR binding assay.* Data represent the mean \pm S.D. of two experiments performed with triplicate samples (*, $p < 0.01$).

3.6. Addendum

Our initial focus on ENT1-dependent mediation of Ara-C drug resistance highlights the role of drug permeability factors in chemotherapy and the necessity for drug access to permit cytotoxic drug action. Much research is dedicated to the understanding of drug transporter-dependent effects on drug pharmacokinetics, pharmacodynamics, and drug-induced cell death. Dysfunction of active transport is a common mechanism underlying resistance to antimetabolite therapy, as indicated by numerous clinically relevant publications describing these mechanisms [91]. However, transporter-independent resistance mechanisms are also prominent in leukemia.

Our studies of ENT1 expression and activity in drug-resistant cell lines uncovered examples where ENT activity did not correlate with sensitivity to nucleoside analogs. Notably, we measured ENT1 and ENT2 expression and activity in the BCR-Abl-transformed mouse 32Dp185 cell line that was made resistant to the BCR-Abl inhibitor imatinib [232]. The primary findings of this study were that 1) imatinib treatment of the imatinib-sensitive 32Dp185 cells decreased ENT1 mRNA expression and transport activity and 2) the imatinib-resistant 32Dp185 STI-R cells were sensitive to gemcitabine treatment even though these cells express less ENT1 and ENT2 and display low ENT activity [232]. Thus, ENT expression was not a limiting factor to the cytotoxic effects of nucleoside analog treatment and did not correlate with gemcitabine sensitivity.

Furthermore, we found that a human chronic myelogenous leukemia (CML) cell line resistant to imatinib, termed MYL-R, were cross-resistant to the nucleoside

analogs gemcitabine and Ara-C. Cells were resistant to drug in a dose-dependent manner as assessed by MTS assay and caspase 3 activity (Fig. 3.7.1A-D). Therefore, we measured ENT1 and ENT2 expression using qRT-PCR to determine whether dysregulation of these transporters caused resistance to these nucleoside analogs. Surprisingly, both ENT1 and ENT2 mRNA expression was similar between the drug-sensitive MYL cells and the drug-resistant MYL-R cells (Fig. 3.7.2A). In agreement with the qRT-PCR data, ENT1 expression was not significantly different between the two cell lines as assessed by [^3H]-NBMPR binding (Fig. 3.7.2B).

To measure transporter activity, [^3H]-uridine uptake was determined in MYL and MYL-R cells. Pre-incubation with NBMPR, an ENT1-selective inhibitor, or dipyridamole, an ENT1 and ENT2 inhibitor, was performed to assess the individual contribution of these transporters. As shown in Figure 3.7.3A, overall transport of [^3H]-uridine was not significantly different between MYL and MYL-R cells. After inhibition of ENT1 only or both ENT1 and ENT2, [^3H]-uridine uptake remained similar between the cell lines (Fig. 3.7.3A). In addition, our data suggest that ENT1- and ENT2-mediated transport accounted for approximately 50% of [^3H]-uridine uptake in both cell lines; non-ENT-mediated transport of the substrate may be due to the presence of CNTs or other dipyridamole insensitive transporters. To confirm the [^3H]-uridine transport data, we measured the uptake of [^3H]-Ara-C. In order to exclude the contribution of CNTs to uptake, we performed this experiment in the absence of sodium, which is necessary for CNT-mediated transport. Similar to the [^3H]-uridine uptake data, transport of [^3H]-Ara-C was not significantly different between MYL and

MYL-R cells (Fig. 3.7.3B). Thus, unlike the CCRF-CEM model, ENT expression and activity did not correspond with drug resistance in MYL-R cells.

Collectively, these findings suggest that ENT expression may not always be prognostic for drug resistance. Thus, as discussed in Chapter 4, we focused on kinase-dependent mechanisms of drug resistance through regulation of apoptotic machinery.

3.7. Addendum figures

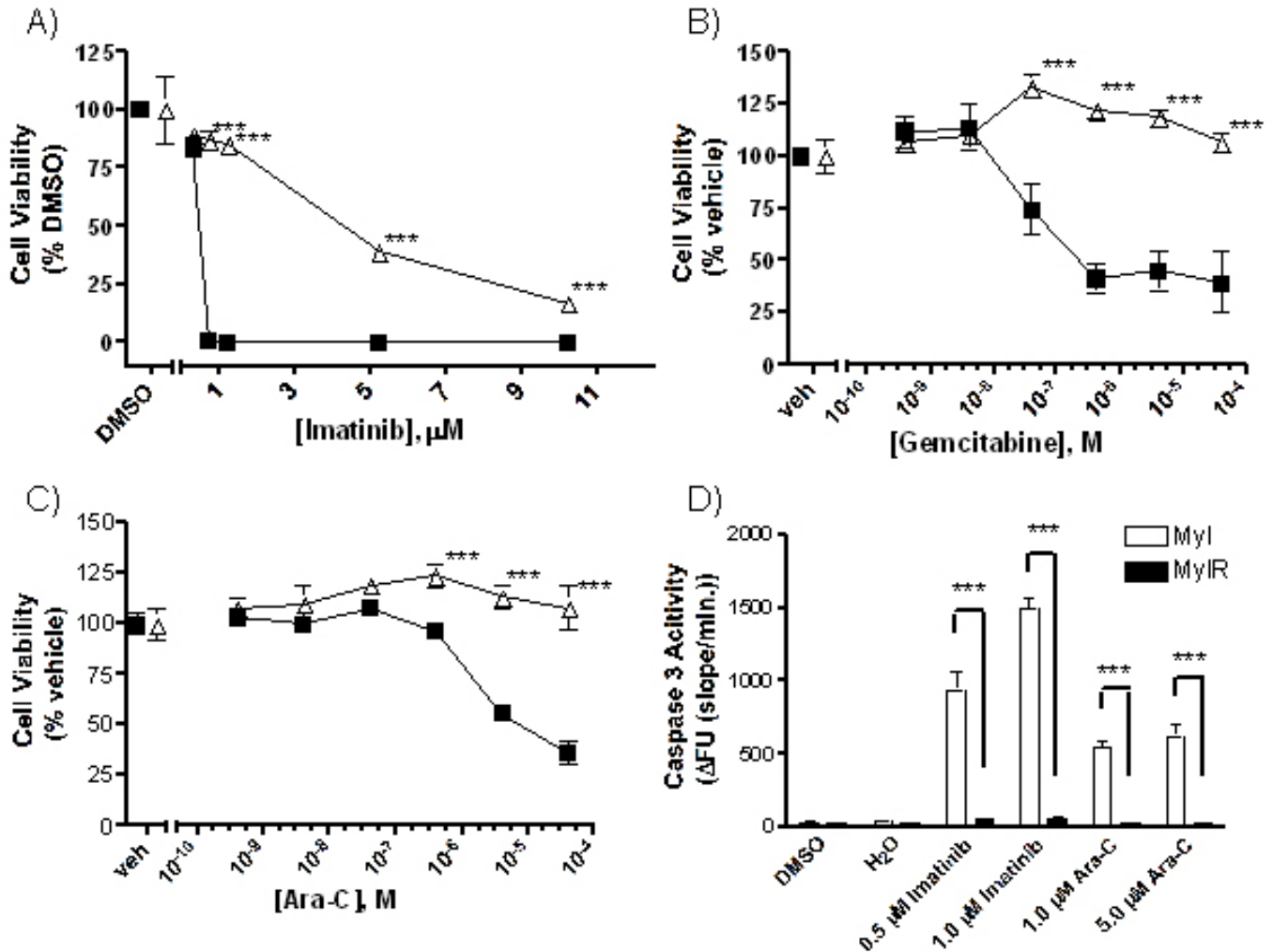


Figure 3.7.1. MYL-R cells are resistant to imatinib and nucleoside analogs. A-C) MYL (open triangles) and MYL-R (closed squares) cells were plated in 96-well plates (1.2×10^4 /well) and cultured in the presence of vehicle or drug at the indicated concentrations for 48 hr. Cell viability was determined using the MTS assay as described in Section 2.2.2. *Cell viability assay*. Data represent the mean \pm S.D. of triplicate samples (***, $p < 0.001$). D) Cells were plated in a 96-well plate (6×10^5) and cultured with vehicle or drug for 24 hr. Caspase 3 activity was measured as described in Section 2.2.3. *Caspase 3 activity assay*. Data represent the mean \pm S.D. of triplicate samples (***, $p < 0.001$).

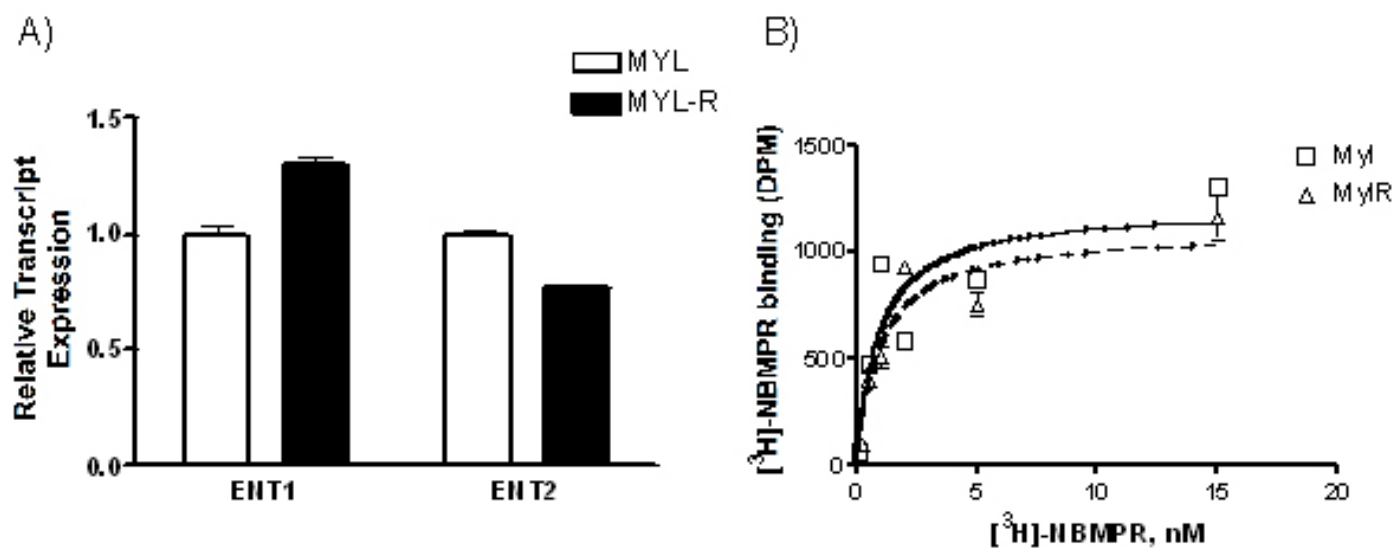


Figure 3.7.2. ENT expression is similar between MYL and MYL-R cells. A) Total RNA was extracted from 2×10^6 cells and mRNA expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples. B) 6×10^5 cells were incubated with the indicated amount of [3 H]-NBMPR and the amount of bound [3 H]-NBMPR was determined by scintillation counting. Data represent the mean \pm S.D. of triplicate samples.

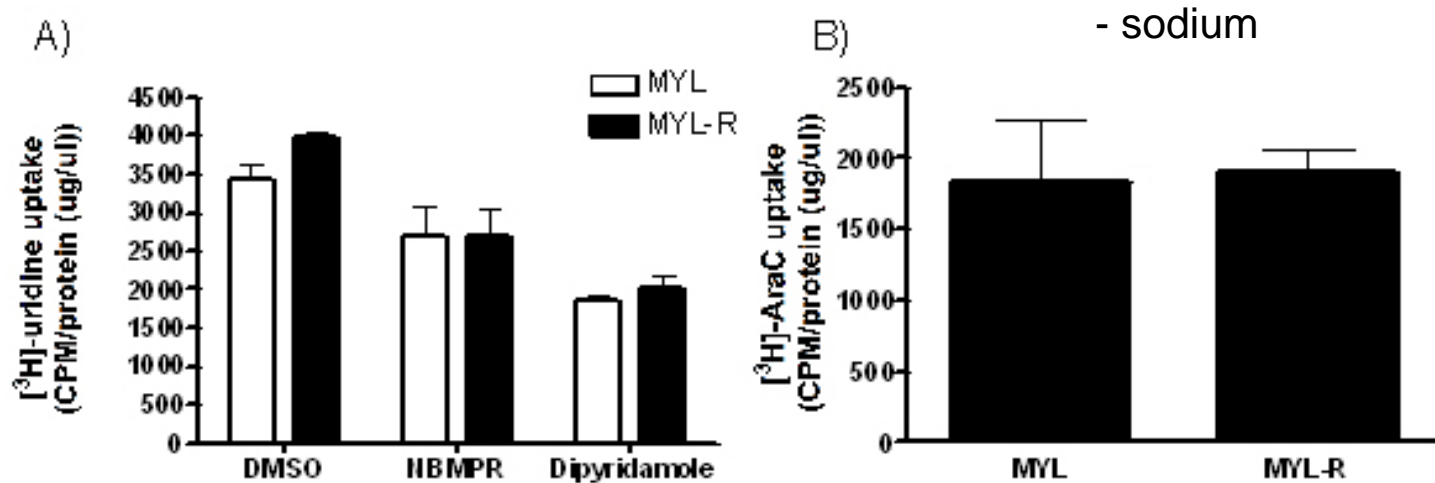


Figure 3.7.3. MYL and MYL-R cells have similar transport activity. A) 3.0×10^5 cells were treated with DMSO, $1.25 \mu\text{M}$ NBMPR, or $2.0 \mu\text{M}$ dipyridamole for 15 min and then were incubated with $[^3\text{H}]$ -uridine for 10 min in the presence of inhibitor. B) 3.0×10^5 cells were incubated with $[^3\text{H}]$ -Ara-C for 45 min and the uptake of radioactive substrate was determined by scintillation counting. Data represent the mean \pm S.D. of triplicate samples.

CHAPTER IV

Lyn Kinase-dependent Regulation of miR181 and Mcl-1 Expression: Implications for Drug Resistance in Myelogenous Leukemia

Elements of the work referenced in this chapter have been published in:

Zimmerman EI, Dollins CM, Crawford M, Grant S, Nana-Sinkam SP, Richards KL, Hammond SM, and Graves LM. Lyn kinase-dependent regulation of miR181b and Mcl-1 expression: implications for drug resistance in myelogenous leukemia. *Mol Pharm* 2010 Nov;78(5): 811-7.

All figures contributed by Eric I. Zimmerman.

4.1. Abstract

The BCR-Abl inhibitor imatinib is a successful front-line treatment for chronic myelogenous leukemia (CML). However, despite the success of imatinib, multiple mechanisms of resistance remain a problem, including over-expression of Lyn kinase (Lyn) and Bcl-2 family anti-apoptotic proteins. Profiling miRNA expression in a model of Lyn-mediated imatinib-resistant CML (MYL-R) identified approximately 30 miRNAs whose expression differed >2-fold in comparison to drug-sensitive MYL cells. In particular, the expression of the miR181 family (a-d) was significantly reduced (~11-25 fold) in MYL-R cells. Incubation of MYL-R cells with a Lyn inhibitor (dasatinib) or nucleofection with Lyn-targeted siRNA increased miR181b and miR181d expression. A similar Lyn-dependent regulation of miR181b and miR181d was observed in imatinib-resistant K562 CML cells. Sequence analysis of potential targets for miR181 regulation predicted Mcl-1, a Bcl-2 family member whose expression is increased in MYL-R cells and drug-resistant leukemias. Inhibition of Lyn or rescue of miR181b expression reduced Mcl-1 expression in the MYL-R cells. To further investigate the mechanism of Mcl-1 repression by miR181, a luciferase reporter construct incorporating the Mcl-1 3' UTR was tested. Over-expression of miR181b reduced luciferase activity, whereas these effects were ablated by mutation of the seed region of the miR181 target site. Lastly, stimulation of Lyn expression by 1,25-dihydroxyvitamin D₃ treatment in HL-60 cells, a cell model of acute myelogenous leukemia, decreased miR181b expression and increased Mcl-1 expression. In summary, our results suggest that Lyn-dependent regulation of miR181 is a novel mechanism of regulating Mcl-1 expression and cell survival.

4.2. Introduction

The discovery and application of the BCR-Abl inhibitor imatinib has been a major hallmark in the development of kinase inhibitors for cancer chemotherapy. However, despite the success of imatinib for the treatment of chronic myelogenous leukemia (CML) and other cancers, multiple mechanisms of imatinib resistance have been identified. These include BCR-Abl mutations that prevent imatinib binding (i.e. T315I) [233, 234], BCR-Abl over-expression [233], and increased expression and activity of Src family kinases (SFKs) or other pro-survival proteins [124, 235]. Recently, Lyn kinase (Lyn) has been implicated in imatinib resistance in both CML cells and patient samples. Over-expression of Lyn, the most abundant SFK in myeloid cells, may contribute to drug resistance through increased STAT5 phosphorylation, Bcl-2 expression, and other pro-survival responses [128, 129, 154]. Inhibition of Lyn with SFK inhibitors reduced pro-survival signaling and reversed imatinib resistance in CML cells [130, 154]. Moreover, dual-BCR-Abl/SFK inhibitors (i.e. dasatinib, nilotinib) effectively treat patients that are non-responsive to imatinib therapy [131, 235].

MicroRNAs (miRNAs) are small (22-24 nt) non-coding RNA molecules that are key regulators of protein expression through their targeted binding to specific mRNAs. By forming a double-stranded RNA duplex with target mRNAs in the RNA-induced silencing complex (RISC), miRNAs trigger the degradation of the mRNA transcript or directly inhibit protein translation [157]. Over 700 miRNAs have been described in humans, and patterns of deletion, down-regulation, or up-regulation of specific miRNAs have been characterized in B-cell chronic lymphocytic leukemia

(CLL), acute myelogenous leukemia (AML), and CML [236-238]. Recent studies have demonstrated the importance of the miR181 family (a-d) expression in AML and CLL. MiR181a is involved in hematopoietic differentiation [175], and loss of miR181 strongly correlates with a common AML morphological subtype [239]. Moreover, high miR181 (a-d) expression is prognostic for the achievement of complete remission and event free survival in AML patients [164, 176, 177].

Bcl-2 family members are important pro-survival regulators of apoptosis that have been implicated in the promotion of drug resistance in cell models of leukemia [128, 240, 241]. Myeloid Cell Leukemia-1 (Mcl-1) is a Bcl-2 family protein shown to correlate with leukemic relapse in AML and has been directly linked to resistance to chemotherapy [242]. In addition, Mcl-1 was recently implicated in AML survival in response to FLT-3 internal tandem duplication (FLT3-ITD), a common mechanism of resistance in AML that involves the activation of Lyn [152, 204]. Mcl-1 functions at the mitochondria by sequestering the pro-apoptotic BH3-only proteins Bim and Noxa, thereby preventing cytochrome c release and cell death [243]. Mechanisms to inhibit Mcl-1 function include ubiquitination and degradation directed by the MULE/LASU1 E3-ligase [195], and targeting of Mcl-1 mRNA for degradation by miRNA has been described [171, 173, 174].

In this study, we describe a novel mechanism by which Lyn may confer multi-drug resistance in a cell model of CML by repressing the expression of miR181. As our data show, miR181b directly represses Mcl-1 expression and the Lyn-dependent loss of miR181 results in enhanced Mcl-1 levels and increased drug resistance. Thus, this is the first demonstration, to our knowledge, of Mcl-1 as a *bona fide* target

of miR181 and suggests that the regulation of miR181 by Lyn in drug-resistant cells may contribute to this important anti-apoptotic mechanism.

4.3. Results

4.3.1. Lyn kinase over-expression in MYL-R cells confers drug resistance

Previous studies have demonstrated that Lyn is important for MYL-R survival and imatinib resistance [130]. To compare Lyn expression and activity, lysates of MYL and MYL-R cells were Western blotted with antibodies that recognize total Lyn, active Lyn (P-Y397 (P-Y416 Src)), or inactive Lyn (P-Y507 (P-Y527 Src)). As shown in Figure 4.5.1A, the Lyn B splice form (53 kDa) was significantly over-expressed and phosphorylated on the activation loop (Y397), indicating increased activity in MYL-R cells. Similarly, the Lyn A splice form (56 kDa), though reduced in expression, was also more highly phosphorylated on this residue in the MYL-R cells (Fig. 4.5.1A). Loss of the inactivating Y507 phosphorylation was observed only for Lyn B, suggesting the elevated activity state of this kinase in MYL-R cells. Importantly, the increase of Lyn activity was confirmed in these cells using a novel peptide-based fluorescent Lyn biosensor [221] (Supplemental Fig. S4.6.1).

Lyn hyper-activation has been attributed to imatinib resistance in MYL-R cells [130]. In addition, incubation of MYL-R cells with gemcitabine, Ara-C, adaphostin, or Immucillin H failed to activate caspase 3 in these cells, demonstrating that MYL-R are highly anti-apoptotic (Fig. 3.6.1 and data not shown). To investigate the importance of Lyn in cell survival, MYL-R cells were transfected with Lyn-directed siRNA or non-targeting control siRNA and then exposed to 1 μ M imatinib. This

treatment resulted in a partial knockdown of Lyn and a significant increase in caspase 3 activation (Fig. 4.5.1B-C). However, the magnitude of caspase activation was less than in MYL cells exposed to the same dose of imatinib (data not shown); this may be due to incomplete silencing of Lyn expression. In addition, pre-treatment with PP2, a SFK inhibitor, significantly elevated caspase activity in MYL-R cells treated with imatinib, an effect not observed after pre-treatment with the inactive inhibitor analog, PP3 (Supplemental Fig. S4.6.2).

4.3.2. MiR181 expression is reduced in MYL-R cells

MiRNAs are small (20-22 nt) non-coding RNA regulators of protein expression that have been implicated in the progression and survival of numerous cancers [244]. To identify miRNAs involved in anti-apoptosis, MYL and MYL-R cells were profiled for miRNA expression using the Human Cancer RT² miRNA qPCR Array. This qRT-PCR-based assay analyzed the expression of 88 known human miRNAs previously associated with cancer. Duplicate experiments demonstrated that 15 miRNAs had a >2-fold increase in expression in MYL-R cells relative to MYL cells (Fig. 4.5.2A) and that 15 miRNAs showed a >2-fold decrease in relative expression (Fig. 4.5.2B). Importantly, we observed a strong down-regulation of the miR181 family (a-d) of miRNAs in MYL-R cells (Fig. 4.5.2B, C). This family of miRNA is highly conserved (Fig. 4.5.2D), and loss of miR181 expression is prognostic for aggressive AML [164, 177]. To confirm the array results, individual qRT-PCR of miR181b and miR181d was performed; the results of these analyses showed that the expression of both miRNAs was reduced approximately 10-fold in MYL-R cells (Supplemental Fig. S4.6.3).

4.3.3. Lyn kinase inhibition increases miR181 expression in MYL-R cells

To determine whether hyper-activation of Lyn regulated miR181 expression in MYL-R cells, cells were treated with dasatinib and miRNA expression was determined by qRT-PCR. As shown in Figure 4.5.3A-B, dasatinib treatment inhibited Lyn and increased miR181b expression in MYL-R cells in a dose-dependent manner. Dasatinib (1 nM) treatment produced minimal effects on cell viability (~15-20% cell loss), as determined by MTS assay and cell proliferation (data not shown). The effects of Lyn inhibition on miR181b expression were confirmed by treatment with PP2, whereas PP3 had minimal effects on miR181b expression (Supplemental Fig. S4.6.4). To determine whether Lyn specifically regulated miR181b expression, we nucleofected MYL-R cells with Lyn-targeted siRNA. We observed a significant elevation in miR181b expression in comparison to control siRNA-treated cells, as assessed by the miR181b Taqman qRT-PCR assay and an independent qRT-PCR method [245] (Fig. 4.5.3C and Supplemental Fig. 4.6.5). In addition, we over-expressed Lyn in HEK293T cells and observed a significant loss of miR181b expression in comparison to cells expressing the control plasmid, confirming our siRNA data (Supplemental Fig. S4.6.6).

K562-R cells are a model of imatinib-resistant CML, and over-expression of Lyn has been attributed to the mechanism of resistance in these cells [128, 129]. To further determine whether Lyn regulates miR181 expression, we tested two independently subcloned imatinib-resistant K562-R cell lines, termed K562-R(1) and K562-R(2). Similar to MYL-R cells, dasatinib treatment of K562-R cells inhibited Lyn activity and increased miR181b expression (Fig. 4.5.3E-G). In addition, dasatinib

treatment increased miR181d expression in each imatinib-resistant cell line (Fig. 4.5.3D-F). These data support our hypothesis that hyper-activation of Lyn represses miR181 expression in imatinib-resistant CML.

4.3.4. Mcl-1 is a target of miR181b

To investigate the potential significance of miR181 to cell survival, we sought to identify mRNA targets for miR181. Public database bioinformatic algorithms (www.targetscan.org) predict a binding site for miR181 on the 3' UTR of Mcl-1, a Bcl-2 family member and anti-apoptotic protein (Fig. 4.5.4A) [243]. Mcl-1 expression inversely correlates with leukemia chemosensitivity [242], and Western blot analysis determined that Mcl-1 expression was higher in MYL-R cells (Fig. 4.5.4B). Furthermore, inhibition of Lyn with dasatinib decreased Mcl-1 expression in a dose-dependent manner, demonstrating the importance of Lyn activity in regulating Mcl-1 expression (Fig. 4.5.4C).

To determine whether miR181b regulates Mcl-1 expression, MYL-R cells were nucleofected with a miR181b-expressing plasmid vector (SDSA-miR181b) or empty vector (SDSA 3.0) control. Expression of GFP confirmed nucleofection and plasmid processing (Fig. 4.5.5A). In addition, qRT-PCR confirmed miR181b expression in these experiments (data not shown). As shown in Figure 4.5.5A, over-expression of miR181b in MYL-R cells significantly decreased Mcl-1 expression in comparison cells expressing the empty vector control.

MiRNAs regulate protein expression by binding to the 3' UTR of target mRNA and initiate mRNA degradation or inhibit translational processing [157]. To determine whether miR181-dependent inhibition of Mcl-1 expression was due to a direct

interaction, HEK293T cells were co-transfected with the miR181b construct or empty vector and a luciferase-reporter construct containing the 3' UTR of Mcl-1 mRNA (Fig. 4.5.5B). Using this approach, we observed that over-expression of miR181b significantly inhibited luciferase activity in comparison to the vector control cells (Fig. 4.5.5C). Because the miRNA “seed region” is critical for the recognition of miRNA targets, complementary nucleotides within this region were mutated in the 3' UTR of the Mcl-1 luciferase reporter (Fig. 4.5.5B). These mutations ablated the miR181b-dependent repression of luciferase activity (Fig. 4.5.5C), whereas mutation of nucleotides outside the seed region failed to affect this process (data not shown). These data suggest that miR181b directly interacts with the 3' UTR of Mcl-1 mRNA to inhibit Mcl-1 protein expression, and nucleotides within the seed region of this binding site are important to mediate this effect.

4.3.5. Lyn kinase regulates miR181b and Mcl-1 expression in AML

The HL-60 cell line is a commonly used cell model for the study of AML. Previously, it was shown that 1,25-D₃ treatment increased the expression of Lyn in these cells [246]. To determine whether manipulation of Lyn expression in HL-60 cells affected miR181b and Mcl-1 expression, we treated these cells with 1,25-D₃ for up to 96 hr. A strong increase in active Lyn expression was observed by Western blotting these samples (Fig. 4.5.6A). In agreement with our data obtained with the MYL-R and K562R cells, the increase in Lyn expression correlated with repression of miR181b and increased Mcl-1 expression in a time-dependent manner (Fig. 4.5.6A-B). Thus, these results indicate that Lyn can affect Mcl-1 expression through modulation of miR181b expression in an AML cell line.

4.4. Discussion

These studies describe three important findings: one, that the hyper-activation of Lyn suppresses the expression of miR181; two, that miR181b represses the expression of a key anti-apoptotic protein, Mcl-1; and three, loss of miR181 upon Lyn activation may represent a novel mechanism of drug resistance in leukemia. This mechanism was observed in cell models of CML and AML, and the specific role of Lyn was confirmed by dasatinib treatment, Lyn-targeted siRNA, and over-expression of Lyn. Considerable evidence now supports the role of Lyn as a “compensatory oncogene” in imatinib-resistant CML and specific subtypes of AML. Lyn is over-expressed in drug-resistant cell lines and patient samples [128, 129, 131] and activated in response to Flt3 activation or Flt3-ITD mutation [152]. Using a model of Lyn-dependent CML, we observed strong repression of the miR181 family of miRNAs. Previously, low miR181 expression has been associated with poor prognosis in AML, whereas high miR181 (a-d) expression is prognostic for event free survival in AML patients [164, 176, 177].

Although our studies primarily focused on miR181b, this family of miRNAs is highly conserved. Previous studies suggest Bcl-2 as a target for repression by miR181 microRNAs [173, 179]. Comparison of the potential 3' UTR targeting sequence of Bcl-2 with that of Mcl-1 demonstrates a remarkable homology between these sequences, which includes conservation within the seed sequence. The seed sequence is a key region of complementarity necessary for the targeting of miRNAs [247]. Our results demonstrated that mutation of the nucleotides complementary to the miR181 seed sequence in the 3' UTR of Mcl-1 ablated the effects of miR181b.

Whether miR181 directly targets Bcl-2 has yet to be confirmed; however, this is would establish the importance of the miR181 family in the regulation of mitochondria-mediated apoptosis.

Previous studies have identified multiple miRNAs that are altered in drug-resistant cancer, suggesting that some miRNAs have tumor suppressive effects, whereas others mediate cell survival [248]. A recent study profiled miRNAs from imatinib-resistant CML patients who did not have BCR-Abl mutations [249]. These authors identified 19 miRNAs that were differentially expressed between drug-resistant patients and drug-responders. In agreement with their results, we observed down-regulation of the expression of miR183 and two members of the Let-7 family (Let-7a, Let-7b). However, in contrast to studies reporting decreased miR10a expression in drug-resistant CML, we observed increased miR10a expression in the MYL-R cells. Many of the other miRNAs reported by San Jose-Eneriz et al. were either unchanged or not present on our arrays.

Previous studies have identified miRNAs that target Mcl-1 [171, 173, 174], and we measured the expression of these miRNAs in our cell lines; however, the expression of these miRNAs were not implicated in the mediation of Mcl-1 regulation in MYL and MYL-R cells, suggesting that they may contribute to Mcl-1 expression in a cell-type specific manner. In addition to the miR181 family, we observed many unique differences in miRNA expression between MYL and MYL-R cells (miR10a, miR128a, miR132, miR150, miR155, miR183, miR196a, and miR212). It is possible that some of these miRNAs may play a role in the mediation of drug resistance, and this will be a focus of future research.

Recently, Chen et al. demonstrated that miR181a over-expression sensitized cells to radiation [173]. Moreover, Studzinski and colleagues have demonstrated that 1,25-D₃ treatment of HL-60 cells not only increases Lyn expression, but also decreases miR181 expression and increases Mcl-1 expression [246, 250, 251]. This work was published independently over a course of a decade. Our study is in agreement with their data, and, importantly, we believe our study provides further mechanistic insight to these observations. Finally, Studzinski and colleagues determined that 1,25-D₃ treatment decreases sensitivity to drugs that induce apoptosis [252]; this observation is in agreement with our data suggesting that Lyn-dependent loss of miR181 may contribute to the development of imatinib resistance.

While the mechanism of Lyn repression of miR181 is not known, miRNA expression can be regulated by cell signaling events by both genetic and epigenetic mechanisms. For instance, the role of BCR-Abl in the silencing of miR328 in CML through a MAPK-dependent manner was recently shown [253]. Furthermore, activating mutations of the c-Kit kinase induce a MYC-dependent repression of miR29b in AML [254]. In addition to transcription factor (CEBP α , Myc, etc.)-dependent mechanisms, kinases may regulate miRNA expression through post-transcriptional events, such as that observed with Lin28 [165, 168]. Future studies will aim to determine the mechanism of Lyn-dependent effects on miR181 expression.

4.5. Figures

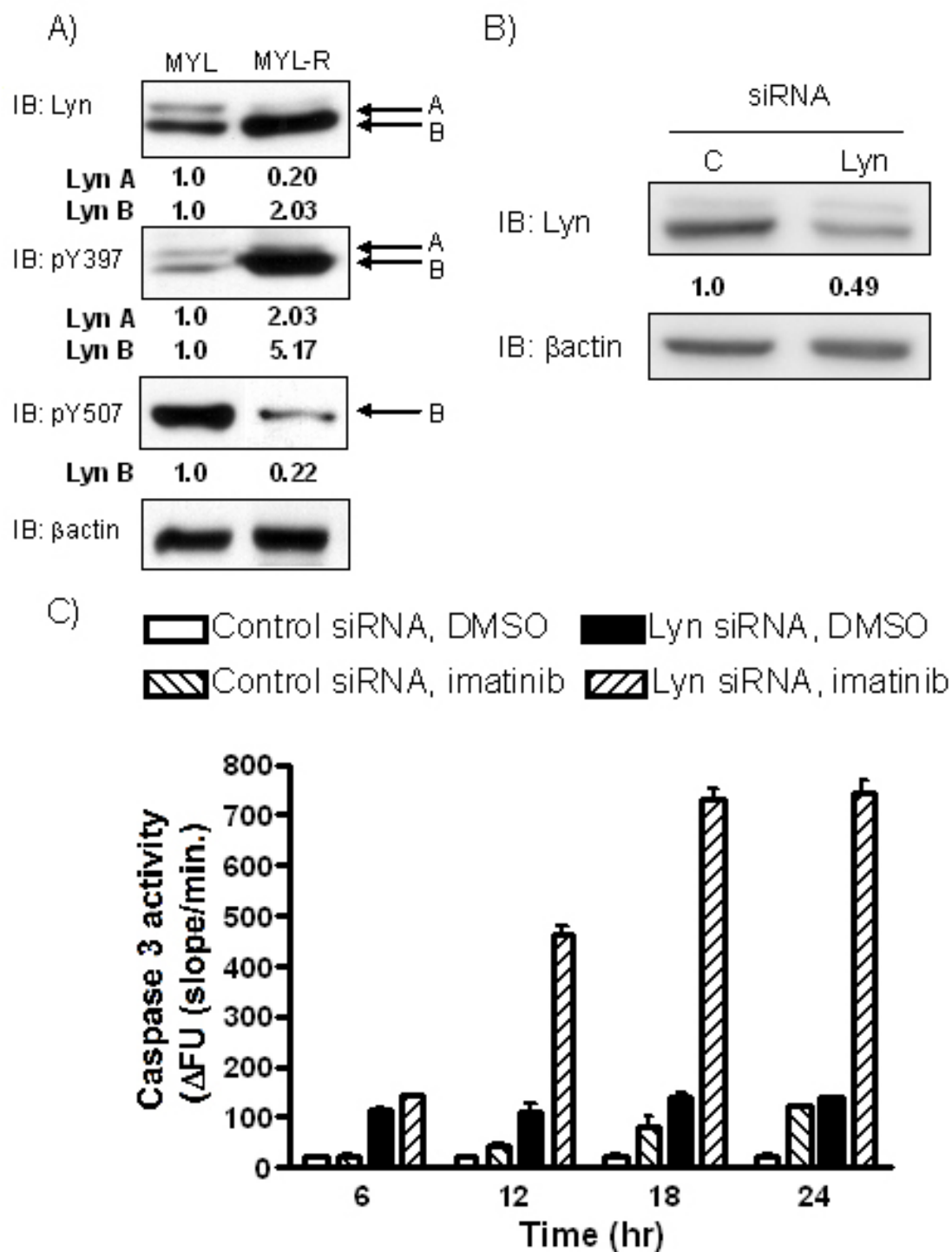
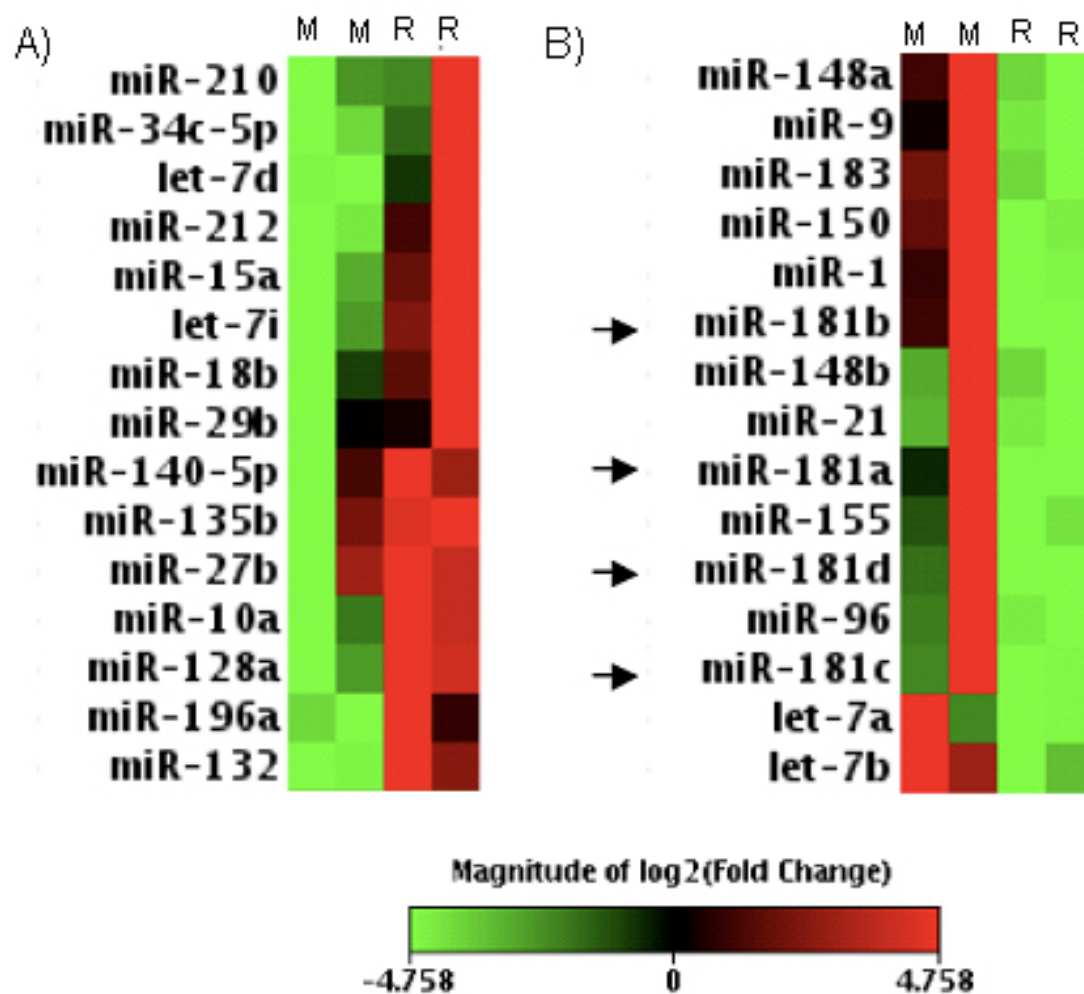


Figure 4.5.1. Lyn kinase confers imatinib resistance in MYL-R cells. A) 2×10^6 cells were lysed and Western analysis was performed using the indicated antibodies (pY507, inhibitory phosphorylation site; pY397, autophosphorylation site) and densitometry was performed. Representative data are shown. B and C) 1×10^6 MYL-R cells were nucleofected with siRNA (100 nM) against Lyn or non-targeting control siRNA (C). B) Forty-eight hr later, cells were lysed and Western blot analysis was performed using the indicated antibodies and densitometry was performed. Representative data are shown. C) Cells were plated in a 96-well plate (6×10^5 /well) and cultured with imatinib (1.0 μ M) or DMSO for the indicated time. Cells were then lysed and caspase 3 activity was measured as described in Section 2.3.4. *Caspase 3 activity assay*. Data represent the mean \pm S.E.M. of triplicate samples.



C)

microRNA	MYL-R Fold change
181a	-11.21
181b	-13.59
181c	-12.95
181d	-25.78

D)

hsa-miR-181b	AACAUUC AUUGCUGUCGGUGGGU
hsa-miR-181d	AACAUUC AUUGUUGUCGGUGGGU
hsa-miR-181a	AACAUUC AACGCUGUCGGUGAGU
hsa-miR-181c	AACAUUC AAC-CUGUCGGUGAGU
	***** ***** **

Figure 4.5.2. Loss of miR181 expression in MYL-R cells. A and B) MYL and MYL-R miRNA expression was determined by qRT-PCR using the Human Cancer RT² miRNA qPCR Array according to the manufacturer's instructions. The heat map depicts fifteen miRNAs that had a >2-fold A) increase or B) decrease in expression in MYL-R cells in comparison to MYL cells. Arrows denote miR181 family members. Data represent duplicate experiments performed with triplicate samples. C) The fold change in miR181 expression in MYL-R cells in comparison to MYL cells. D) Alignment of human miR181 miRNAs (5'→3') using ClustalW software (<http://align.genome.jp/>). An asterisk denotes a conserved nucleotide.

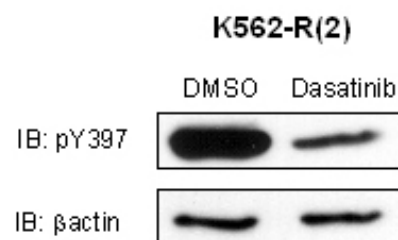
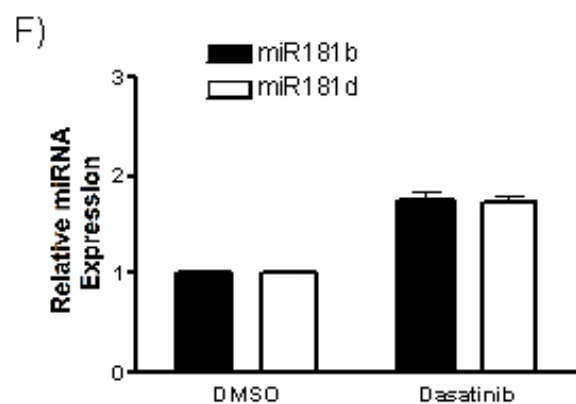
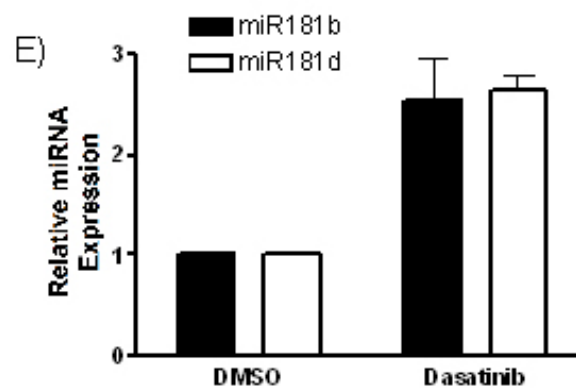
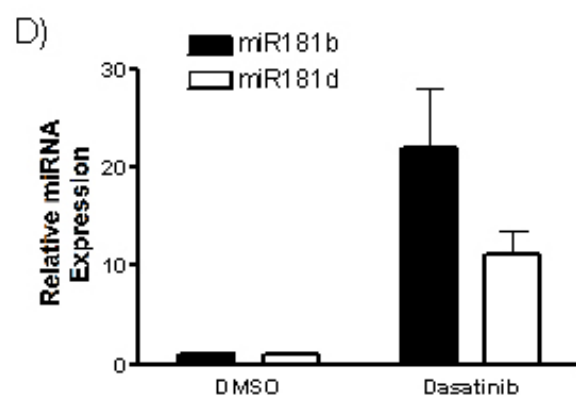
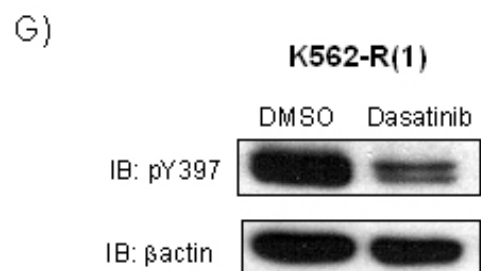
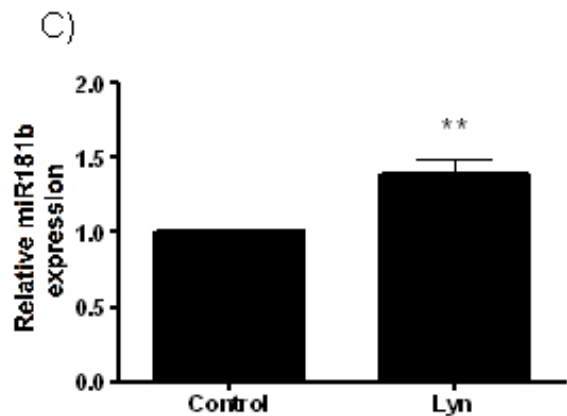
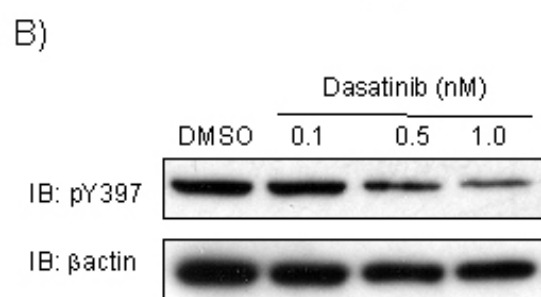
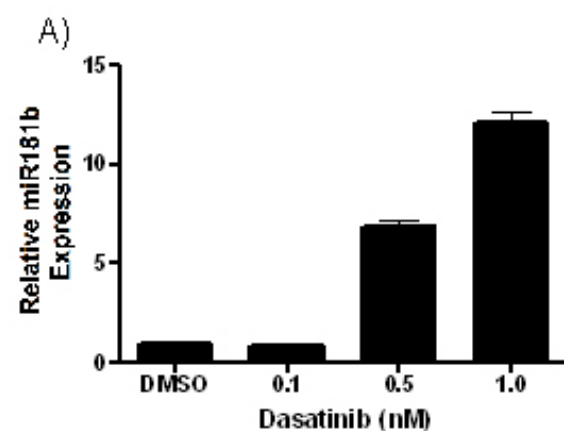


Figure 4.5.3. Lyn kinase inhibition increases miR181 expression. A and B) 2×10^6 MYL-R cells were treated for 24 hr with the indicated dose of dasatinib or DMSO. A) Total RNA was extracted and miR181b expression was measured by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples. B) Cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown. C) 1×10^6 MYL-R cells were nucleofected with siRNA against Lyn or non-targeting control siRNA. Forty-eight hr later, RNA was extracted and miR181b expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of duplicate experiments performed with triplicate samples (**, $p=0.0036$). D-G) 2×10^6 cells were treated for 24 hr with 1 nM dasatinib or DMSO. D-F) Total RNA was extracted from D) MYL-R, E) K562-R(1), and F) K562-R(2) cells and miRNA expression was measured by qRT-PCR. Data represent the mean \pm S.E.M. of duplicate experiments performed with triplicate samples. G) 1×10^6 cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown.

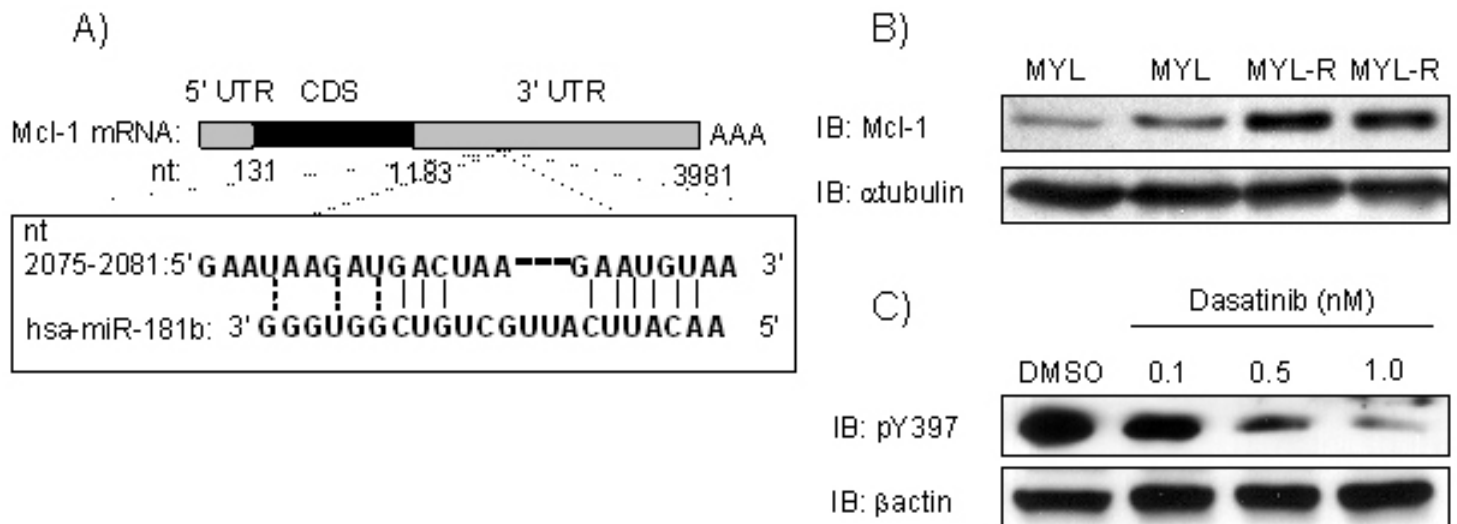


Figure 4.5.4. Mcl-1, a predicted target of miR181, is over-expressed in MYL-R cells. A) Schematic depiction of the predicted miR181 binding site in the Mcl-1 3' UTR (www.targetscan.org). B) 2×10^6 cells were lysed, and Western blot analysis was performed using the indicated antibodies. Samples were loaded in duplicate. Representative data are shown. C) 2×10^6 MYL-R cells were treated for 24 hr with the indicated dose of dasatinib or DMSO. Cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown.

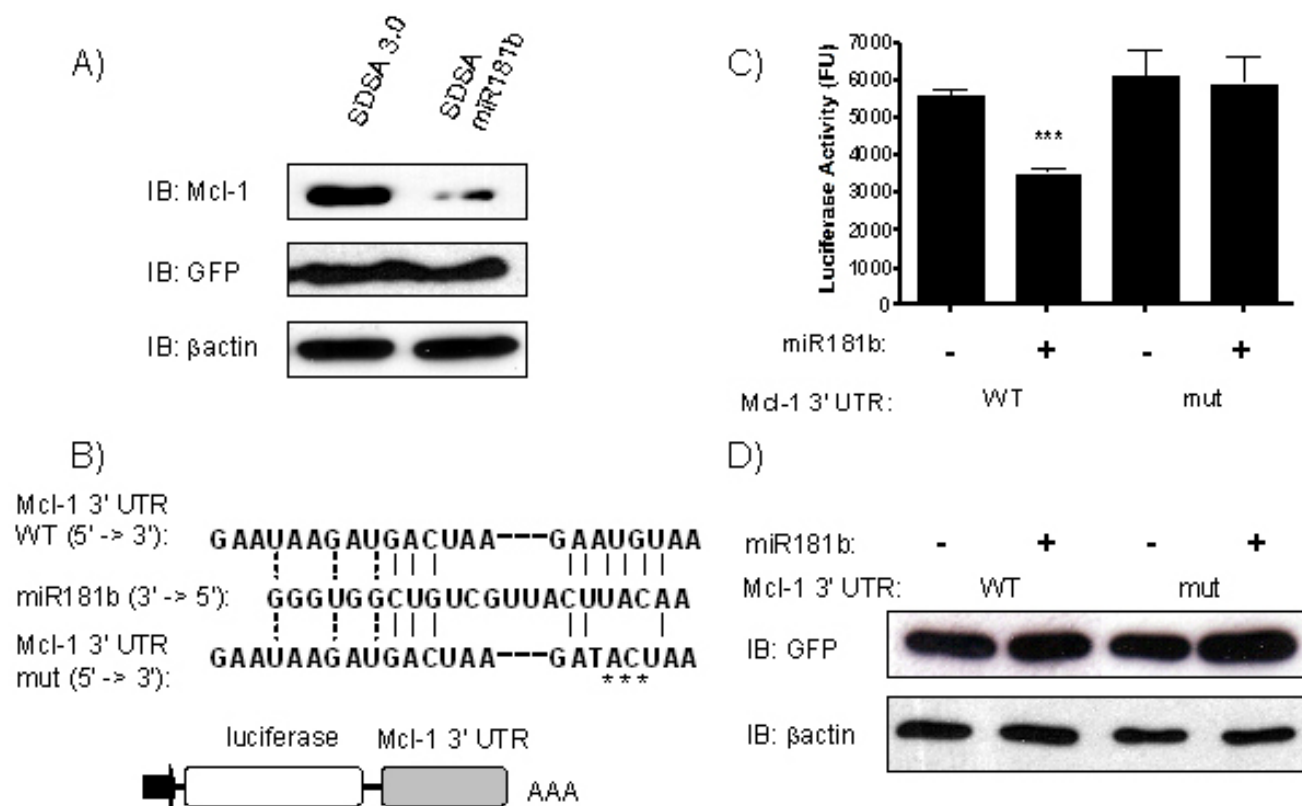


Figure 4.5.5. MiR181b inhibits Mcl-1 expression. A) 1×10^6 MYL-R cells were nucleofected with the SDSA 3.0 miR181b vector or empty vector (SDSA 3.0). Forty-eight hr later, cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data is shown. B) Schematic depiction of luciferase constructs containing the Mcl-1 3' UTR. Alignment of miR181b with the WT and mutant Mcl-1 3' UTR sequences. An asterisk denotes a nucleotide mutation in the predicted miR181b binding site. C and D) HEK293T cells (3×10^5) were co-transfected with the indicated SDSA 3.0 and psicheck Mcl-1 3' UTR vectors. Twenty-four hr later cells were lysed. C) Luciferase activity was measured as described in Section 2.3.8. *Transfection and luciferase activity assay*. Data represent the mean \pm S.E.M. of duplicate experiments performed with triplicate samples (***, $p < 0.001$). D) Western blot analysis was performed with the indicated antibodies. Representative data is shown.

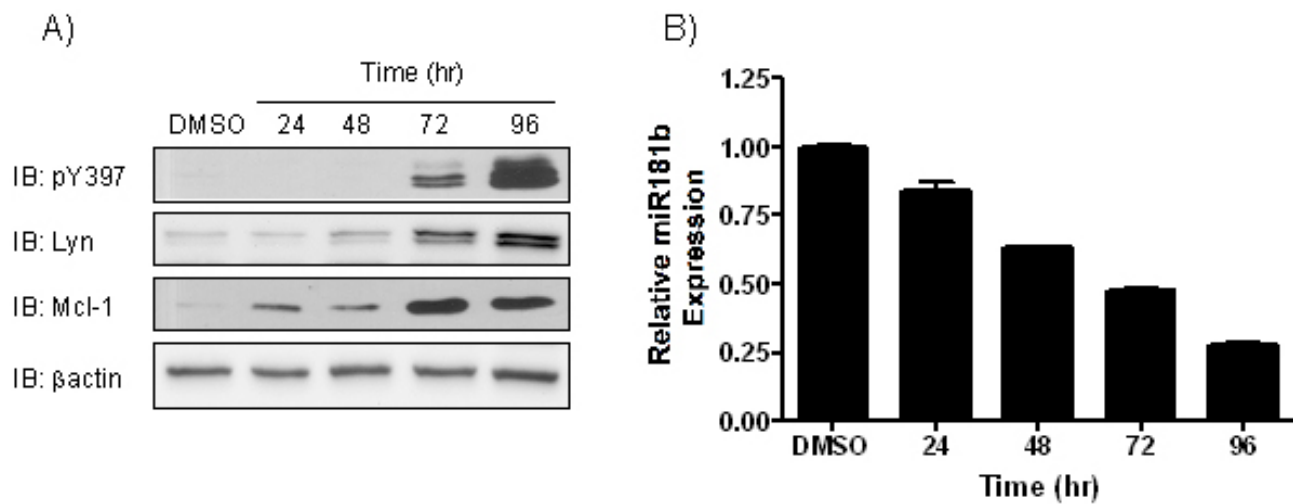
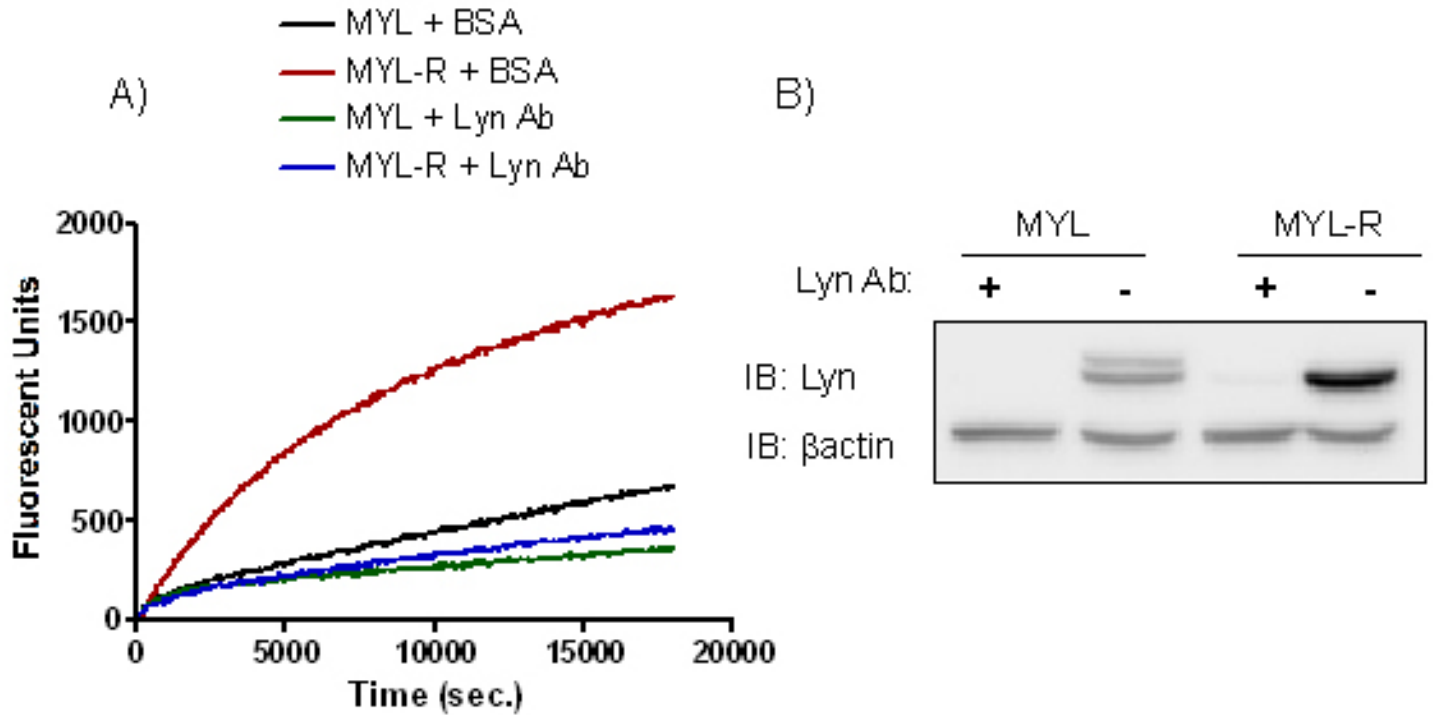
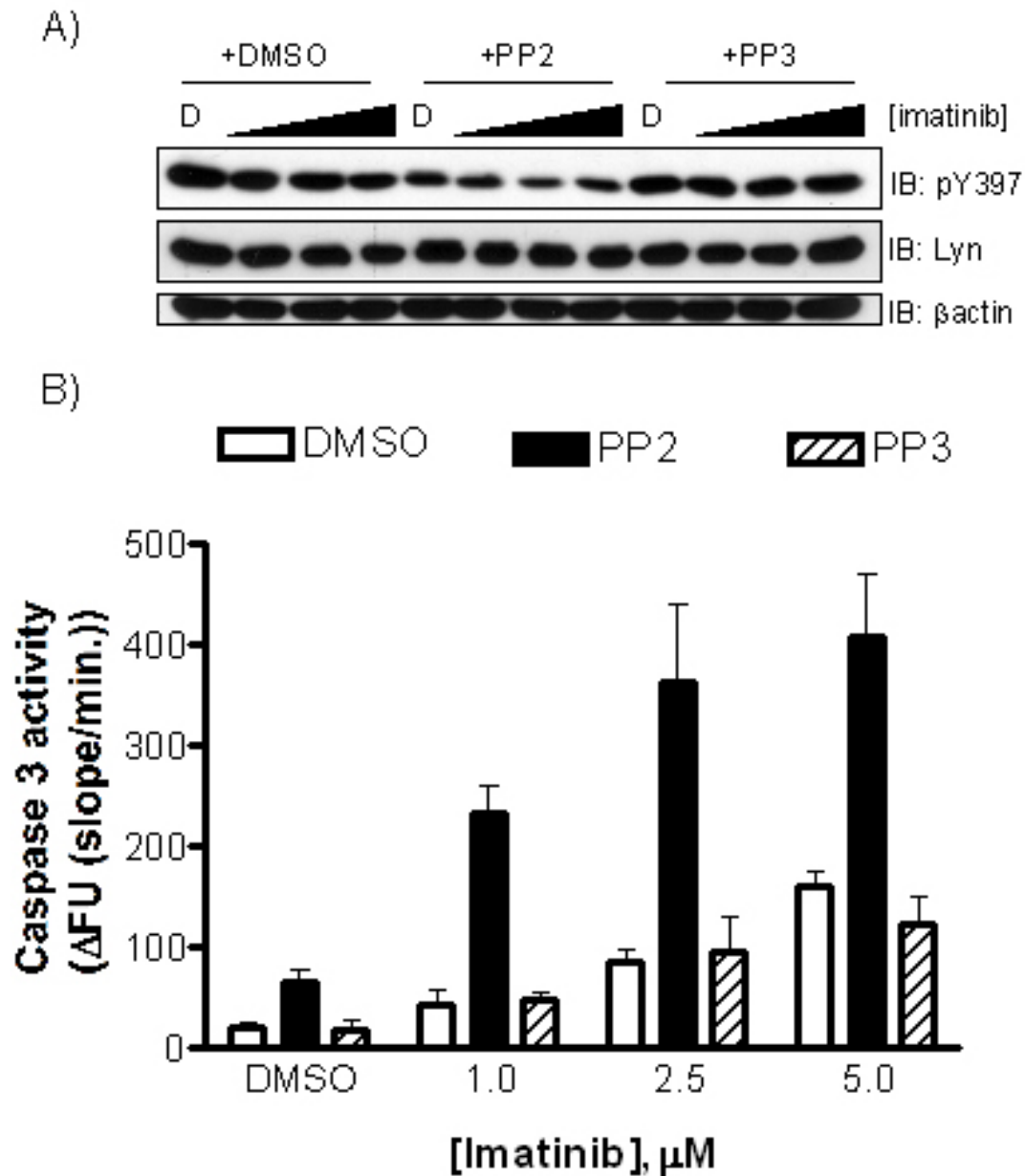


Figure 4.5.6. Up-regulation of Lyn kinase expression in HL-60 cells decreases miR181b expression and increases Mcl-1 expression. A and B) 1×10^6 HL-60 cells were treated with DMSO (96 hr) or 10 ng/ul 1,25-D₃ for the indicated time. A) Cells were lysed, and Western blot analysis was performed using the indicated antibodies. Representative data is shown. B) Total RNA was extracted and miR181b expression was measured by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples.

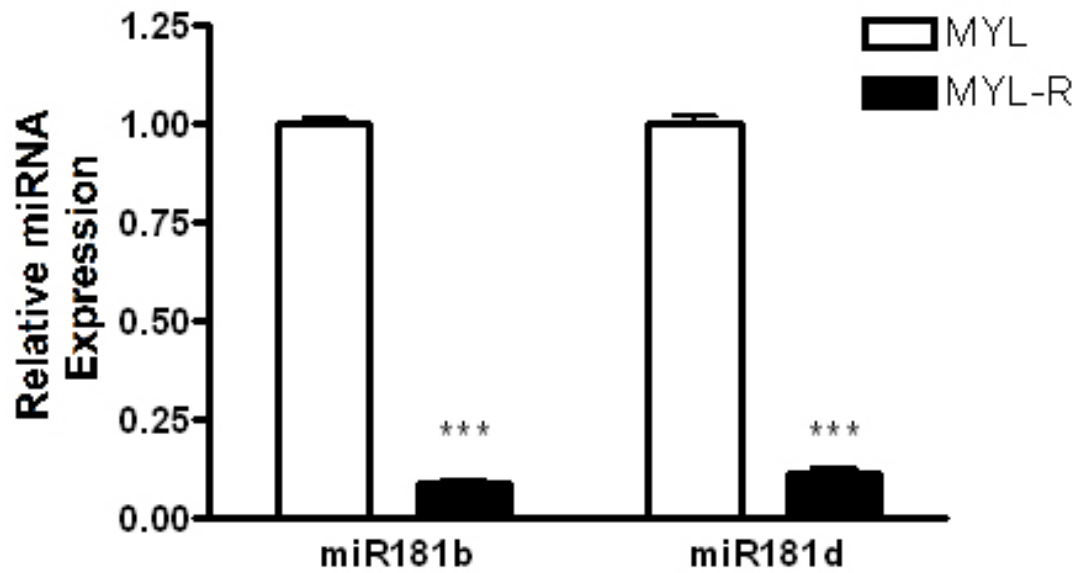
4.6. Supplemental figures



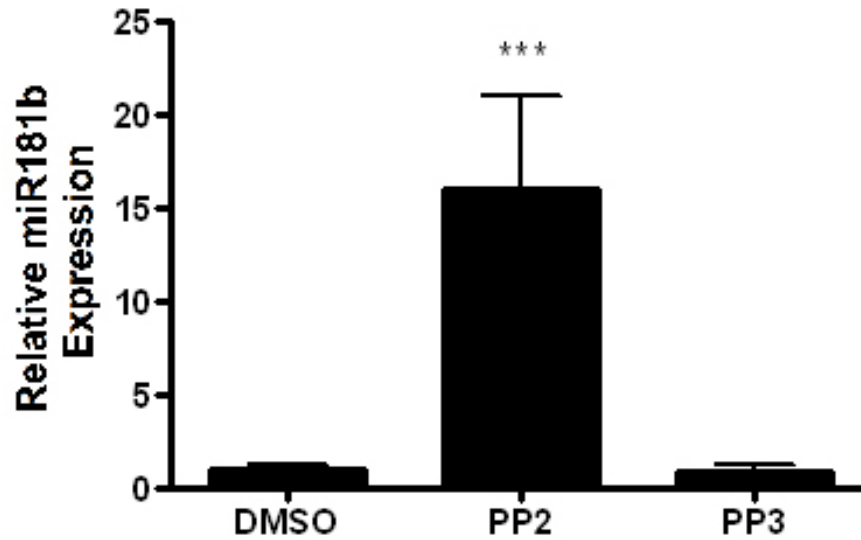
Supplemental Figure 4.6.1. Lyn kinase is hyper-activated in MYL-R cells. A and B) Cells (2×10^6) were lysed and 500 μg of total cell lysate was incubated with agarose beads conjugated to anti-Lyn antibodies or BSA at 4°C for 2 hr. Samples were centrifuged and the supernatant was collected. A) Supernatant ($2.5 \mu\text{g}/\mu\text{l}$) was incubated with a fluorophore-conjugated Lyn peptide sensor (Ac-E-K-E-I-Y-G-E-I-E-A-NH₂) and fluorescence was recorded on a plate reader. B) Western blot analysis was performed on the supernatant using the indicated antibodies. Representative data are shown. Adapted from [221].



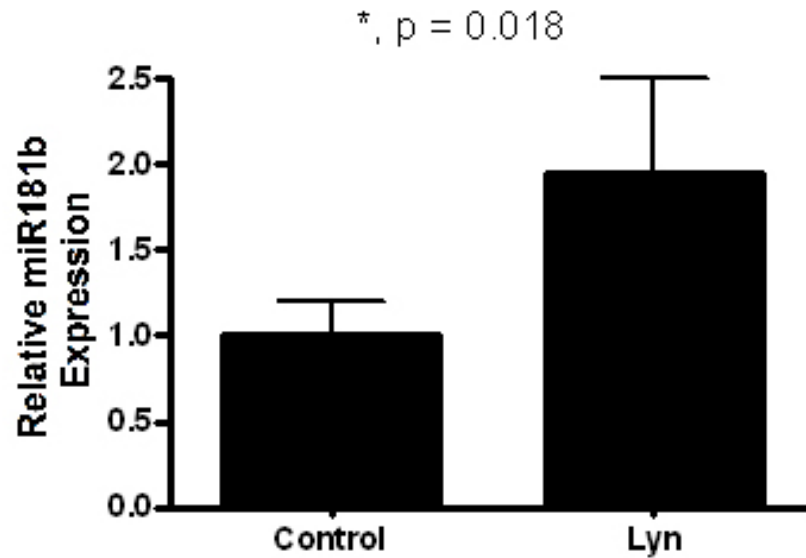
Supplemental Figure 4.6.2. Lyn kinase inhibition sensitizes MYL-R cells to imatinib treatment. A and B) MYL-R cells (2×10^6) were incubated with DMSO, PP2 (5 μ M), PP3 (5 μ M), or imatinib or a combination thereof for 24 hr. A) Cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown. B) Cells were lysed and caspase 3 activity was measured as described in Section 2.3.4. *Caspase 3 activity assay*. Data represent the mean \pm S.E.M. of triplicate samples.



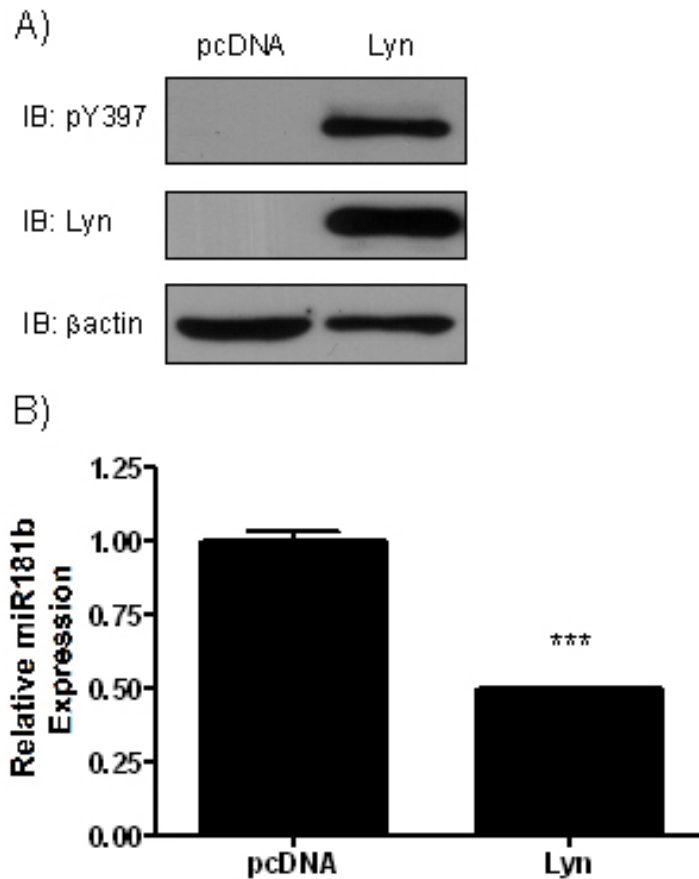
Supplemental Figure 4.6.3. Quantitative RT-PCR of miR181b and miR181d expression. Total RNA was extracted from 2×10^6 cells and miRNA expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate experiments performed with triplicate samples (***, $p < 0.001$).



Supplemental Figure 4.6.4. Lyn kinase inhibition increases miR181b expression. MYL-R (2×10^6) cells were treated with DMSO, PP2 (5 μ M), or PP3 (5 μ M) for 24 hr. Total RNA was extracted and miR181b expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples (***, $p < 0.001$).



Supplemental Figure 4.6.5. Treatment with Lyn kinase-directed siRNA increases miR181b expression. MYL-R (1×10^6) cells were nucleofected with 100 nM non-targeted control or Lyn-directed siRNA. Forty-eight hr later, total RNA was extracted and miR181b expression was determined by qRT-PCR using the method of Thomson et al. 2006. Data represent the mean \pm S.E.M. of duplicate experiments performed with duplicate samples.



Supplemental Figure 4.6.6. Lyn kinase over-expression decreases miR181b expression. A and B) HEK293T cells (2×10^6) were transfected with pcDNA or Lyn-expressing plasmids. Analysis was performed 36 hr post-transfection. A) Cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown. B) Total RNA was extracted and miR181b expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples (***, $p < 0.001$).

CHAPTER V

Elucidating the Mechanism(s) of miR181 Transcriptional Regulation

All figures contributed by Eric I. Zimmerman.

5.1. Abstract

In Chapter 4, we describe the negative regulation of miR181 by Lyn kinase (Lyn). In this chapter, we attempt to determine the mechanism(s) of Lyn-dependent miRNA regulation. Initially, we observed that expression of the miR181b primary transcript was severely reduced in MYL-R cells in comparison to MYL cells. In addition, transcript levels were increased after Lyn inhibition, similar to the effects on mature miRNA expression. Thus, we hypothesized that Lyn regulates miR181 expression at the transcriptional level. After profiling the effects of dasatinib treatment on kinase signaling using the Proteome Profiler Human Phospho-kinase Array, we determined that Lyn regulates the phosphorylation of the transcription factors (TFs) CREB and STAT5 in MYL-R cells. However, subsequent inhibition of these TFs did not affect miR181b expression. In contrast to the relatively uncharacterized promoter elements for miR181b, the miR181c/d promoter contains two CpG islands and treatment with the DNA methyltransferase inhibitor 5-aza-C increased miR181c/d expression. Furthermore, increased histone acetylation after treatment with histone deacetylase inhibitors (HDACi) or dasatinib correlated with enhanced miR181c expression. However, in contrast to dasatinib treatment, HDACi treatment also reduced the expression of Lyn, possibly due to loss of HSP90 activity. Thus, in regard to the miR181c/d gene cluster, Lyn may regulate expression by affecting DNA methylation indirectly through the modification of histones. Future studies will address this hypothesis and elucidate the effects of Lyn on gene expression.

5.2. Introduction

It is speculated that microRNAs (miRNAs) impact the expression of nearly one-third of all protein-coding genes [255], and miR181, in particular, regulates the expression of oncogenes implicated in hematopoietic disorders, such as TCL-1 and p27 [250, 256]. Thereby, miRNAs are involved in numerous developmental and physiological processes. Due to the broad impact of this RNA class, it is important to elucidate the regulation of miRNA expression by cell signaling cascades to better understand human disease. In this chapter, I attempt to address a logical extension of the conclusions from Chapter 4: how is miR181 regulated by Lyn kinase?

MiRNA biogenesis is regulated at both the transcriptional and post-transcriptional levels. These non-coding RNAs are initially transcribed as primary transcripts (pri-miRNA), which are often thousands of nucleotides in length and acquire a stem-and-loop structure [157]. After processing by the RNase Drosha into a shorter (100-500 nt) pre-miRNA form and subsequent export from the nucleus, the RNase Dicer produces the mature species, a molecule typically 20-22 nt in length. MiRNA then bind, by virtue of complementary base-pairing, to mRNA and initiate mRNA silencing by degradation [257], deadenylation [258], or translational repression [259] through association with the RNA-induced silencing complex (RISC).

Many miRNAs are located within largely uncharacterized intergenic regions of the genome. These regions are void of genes and may span kilobases of nucleotides. Therefore, intergenic miRNAs are thought to be under the control of unique promoter elements. By contrast, miRNAs that are located within the introns of

existing mRNA (intronic) are thought to be regulated coordinately with transcription of the host mRNA; however, as recent studies have demonstrated, this assumption may not always be valid [260].

In general, miRNA transcriptional regulation remains poorly understood. Most pri-miRNAs are transcribed by the polymerase II (Pol II) enzyme [261], whereas some pri-miRNAs are transcribed by Pol III [262]. However, a deficit in promoter sequence characterization and annotation of transcriptional start sites (TSSs) is a major limitation to understanding what additional factors regulate miRNA transcription. The observation that certain histone modifications (H3K4me3, H3K9 Ac) are associated with transcriptionally active promoters [263] and that nucleosome activity is depleted within 100-130 base-pairs of the TSS [264] have resulted in efforts to map miRNA promoter signatures [260, 265]. Future studies to discover miRNA promoter elements may be benefited by systems biologic and bioinformatic analyses.

The miR181 family of miRNAs consists of four members (miR181a-d). MiR181a is clustered with miR181b and miR181c is clustered with miR181d, suggesting that these miR181 pairs are coordinately regulated (miRBase; www.mirbase.org). In addition, the miR181a/b cluster is found at two genomic locations (1;q32.1 and 9;q33.3), whereas the miR181c/d cluster is located on chromosome 19 (19; p13.1). Each cluster is within an intergenic region and no family member is associated with a known gene. Additionally, the promoter regions and regulation of miR181 by transcriptional elements are largely undefined.

In this chapter, we took a logical approach to identify the mechanism(s) of Lyn kinase (Lyn)-dependent miR181 regulation. We initially focused on key transcription factors (TFs) responsive to Lyn inhibition and tested whether they could affect miR181b expression. Our findings argue against the regulation of miR181b by the TFs cAMP response element binding protein (CREB) and signal transducer and activator of transcription 5 (STAT5). Because previous studies suggest that the miR181c/d cluster is epigenetically regulated, we determined whether methylation and/or acetylation could explain the Lyn-dependent effects on miR181c/d expression [266, 267]. Our studies confirmed that the miR181c/d cluster is regulated by methylation in MYL-R cells. Interestingly, we found that treatment with the Src family kinase (SFK) inhibitor dasatinib enhanced histone acetylation and methylation, resulting in increased miR181c/d expression, presumably through a loss of DNA methylation. Furthermore, Lyn over-expression decreased histone acetylation, confirming the link between Lyn activity and the regulation of gene expression. These effects were similar to that of histone deacetylase inhibitor (HDACi) treatment; however, HDACi treatment also decreased HSP90 activity, subsequently reducing Lyn expression. Thus, HDACis may affect miR181c/d expression through both genomic and non-genomic mechanisms.

5.3. Results

5.3.1. Lyn kinase inhibition decreases the phosphorylation of CREB and STAT5

To determine the molecular consequences of Lyn inhibition, we treated MYL-R cells with the SFK inhibitor dasatinib and analyzed the activity of multiple kinase

signaling pathways using the Proteome Profiler Human Phospho-kinase Array. Treatment with 10 nM dasatinib for 24 hr resulted in specific changes in kinase activity. Among the SFKs measured on the array, we detected a loss of both Lyn (Y397) and Yes (Y426) phosphorylation, whereas, surprisingly, an increase in Fyn (Y420) phosphorylation was observed (Fig. 5.5.1A-B). In addition, Chk-2 (T68) phosphorylation was increased and phosphorylation of the TFs CREB (S133) and STAT5a/b (Y699) was decreased (Fig. 5.5.1A-B). Dose-dependent confirmation of the loss of CREB and STAT5 phosphorylation was obtained by Western blot analysis (Fig. 5.5.1C). Interestingly, dasatinib not only reduced STAT5 phosphorylation, but also decreased STAT5 expression (Fig. 5.5.1C).

Studies in our lab have shown that dasatinib-induced Chk-2 phosphorylation in MYL-R cells is due to activation of ataxia telangiectasia mutated (ATM) kinase (data not shown), probably as a result of a general stress response. Therefore, we focused on the relationship between Lyn and CREB and STAT5 activity. In this regard, analysis of the phosphorylation of CREB (S133) and STAT5 (Y694) between MYL-R cells and low Lyn-expressing MYL cells demonstrated that CREB and STAT5 phosphorylation corresponded with Lyn expression and activity (Fig. 5.5.1D).

5.3.2. Pri-miR181b expression is regulated by Lyn kinase

In Chapter 4, we describe that inhibition of Lyn rescues mature miR181 expression. To determine whether Lyn regulates miR181 at the transcriptional level, we measured the expression of pri-miR181b using non-quantitative RT-PCR. Primers were designed against both the pri-miR181b-1 (Chr 1;q32.1) and pri-miR181b-2 (Chr 9;q33.3) transcripts. Initially, we profiled primary transcript

expression in MYL and MYL-R cells and found a profound loss of both miR181b-1 and miR181b-2 in the MYL-R cells (Fig. 5.5.2A-B). To test whether loss of primary transcript expression was due to enhanced Lyn activity, we treated MYL-R cells with dasatinib and observed a dose-dependent increase in pri-miR181b-2 expression (Fig. 5.5.2C-D). Though we cannot rule out the possible effects of Lyn on post-transcriptional miRNA regulation, the primary transcript data suggest that Lyn strongly affects miR181 transcription.

5.3.3. CREB and STAT5 do not regulate miR181b expression in MYL-R cells

We tested whether the TFs CREB and STAT5, which are responsive to Lyn activity, could regulate miR181 expression. CREB is phosphorylated and activated by several signaling pathways in myeloid cells; SFKs [268], cAMP-dependent protein kinase (PKA) [269], protein kinase C (PKC) [270], and the mitogen-activated kinase (MAPK) MEK [271] directly or indirectly increase CREB S133 phosphorylation, leading to TF activation. However, the pathway(s) responsible for CREB activation in MYL-R cells is unknown. Therefore, we measured CREB phosphorylation after treatment with the inhibitors Go6983 (PKC; 5 μ M), H89 (PKA; 10 μ M), and U0126 (MEK; 10 μ M). We found that only PKC inhibition reduced CREB phosphorylation in MYL-R cells and that these effects were similar to dasatinib (1 nM) treatment (Fig. 5.5.3A). However, in contrast to dasatinib treatment, PKC inhibition did not affect the expression of miR181b at the time points tested, suggesting that CREB does not regulate miR181 transcription in response to Lyn activation (Fig. 5.5.3B).

STAT5 promotes myelogenous leukemia cell survival and malignancy and is a substrate of multiple tyrosine kinases, including Lyn [272]. Because dasatinib

treatment reduces the phosphorylation of STAT5 in MYL-R cells, we tested whether inhibition of STAT5 affected miR181 expression. We treated MYL-R cells with siRNA directed against STAT5 and measured mature miR181b expression using qRT-PCR. Though a profound (60-70%) and sustained knockdown of STAT5 was achieved, loss of STAT5 minimally affected miR181b expression (Fig. 5.5.3C-D), suggesting that STAT5 is not involved in Lyn-dependent miR181 regulation. In summary, though Lyn (and PKC)-dependent activation of CREB and Lyn-dependent activation of STAT5 is up-regulated in MYL-R drug-resistant cells, these signaling pathways may have consequences other than the regulation of miR181b expression.

5.3.4. MiR181c/d expression is increased after treatment with inhibitors against DNMT and HDAC

In contrast to the relatively uncharacterized promoters for the miR181a/b clusters, the region upstream of the miR181c/d cluster contains two CpG islands (Fig. 5.5.4A). A recent report by Hashimoto et al. identified the miR181c/d TSS and described the regulation of miR181c by methylation of the upstream CpG islands [267]. We found that Lyn inhibition not only increased miR181b expression, but also increased the expression of the miR181c and miR181d in MYL-R cells, suggesting that the miR181c/d cluster is under the regulation of this kinase (Fig. 5.5.4B and 4.5.4D). Therefore, we determined whether Lyn could regulate miR181c and miR181d expression through epigenetic mechanisms.

We initially determined whether the miR181c/d cluster could be regulated by methylation in MYL-R cells. Cells were treated with the DNA methyltransferase (DNMT) inhibitor 5-aza cytidine (5-aza-C) and, in both a dose- and time-dependent

manner, an increase in miR181c and miR181d expression was observed (Fig. 5.5.4C-D). In agreement with Hashimoto et al., the degree of miR181c increase was much higher than miR181d, possibly indicating a difference in post-transcriptional regulation of the two miRNAs [267].

Though these data demonstrate that miR181c/d is regulated by DNMT activity, the discrepancy between the effects of dasatinib and 5-aza-C at 24 hr (Fig. 5.5.4B vs. Fig. 5.5.4D) suggests that Lyn does not affect miR181 expression through regulation of DNMTs. This is reinforced by the fact that DNMT activity is replication-dependent and the cell must go through several rounds of replication for 5-aza-C to be functional. However, MYL-R doubling time requires approximately 22-24 hours (unpublished observations). Therefore, the effects of 5-aza-C on miR181c/d expression are minimal. Thus, Lyn may affect DNA methylation, and thereby miR181c/d expression, through a mechanism not involving DNMTs.

Several studies have described active demethylation of DNA after treatment with HDAC inhibitors (HDACi) [273-275]. This mechanism is replication-independent and does not depend on the activity of DNMTs; it is thought that active demethylation involves the recruitment of active demethylases, such as MBD2, after hyper-acetylation of histones, which subsequently leads to an increase in gene expression [273]. Therefore, we determined the effects of HDACi treatment on miR181c expression. Treatment with the HDACi trichostatin A (TSA) increased acetylation of histone H3 in a dose-dependent manner (Fig. 5.5.4E) with a moderate increase in miR181c expression (Fig. 5.5.4F). Though DNA methylation was not measured directly, we observed a concomitant increase in histone H3K4

dimethylation (H3K4me2), a marker of DNA hypo-methylation (Fig. 5.5.4E) [276]. Thus, these data demonstrate that HDACi treatment increases miR181c expression, possibly through active demethylation of the promoter region.

5.3.5. Lyn kinase regulates histone acetylation and methylation

As a logical extension of the HDACi data, we tested whether inhibition of Lyn affected histone modification. Similar to HDACi treatment, dasatinib treatment increased histone H4 acetylation and these effects corresponded with an increase in miR181c expression (Fig. 5.5.5A-B). To more thoroughly profile the effects of dasatinib on histone modification, we treated cells with various concentrations of dasatinib and Western blotted for histone acetylation and methylation. As shown in Figure 5.5.5C, dasatinib dose-dependently increased the acetylation of histones H4 and H3. In addition, dasatinib treatment increased H3K4 dimethylation (Fig. 5.5.5C). Finally, over-expression of Lyn reduced histone H4 acetylation in HEK293 cells, indicating that the effects on histone modification are due to Lyn activity, specifically (Fig. 5.5.5D). In summary, these data suggest that Lyn regulates histone acetylation and methylation, which, in turn, may affect the expression of genes, such as the miR181c/d cluster.

5.3.6. HDAC inhibition decreases Lyn kinase expression and activity

As a control experiment, we determined whether HDACi treatment could affect Lyn signaling. To our surprise, we found that TSA dose-dependently decreased Lyn phosphorylation and expression (Fig. 5.5.6A). Since previous studies reported that acetylation of HSP90 decreases activity, resulting in the reduced

stability of HSP90 client proteins [277], we tested whether HDACi treatment affected HSP90 activity.

SNX5422 is a potent HSP90 inhibitor that binds to the active conformation of HSP90 [278]. Therefore, SNX5422-conjugated beads could be used to determine the amount of HSP90 activity. To test the ability of SNX5422 to recognize active HSP90, we treated cells with the HSP90 inhibitor 17-AAG and then incubated cell lysate with the SNX5422 beads. A dose-dependent reduction of HSP90 precipitation was observed, indicating a loss of HSP90 activity due to 17-AAG treatment (Fig. S5.6.1). HSP90 expression remained constant in the non-precipitated samples, demonstrating that 17-AAG treatment did not decrease protein stability (Fig. S5.6.1). SNX5422 beads were then incubated with TSA-treated MYL-R cell lysate. As expected, we observed a moderate dose-dependent reduction in HSP90 precipitation after TSA treatment, indicating a loss of HSP90 activity (Fig 5.5.6B). Thus, the effects of HDACi treatment on Lyn activity and protein expression may be due to inhibition of HSP90 activity.

5.4. Discussion

In this chapter, I attempted to determine the mechanism(s) of miR181 regulation by Lyn. From our studies, the following key observations were made: 1) Lyn regulates miR181b expression at the transcriptional level; 2) miR181b is not regulated by the TFs CREB and STAT5; 3) Lyn regulates histone acetylation and methylation, which may affect miR181c and miR181d expression; and 4) the mechanism of miR181c/d regulation by HDACi may involve both an increase in histone acetylation and a loss of Lyn signaling. Though the exact nature of miR181

regulation was not fully elucidated, these results provide a foundation for future experiments and insight into Lyn function in drug-resistant CML.

Figure 1 demonstrates that MYL-R cells are not only deficient in mature miR181b expression, but also exhibit a loss of pri-miR181b. Furthermore, dasatinib treatment increases the amount of pri-miR181b expression. The increase in pri-miR181b was approximately 3-fold at the highest dose of dasatinib tested; this is in contrast to an approximate 12-fold increase in mature miRNA expression (Fig. 5.5.2C-D vs. 4.4.5A). Only one miR181b transcript (pri-miR181b-2) was measured, so a coordinate increase in pri-miR181b-1 may explain the apparent discrepancy between primary transcript and mature miR181 levels. In addition, post-transcriptional regulation of miR181b may amplify mature miRNA expression. For instance, RNA-binding proteins have been found to regulate miRNA processing in a miRNA-specific manner. Lin-28 directly binds let-7 miRNA [165] and regulates let-7 maturation by inhibiting pri-miRNA and pre-miRNA cleavage by Drosha and Dicer, respectively [166, 279]. Other RNA-binding proteins, such as hnRNP A1, can enhance miRNA processing by inducing a conformational change preferred by these nucleases [280]. The post-transcriptional regulation of miR181 is unknown and future studies should determine whether miR181 undergoes alternative processing.

Nevertheless, the profound loss of pri-miR181b in MYL-R cells suggests that transcriptional regulation is the predominant mechanism of miR181b regulation in these cells. Furthermore, we show that the loss of activity of the TFs CREB and STAT5 are consequences of dasatinib treatment. Both TFs have been previously implicated in myeloid disorders; CREB is often over-expressed in acute

myelogenous leukemia (AML) and mediates growth factor-independent survival and proliferation of cells [281, 282], whereas STAT5 is a substrate of multiple hematopoietic kinase oncogenes (i.e. Lyn, BCR-Abl, and JAK) and is often associated with imatinib-resistant CML [283]. Due to the lack of miR181a/b promoter characterization we could not determine the presence of predicted STAT or CREB binding sites upstream of the miRNA sequences. Regardless, we tested whether down-regulation of these TFs affected miR181b expression.

Of the signaling pathways known to phosphorylate and activate CREB, only PKC and Lyn were found to regulate CREB phosphorylation in MYL-R cells. Therefore, we treated with selective inhibitors of these pathways and measured miRNA expression. In contrast to dasatinib treatment, treatment with the broad spectrum PKC inhibitor Go6983 did not affect miRNA expression at the time points tested. Thus, though PKC signaling is affected by Lyn inhibition, it is not involved in miR181b regulation.

We tested whether STAT5 could regulate miR181 expression by treating MYL-R cells with STAT5-directed siRNA. Though an efficient and sustained knockdown of STAT5 expression was obtained, this did not affect miR181b expression. While it is possible that the residual STAT5 still active during siRNA treatment maintained miRNA repression, our initial results suggest that this TF is not involved in miR181 regulation. Admittedly, a more comprehensive approach to determine the effects of Lyn inhibition on TF activity and signaling pathways is better suited to determine the mechanism of miR181 regulation; alternative approaches are described in more detail in Chapter 6.

In contrast to the miR181a/b clusters, the TSS is known for the miR181c/d cluster. Furthermore, it has been shown that these miRNAs are epigenetically regulated due to the presence of conserved CpG islands within the promoter region. Hashimoto et al. found that 5-aza-C treatment of gastric cancer cell lines decreased DNA methylation within the CpG islands and increased the expression of miR181c and miR181d [267]. We replicated these results in the MYL-R cells. Thus, these data suggest that Lyn may regulate miR181 expression by affecting DNA methylation status. However, the time course of the effects of DNMT inhibition on miR181 expression did not match the time course of dasatinib-mediated effects (Fig. 5.5.4B vs. 5.5.4D). Multiple cell replications are necessary for DNMT-mediated transfer of epigenetic information and for 5-aza-C-mediated inhibition of DNMTs; however, apparently, cell replication is not necessary for Lyn-mediated effects on miR181 expression because the MYL-R doubling time is approximately 22-24 hours. Therefore, we tested whether active demethylation could affect miR181c expression.

Replication-independent active demethylation occurs upon epigenetic modification of histones. Specifically, an increase in histone acetylation decreases heterochromatin formation, exposing DNA to demethylating enzymes, such as MBD2, and results in an increase in gene transcription [284]. Thus, numerous reports have shown that HDACi treatment increases gene transcription, at least in part, through DNA demethylation, molecular justification for the combined use of HDAC inhibitors and DNMT inhibitors for cancer therapy [285]. Furthermore, a study by Fayyad-Kazan et al. showed that treatment with the HDACi VPA resulted in an increase in miR181c expression in CD4⁺ T-cells [266]. Indeed, in the MYL-R cells

both VPA and TSA treatment resulted in an increase in miR181c expression. To determine whether this resulted in changes in methylation, we measured the dimethylation of histone H3 at lysine 4 (K4), a marker of DNA hypo-methylation and gene transcription [276]. As predicted, inhibition of HDACs increased methylation, suggesting that Lyn may alter histone modification to indirectly affect miR181c/d cluster expression.

To further test this hypothesis, we determined the effects of Lyn inhibition on histone modification. Importantly, we showed that dasatinib treatment increased histone acetylation, as measured by histone H4 and H3 acetylation. Furthermore, histone methylation was increased, recapitulating the results of HDACi treatment. Finally, Lyn over-expression resulted in a loss of histone H4 acetylation.

Lyn regulation of histone modification has broad implications and may provide a mechanism for not only miR181c/d regulation, but also the regulation of multiple other genes shown to be epigenetically sensitive. Whether Lyn promotes histone acetylation through inhibition of HDAC function is an important question for future studies. HDAC1 is phosphorylated on tyrosine residue 221, which is conserved in HDAC2 and HDAC3 (Fig. S5.6.2); however, the biological context of this event has not been determined [286]. In addition, it has been shown that HDAC3 co-localizes with Src kinase and is a substrate of this kinase [287]. Whether HDACs are directly phosphorylated by Lyn and whether this modification is relevant to HDAC function is currently being tested.

As a control experiment, we determined whether Lyn, itself, was affected by HDACi treatment. To our surprise, we found that TSA dose-dependently reduced

Lyn activity and expression. Though we have not measured Lyn transcription after HDACi treatment, previous reports have described HDACi-dependent enhancement of HSP90 acetylation, which reduces HSP90 activity. Acetylation-dependent loss of HSP90 function results in a reduction in the stability of HSP90 client proteins, such as BCR-Abl and c-Kit [288, 289]. This effect has been attributed to inhibition of HDAC6 [290], which is primarily cytoplasmic and associates with microtubules [291]. Because Lyn is an HSP90 client (data not shown), we hypothesized that the HDACi-dependent loss of Lyn expression is due to a reduction in HSP90 activity. This was confirmed by precipitation of HSP90 with SNX5422, an HSP90 inhibitor that binds only active HSP90, which demonstrated that HDACi treatment dose-dependently decreased HSP90 activity, presumably due to increased acetylation. Therefore, the effects of HDACi treatment on miR181c expression may be due to both genomic and non-genomic mechanisms. Future studies using selective HDAC6 inhibitors, such as tubastatin A [292], should be performed to determine the contribution of HSP90-dependent processes to miR181c/d cluster regulation.

Though this chapter does not fully elucidate the mechanism(s) of Lyn-dependent miR181 regulation, our data shed light on some interesting aspects of Lyn function. Studies designed to interrogate the mechanism of dasatinib-induced histone acetylation and the non-genomic functions of specific HDAC isoforms will increase our knowledge of cellular adaptation to drug treatment and may have broad implications for gene and protein regulation.

5.5. Figures

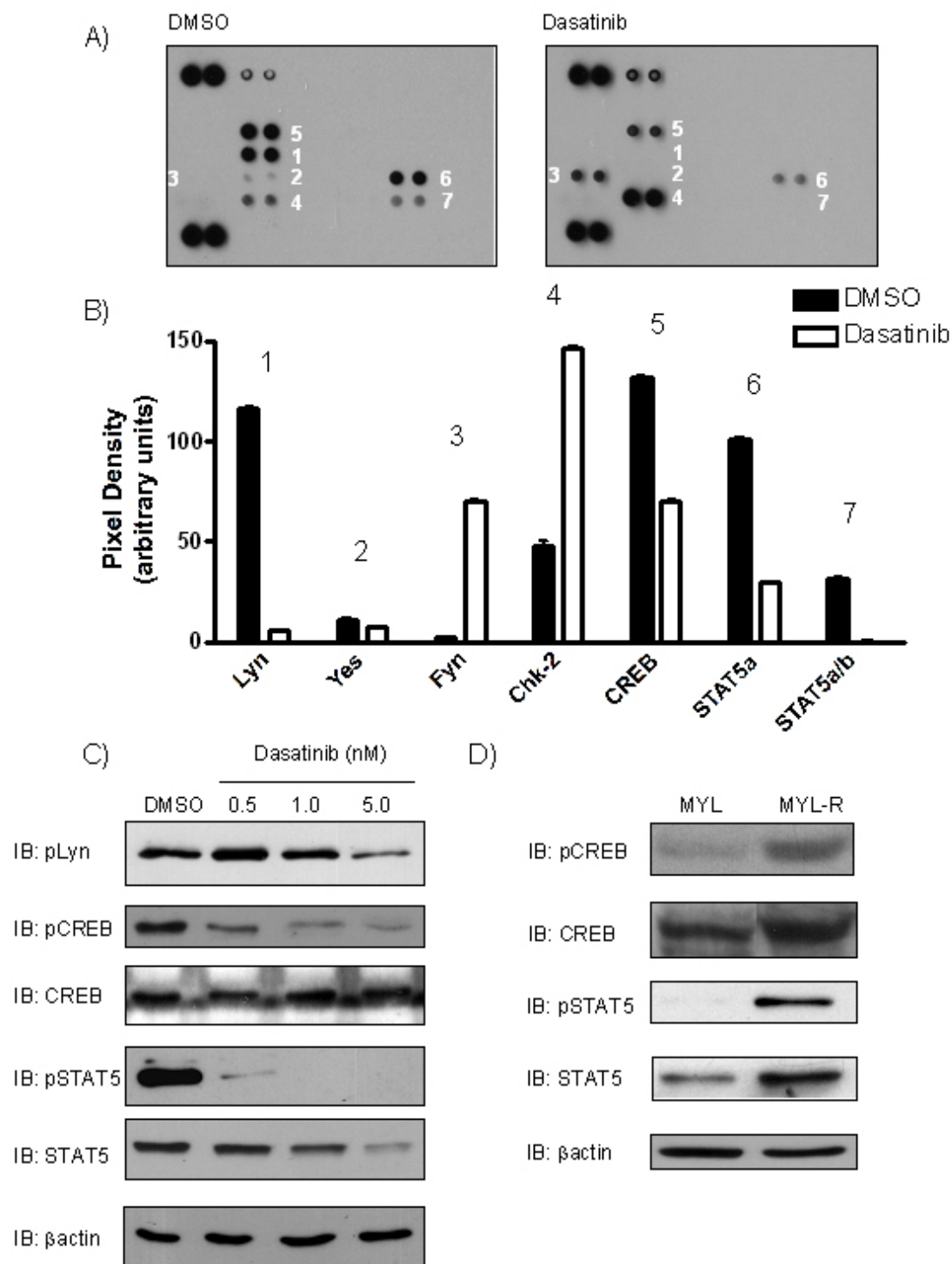


Figure 5.5.1. Lyn kinase inhibition decreases CREB and STAT5 activity. A) MYL-R cells (4×10^6) were treated with DMSO or 10 nM dasatinib for 24 hr. Analysis of protein phosphorylation was performed using the Proteome Profiler Human Phospho-kinase Array according to the manufacturer's instructions. B) Densitometry was performed using a FluorChem FC2 imager. C) MYL-R cells (2×10^6) were treated with DMSO or dasatinib for 24 hr. Cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown. D) Cells (2×10^6) were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown.

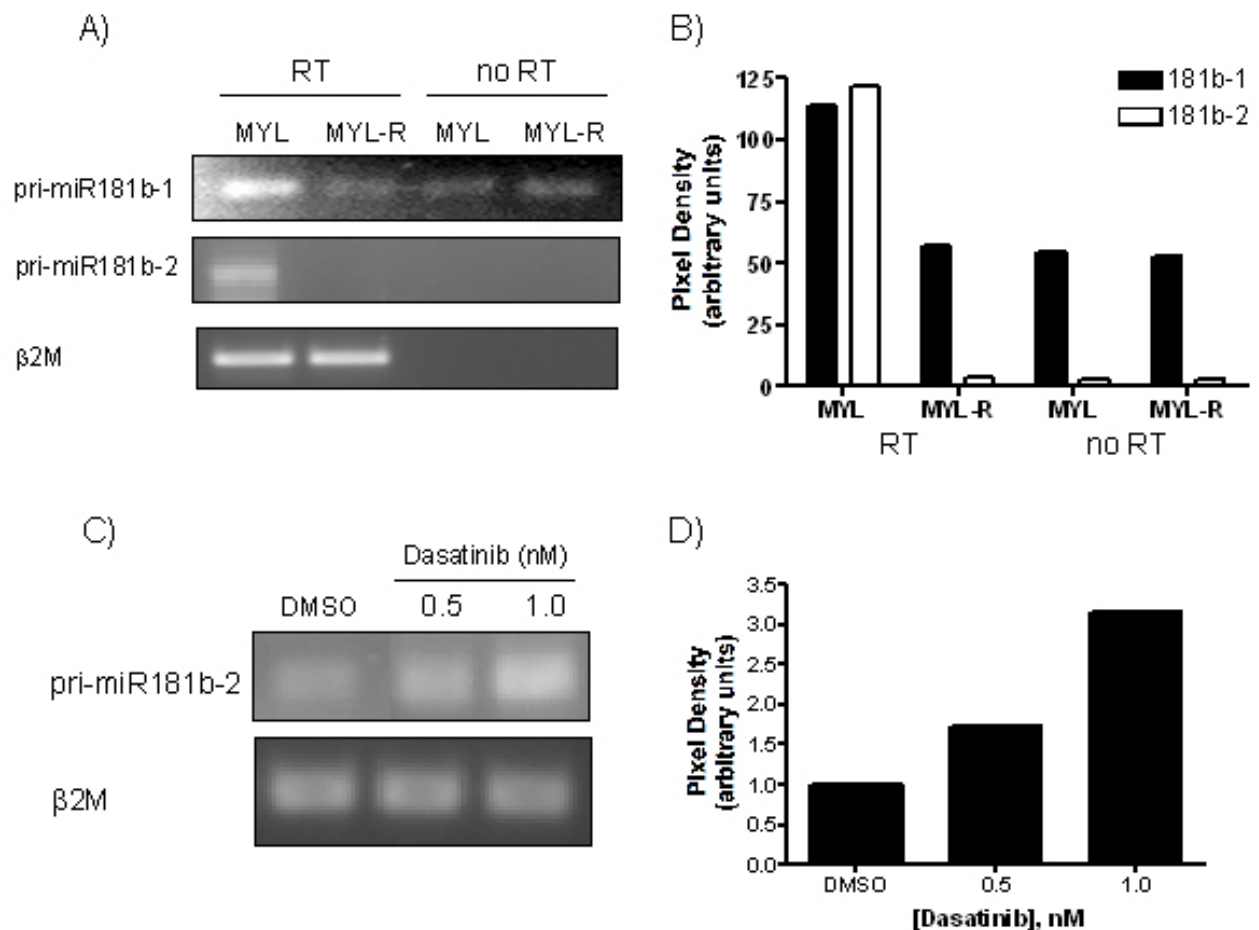


Figure 5.5.2. Regulation of pri-miR181b by Lyn kinase. A) Total RNA was extracted from 2×10^6 cells. Non-quantitative RT-PCR was performed using primers for pri-miR181b. Representative data are shown. B) Densitometry was performed using a FluorChem FC2 imager. C) MYL-R cells (2×10^6) were treated with DMSO or dasatinib for 24 hr. Total RNA was extracted and non-quantitative RT-PCR was performed using primers for pri-miR181b-2. Representative data are shown. D) Densitometry was performed using a FluorChem FC2 imager.

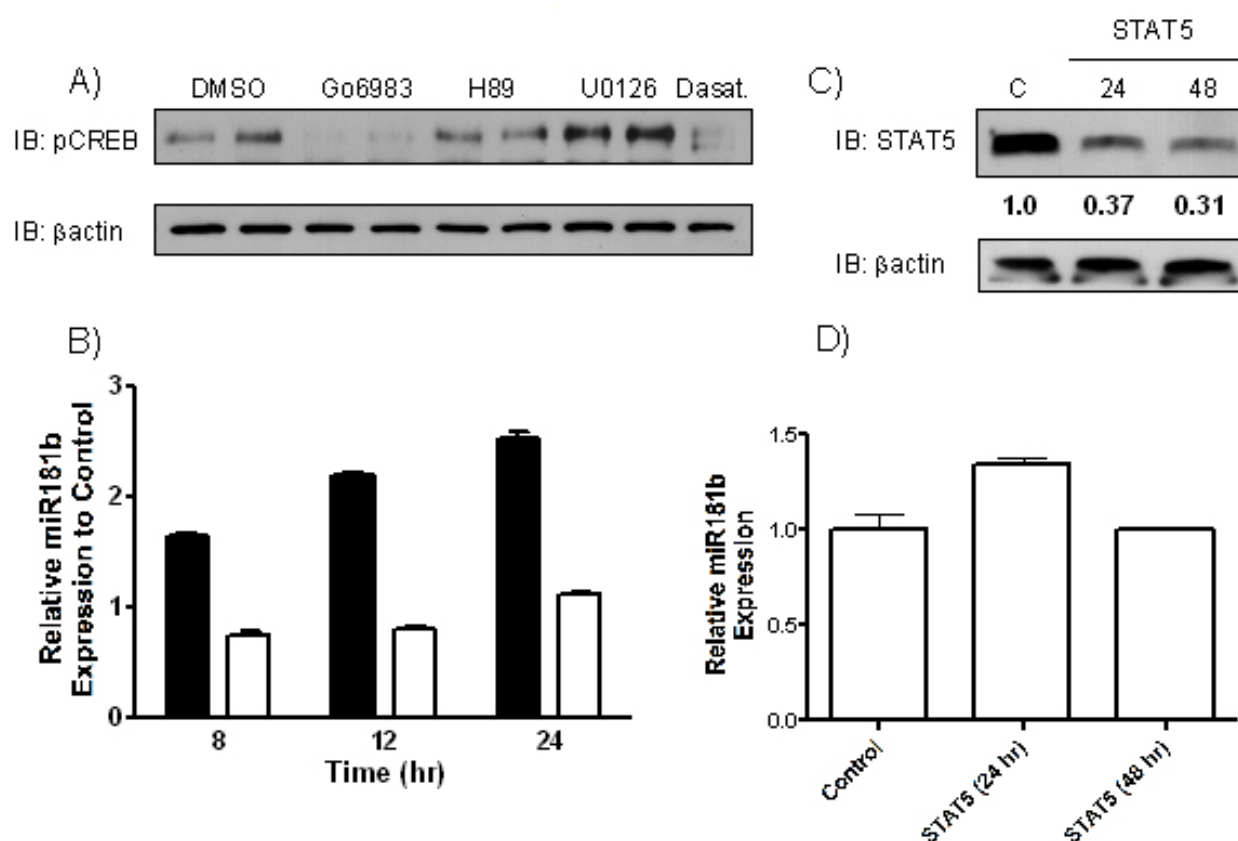


Figure 5.5.3. CREB and STAT5 do not regulate miR181b expression. A) MYL-R cells (4×10^6) were treated with DMSO, Go6983 ($5 \mu\text{M}$), H89 ($10 \mu\text{M}$), U0126 ($10 \mu\text{M}$), or dasatinib (1 nM) for 24 hr. Cells were lysed and Western blot analysis was performed using the indicated antibodies. Samples were loaded in duplicate. Representative data are shown. B) MYL-R cells (4×10^6) were treated with DMSO (24 hrs), Go6983 ($5 \mu\text{M}$) or dasatinib (1 nM) for 8, 12, and 24 hr. Total RNA was extracted and miR181b expression was determined by qRT-PCR. Data are normalized to DMSO (24 hrs) and represent the mean \pm S.E.M. of triplicate samples. C and D) MYL-R cells (1×10^6) were nucleofected with 100 nM non-targeted control siRNA (C) or siRNA against STAT5. Analysis was performed twenty-four and forty-eight hr later. C) Cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown. D) Total RNA was extracted and miR181b expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples.

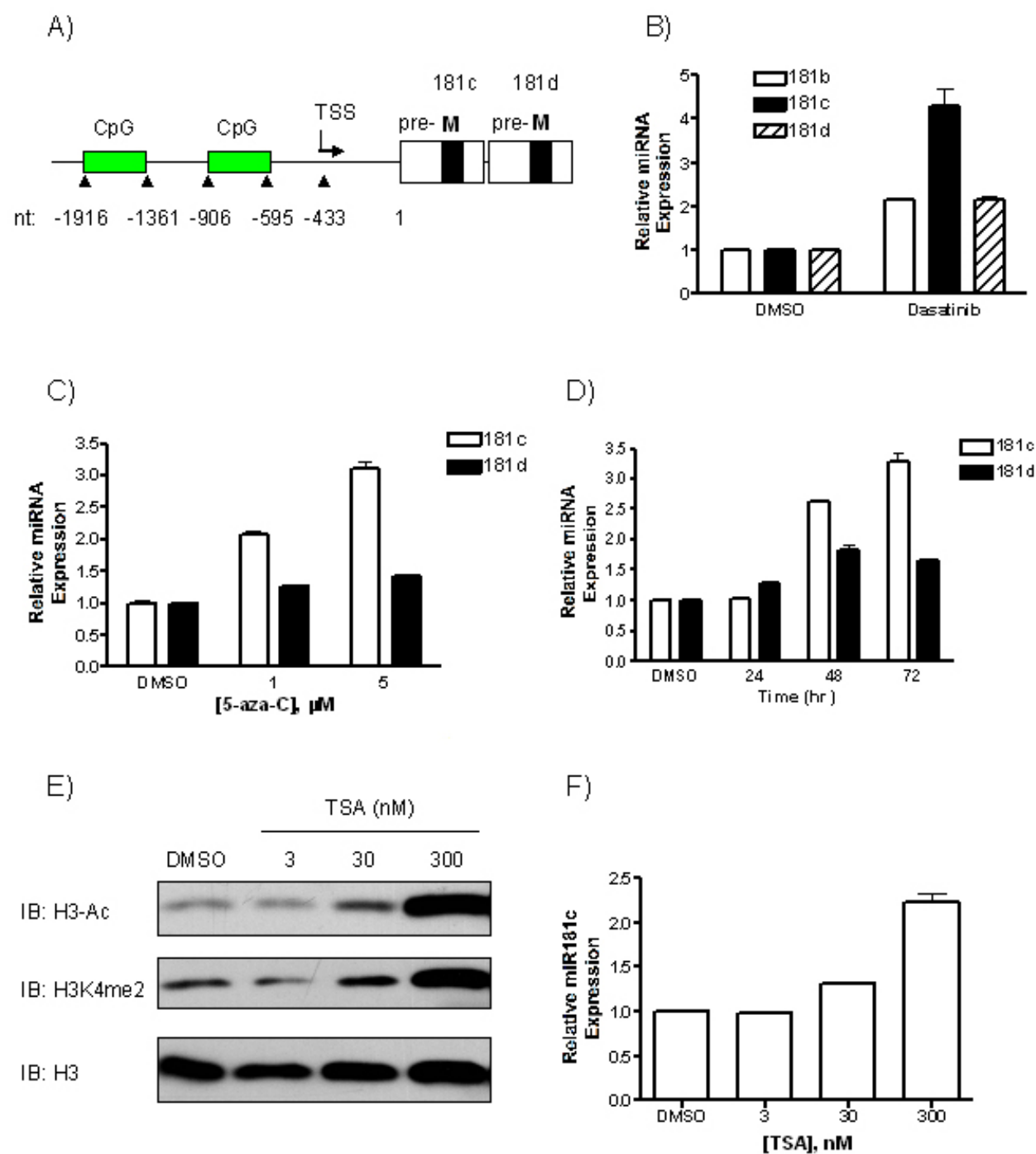


Figure 5.5.4. miR181c/d expression is increased by DNMT and HDAC inhibition. A) Schematic depiction of the miR181c/d gene cluster (nt, nucleotide; TSS, transcriptional start site; pre-, pre-miRNA sequence; M, mature miRNA sequence). B) MYL-R cells (2×10^6) were treated with DMSO or 1 nM dasatinib for 24 hr. Total RNA was extracted and miRNA expression was determined by qRT-PCR. Data represent the mean \pm SEM of triplicate samples. C) MYL-R (2×10^6) cells were treated with DMSO or 5-aza-C for 72 hrs. Total RNA was extracted and miRNA expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples. D) MYL-R cells (2×10^6) were treated with DMSO (72 hr) or 5-aza-C for 24, 48, and 72 hr. Total RNA was extracted and miRNA expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples. E and F) MYL-R cells (4×10^6) were treated with DMSO or TSA for 24 hr. E) Cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown. F) Total RNA was extracted and miR181c expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples.

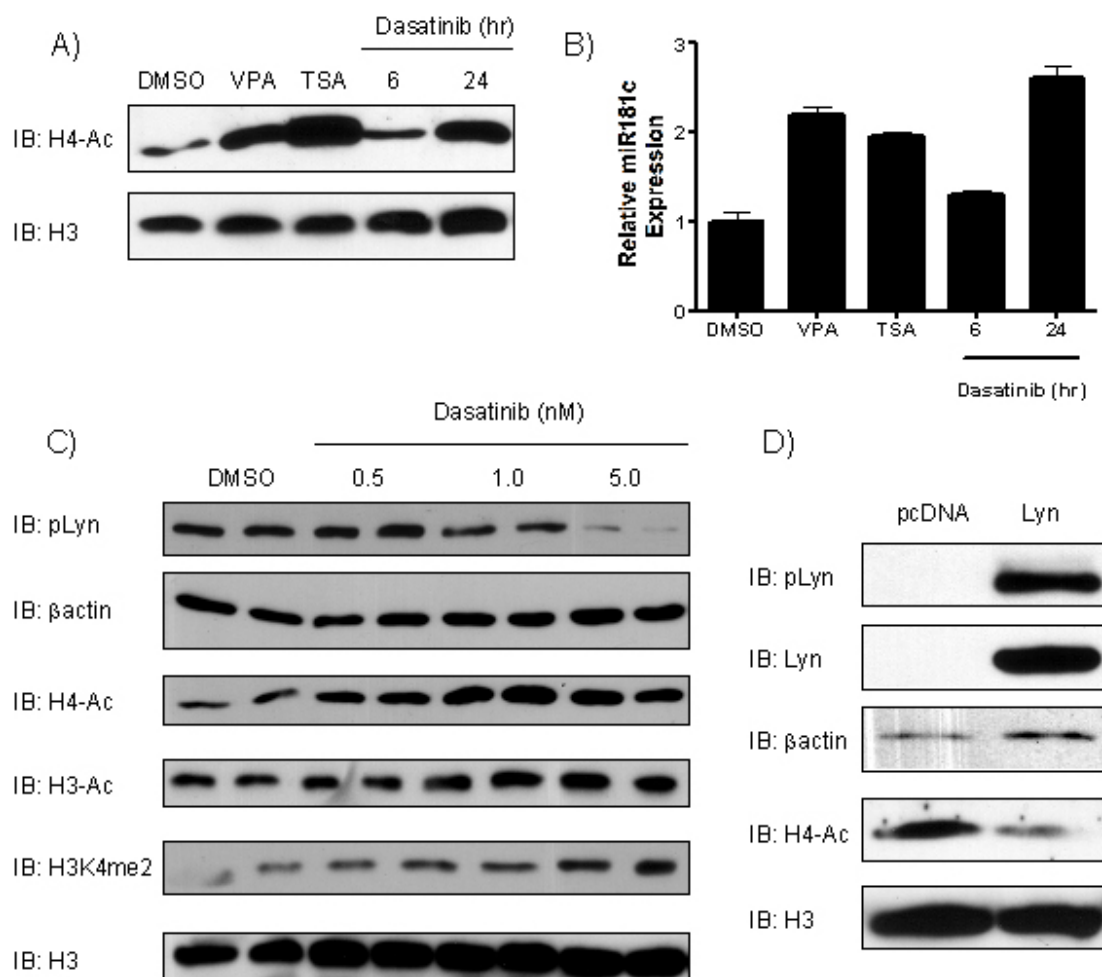


Figure 5.5.5. Lyn kinase regulates histone post-translational modification. A and B) MYL-R cells (4×10^6) were treated with DMSO, VPA (1 mM), TSA (300 nM), or dasatinib (1 nM) for 6 hr or as indicated. A) Cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown. B) Total RNA was extracted and miR181c expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples. C) MYL-R cells (2×10^6) were treated with DMSO or dasatinib for 24 hr. Cells were lysed and Western blot analysis was performed using the indicated antibodies. Samples were loaded in duplicate. Representative data are shown. D) HEK293T cells (2×10^6) were transfected with pcDNA or Lyn-expressing plasmids. Thirty-six hr later cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown.

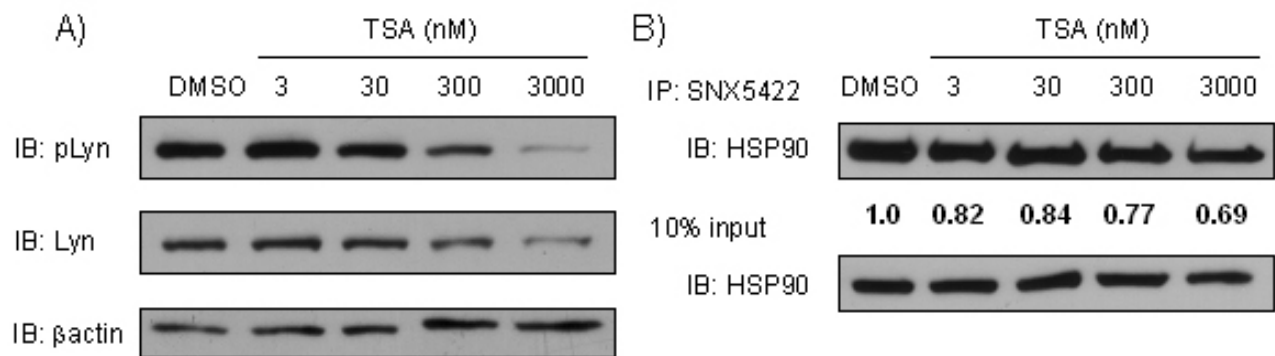
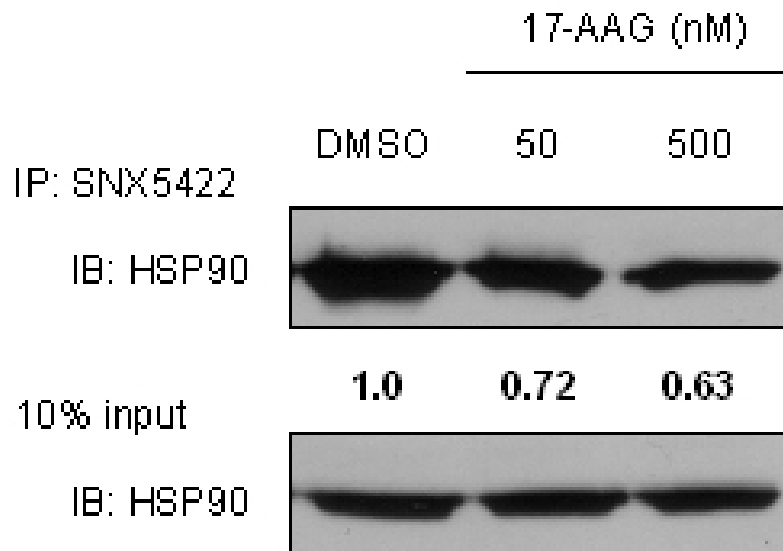


Figure 5.5.6. HDAC inhibition decreases HSP90 activity and Lyn kinase expression. A and B) MYL-R cells (2×10^6) were treated with DMSO or TSA for 24 hr and lysed. A) Western blot analysis was performed using the indicated antibodies. B) Lysate was incubated with SNX5422-conjugated beads for 2 hr at 4°C . Western blot analysis was performed on the eluent and 10% of the lysate input using the indicated antibodies. Representative data are shown.

5.6. Supplemental figures



Supplemental Figure 5.6.1. SNX5422-conjugated beads precipitate active HSP90. MYL-R cells (2×10^6) were treated with DMSO or 17-AAG for 24 hr. 250 μg of total cell lysate was incubated with SNX5422-conjugated beads for 2 hr at 4°C in a total volume of 200 μl . Afterward, beads were washed 3 times with RIPA buffer (no SDS) and protein was eluted using 1X sample buffer. Western blot analysis was performed on the eluent and 25 μg of lysate for each sample using the indicated antibodies. Representative data are shown.



HDAC1	A	G	K	G	K	Y	Y	A	V	N	Y	P	L	R	D	G
HDAC2	A	G	K	G	K	Y	Y	A	V	N	F	P	M	R	D	G
HDAC3	A	E	S	G	R	Y	Y	C	L	N	V	P	L	R	D	G
	*		*		*	*		*			*		*	*	*	

Supplemental Figure 5.6.2. Sequence alignment of human HDAC1-3. Alignment of amino acids 216-231 of human HDAC1 with HDAC2 and HDAC3 using ClustalW software (<http://align.genome.jp/>). An asterisk denotes a conserved residue. An arrow denotes tyrosine 221.

CHAPTER VI

Summary and Future Directions

All figures contributed by Eric I. Zimmerman.

Data contributed to Fig. 6.4.1. and 6.4.2. by Brian Dewar, James Duncan, Marty Whittle, and Matt Cooper.

6.1. Summary

My dissertation research investigated mechanisms of resistance to chemotherapy in cell models of leukemia. These studies initially focused on ENT1, a nucleoside transporter protein responsible for mediating nucleoside analog membrane permeability. Evidence suggests that the activity of ENTs is the limiting factor for nucleoside analog-induced cytotoxicity, and the expression of ENTs correlates with sensitivity of leukemia patients to therapy [293, 294]. Mutation, alternative splicing or other dysfunctions of ENT genes that affect transporter function may lead to drug resistance [52, 89]. For instance, Nishio et al. found that mislocalization of ENT2 away from the plasma membrane was responsible for gemcitabine resistance in pancreatic cancer cells [88]. Furthermore, cytarabine resistance was conferred in leukemia cell lines by a nonsense point mutation of Y11 in ENT1, resulting in translational arrest [89].

In Chapter 3 I describe a mechanism by which dysfunction of ENT1 confers resistance to Ara-C in a cell model of T-cell acute lymphoblastic leukemia (ALL). My studies revealed that a single point mutation (G24R) in the TM1 portion of the protein disrupts ENT1 transport activity, even though the protein was properly localized to the plasma membrane. Previous reports have focused on the TM1 region of this protein and discovered residues (M33, W29) important for substrate and inhibitor recognition [55, 56]. My data extends the findings of these studies and provides novel information on the determinants of ENT1 transporter activity within TM1.

In Chapter 4 we initiated a new study that focused on kinase regulation of drug resistance in an imatinib-resistant cell model of chronic myelogenous leukemia (CML), termed MYL-R. The MYL-R cells are resistant to multiple drugs with distinct modes of action, including nucleoside analogs, suggesting that cell death is prevented at a broad, non-specific level as opposed to alteration of BCR-Abl signaling or changes in nucleoside transport. In agreement with these predictions, our initial studies eliminated the contribution of BCR-Abl up-regulation or mutation as well as p-glycoprotein up-regulation to the mechanism of resistance. Moreover, absence of dysfunctional ENT activity eliminated differential transporter function from the mechanism of drug resistance in these cells.

Previous studies implicated the Lyn tyrosine kinase (Lyn) as an important mediator of drug resistance in MYL-R cells [130]. Thus, our study focused on Lyn-dependent molecular signaling events. In this thesis I describe the elucidation of a novel signaling pathway by which Lyn activation up-regulates expression of the pro-survival protein Mcl-1. A screen for miRNA expression demonstrated that the family of miR181 miRNAs was highly down-regulated in the MYL-R cells. Subsequent experiments determined that these miRNAs were suppressed by Lyn activity. Based on the predicted miR181 binding site within the Mcl-1 3' UTR we tested whether miR181 targeted Mcl-1 for degradation. Indeed, rescue of miR181b expression in MYL-R cells decreased Mcl-1 expression. Finally, we determined that miR181 targeted Mcl-1 mRNA using a luciferase reporter assay, providing molecular evidence for the direct interaction of these molecules.

Our investigations into the effects of miR181 on Mcl-1 complement recent work uncovering miR181-dependent degradation of Bcl-2, a protein related to Mcl-1 [178]. Originally described by Phil Sharp and colleagues, miR181 interacts directly with the Bcl-2 3' UTR to suppress Bcl-2 expression [179]. In fact, the miR181 “seed” region binding site is completely conserved between Bcl-2 and Mcl-1 (www.targetscan.org). Therefore, the miR181 family may be a master regulator of pro-survival Bcl-2 proteins, implicating this miRNA as a potentially important regulator of Bcl-2- or Mcl-1-dependent drug resistance.

Recent studies have shown that miR181 expression affects sensitivity to anti-cancer therapy. Over-expression of miR181a sensitized glioma cells to radiation-induced cell death [178] and in non-small cell lung cancer cells, miR181a over-expression increased sensitivity to cisplatin treatment [295]. These effects were concomitant with increased Bax oligomerization and pro-caspase cleavage, indicating miR181 alteration of mitochondria-mediated apoptosis, which is in agreement with its ability to affect Mcl-1 and Bcl-2 expression [295].

Interestingly, studies suggest that the miR181 family functions both as a tumor suppressor and oncogene depending its cellular context. In addition to promoting hepatocarcinogenesis [296], these miRNAs are elevated in breast [297] and colon tumors [298] as well as pancreatic cancer [299]. However, miR181 acts as a tumor suppressor in glioblastoma [300] and aggressive CLL [301]. A major determinant as to whether miR181 acts as an oncogene or tumor suppressor may be the array of mRNAs targeted by this miRNA for degradation. To date, ten specific targets for miR181, including p27 and TCL-1, have been reported in various cell

types [250, 256]. However, potentially hundreds of more targets exist for this miRNA family. To better evaluate the specific role of miR181 in cancer, additional analysis of miR181 targets must be performed.

The importance of miR181 to cancer biology is apparent. Herein, I propose two future directions for the research described in the previous chapters. These proposed studies will extend our knowledge of miR181 regulation and may impact the development of therapies for drug-resistant leukemia patients.

6.2. Future Direction #1: Determine the kinome response to Lyn inhibition

In general, the effects of molecular signaling pathways on miRNA expression are poorly understood. Exceptions include MAPK regulation of let-7 [302] and BCR-Abl-dependent regulation of miR328 [253], pathways which are of high impact and lend insight to cancer biology. Our data suggest that Lyn regulates miR181 transcription; however, the mechanistic details of this pathway are unknown. Elucidation of this process has important biological and therapeutic ramifications.

As outlined in Chapter 5, we attempted to understand the transcriptional regulation of the miR181a/b and miR181c/d gene clusters. Though we determined that miR181c/d is epigenetically regulated in MYL-R cells, the substrates of Lyn and the cellular machinery necessary for miR181 expression control were not identified. Our initial attempt to determine Lyn-dependent signaling targets using the Phospho-Kinase Array was unsuccessful. Therefore, to better accomplish this goal, an objective and more comprehensive approach to identify the molecular consequences of Lyn inhibition is needed.

As an alternative and novel strategy I propose to determine changes in kinase expression and activity using affinity purification and mass spectrometry. To enrich for kinases, we will incubate cell lysate with beads covalently conjugated to non-selective pan-kinase inhibitors, termed kinase inhibitor beads (KIBs) or KinoBeadsTM (Cellzome, Heidelberg, Germany) (Fig. 6.4.1A). Bound kinases will be eluted, tryptic digested and the peptides labeled using isobaric molecular tags (iTRAQ®; Applied Biosystems) for quantitative mass spectrometry. After separation of peptides by reversed phase HPLC, identification and quantification of kinases will be performed by MALDI-TOF/TOF mass spectrometry equipped with ProteinPilot software. This method will be used to 1) compare kinase expression between MYL and MYL-R cells and 2) determine changes in kinase expression in MYL-R cells after dasatinib treatment and/or Lyn-directed siRNA treatment.

The KinoBeadTM technology was originally described by several German research groups, including Henrik Daub, Mathias Mann and colleagues, and has been implemented in cell systems for the study of kinase networks [303]. These beads are conjugated to pan kinase inhibitors that target the ATP-binding pocket and interact with multiple protein kinases primarily based on 1) kinase affinity, 2) kinase avidity (combined strength of multiple bonds), and 3) kinase expression. In addition, we have observed activity-dependent capture of kinases after treatment with pervanadate, an irreversible protein phosphatase inhibitor (data not shown). This may be due to increased affinity/avidity of activated kinases and studies are now being performed to profile kinase activation-loop phosphorylation using KIB technology. Furthermore, kinases that indirectly interact with the KIBs may be

precipitated as well as ATP-binding non-kinase proteins (i.e. metabolic enzymes). Therefore, KIB technology may be used to study protein complex formation; however, the isolation of proteins that interact indirectly with bead is largely affected by the stringency of bead washes (i.e. salt content) prior to analysis.

With regard to kinase network profiling, KinoBead™ technology has successfully been used to determine global kinase expression in cancer cell lines [303] and primary cancer cells [304]. In addition, this quantitative proteomic approach has been useful to interrogate changes in kinase signaling during the cell cycle [305]. Furthermore, Bantscheff et al. used KinoBeads™ to identify kinase inhibitor targets by competitive binding [306]. In this study, kinases were captured by KinoBeads™ in the presence of different concentrations of BCR-Abl kinase inhibitors (imatinib, dasatinib, and bosutinib). The loss of peptide signal from the KinoBead™ purification indicated competitive binding with the free inhibitor and identified novel inhibitor targets, such as the tyrosine kinase DDR1, that may be important for the therapeutic effects of the drug [306]. Recently, this approach was extended to determine the effect of kinase inhibitors on kinase phosphorylation [307].

Our initial studies compared kinase expression in MYL and MYL-R cells using KIB-mediated kinase enrichment followed by iTRAQ® labeling and MALDI TOF/TOF analysis (Fig. 6.4.1A). Using beads conjugated to two pan-kinase inhibitors (PP58, V116932) we identified approximately 120 unique kinases (Fig. 6.4.1B-C). The enrichment of kinases from multiple different sub-families was observed, demonstrating the relative lack of experimental bias of this technology (Fig. 6.4.1B). The inclusion of KIBs containing other pan-kinase inhibitors should increase the

number of kinases captured in future experiments. Interestingly, in our initial study, more kinases were down-regulated >2-fold than up-regulated >2-fold in MYL-R cells (12.8% vs. 8.5%), whereas expression of the majority of kinases was unchanged (78.7%) (Fig. 6.4.1C). As confirmation of our previous Western blot data, Lyn was found to be highly up-regulated in MYL-R cells (Fig. 6.4.2A).

Subsequent Western blot analysis confirmed important changes in kinase expression between the MYL and MYL-R cells. Of note, the expression of BCR-Abl and c-Kit was highly down-regulated in MYL-R cells (Fig. 6.4.2A-B). These are two imatinib targets and the loss of expression may indicate “oncogene switching” away from these pro-survival kinases. In addition, Western blot analysis of IKK α and MEK2, two kinases up-regulated in MYL-R cells as determined by the KIB experiment, revealed that the expression of total protein was similar between the two cell lines (Fig. 6.4.2A, C). Analysis of the phosphorylated, active form of the kinases indicated that both were increased in activity in MYL-R, indicating that the KIBs may precipitate these kinases in an activity-dependent manner (Fig. 6.4.2C). In summary, this approach provides a robust and large-scale evaluation of kinase expression in leukemia cells and may give insight into kinase activity. Moreover, the finite number of kinases that were different between MYL and MYL-R suggests that these individual kinases could be targeted and evaluated for their specific effects on miR181 expression.

In the proposed experiment, identification of differentially bound kinases after dasatinib treatment will be followed by Western blot analysis of kinase expression and activity. Confirmation of dose-dependent dasatinib effects on the target kinase

will be followed by evaluation of Lyn involvement using Lyn-directed siRNA. Finally, determination of the effects of target kinase inhibition (by siRNA or selective kinase inhibitors) on miR181 expression will be performed. It is anticipated that this “top-down” approach will identify Lyn-dependent kinases signaling pathways and the transcriptional machinery that regulates miRNA expression.

6.3. Future Direction #2: Define miR181 regulation in AML

As opposed to CML, AML is a much more molecularly heterogeneous disease. Approximately 30% of patients have constitutive activation of the receptor tyrosine kinase, Fms-like tyrosine kinase 3 (FLT3) [308], whereas other patients express JAK mutations, transcription factor fusions, etc. An internal domain duplication (FLT3-ITD) or a point mutation within the kinase domain (D835) results in ligand-independent and IL-3 independent FLT3-ITD signaling [309-311]. The majority of patients with this molecular phenotype have a very aggressive disease characterized by a low complete remission rate and are more likely to undergo relapse [33, 312].

Unlike wild-type FLT3, mutant FLT3 activates multiple pro-survival signaling pathways, including STAT5 [313]. In addition, a study by Okamoto et al. found that FLT3, in particular FLT3-ITD, associates with and activates Lyn [152]. Inhibition of Lyn using siRNA or the SFK inhibitor PP2, decreased STAT5 phosphorylation and FLT3-ITD-expressing 32D cell viability. Treatment of mice implanted with FLT3-ITD-expressing 32D cells with PP2 decreased tumor size [152]. These data suggest that Lyn is an integral component of FLT3-ITD signaling and the observation that SFKs are activated by FLT3 and are important to AML cell survival has been confirmed by

independent research groups [151, 314]. Furthermore, a recent study demonstrated that the SFK inhibitor dasatinib is effective against molecularly heterogeneous AML, though this compound may also inhibit FLT3 directly [315].

As explained above, low miR181 expression is prognostic for aggressive AML. A series of studies by Bloomfield and colleagues demonstrate that AML patients with relatively low expression of miR181 experience lower complete remission rates, shorter overall survival, and shorter disease-free survival in comparison with patients with high miR181 expression [164, 176, 177]. In both studies these correlations were more significant for patients characterized by FLT3-ITD and/or NPM1 wild-type.

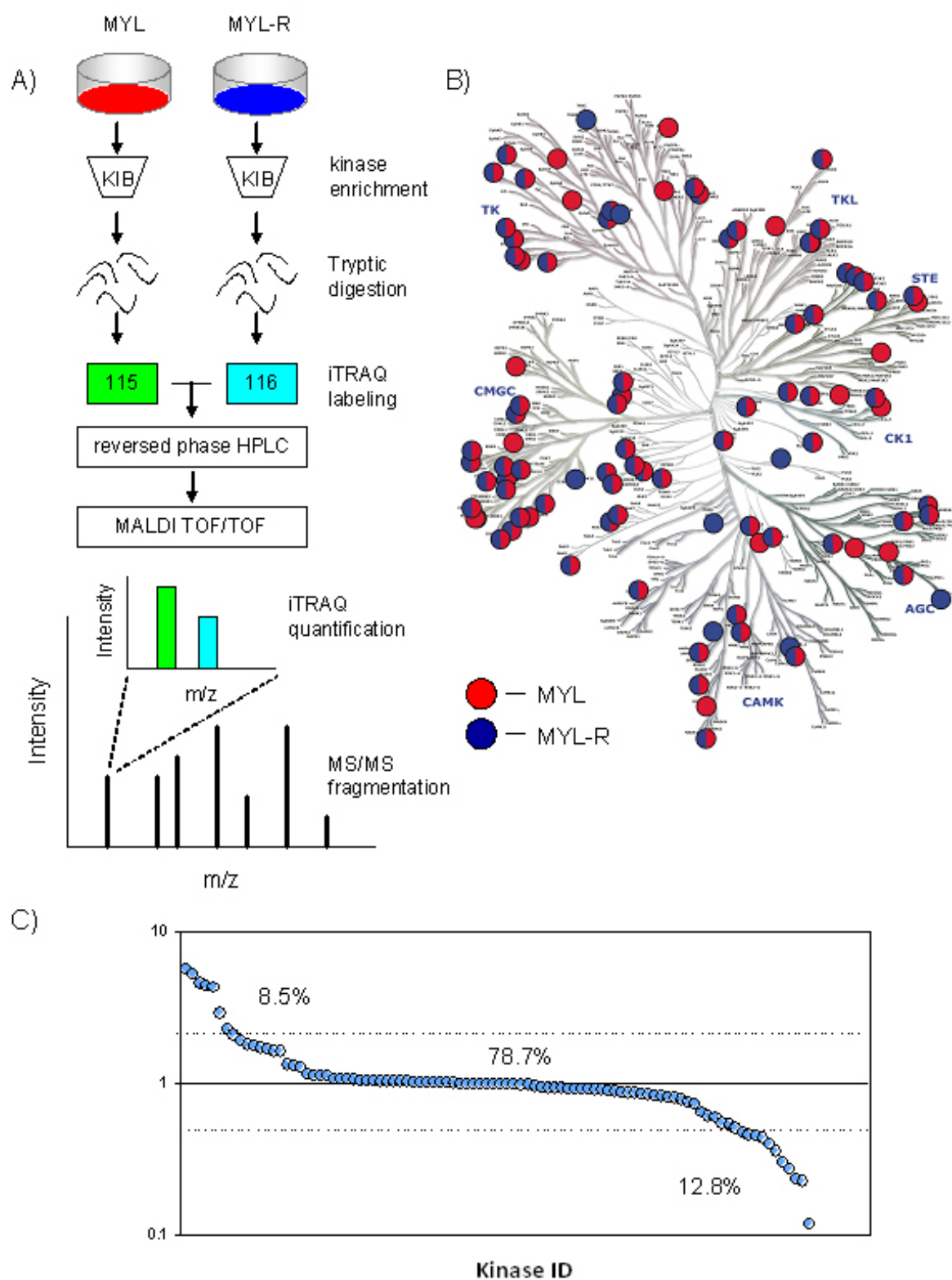
Based on these studies we hypothesize that FLT3-ITD may decrease miR181 expression through the activation of Lyn. In support of this hypothesis, preliminary experiments demonstrate that when FLT3-ITD-expressing MV4-11 cells are treated with the FLT3 inhibitors PKC412 and sorafenib, miR181b expression is increased in a dose-dependent manner (Fig. 6.4.3A-B). Future experiments should determine whether this effect is dependent on Lyn inhibition. These studies will help substantiate the role of SFKs in AML and will provide insight into miR181 regulation in the context of this deadly disease.

6.4. Conclusions

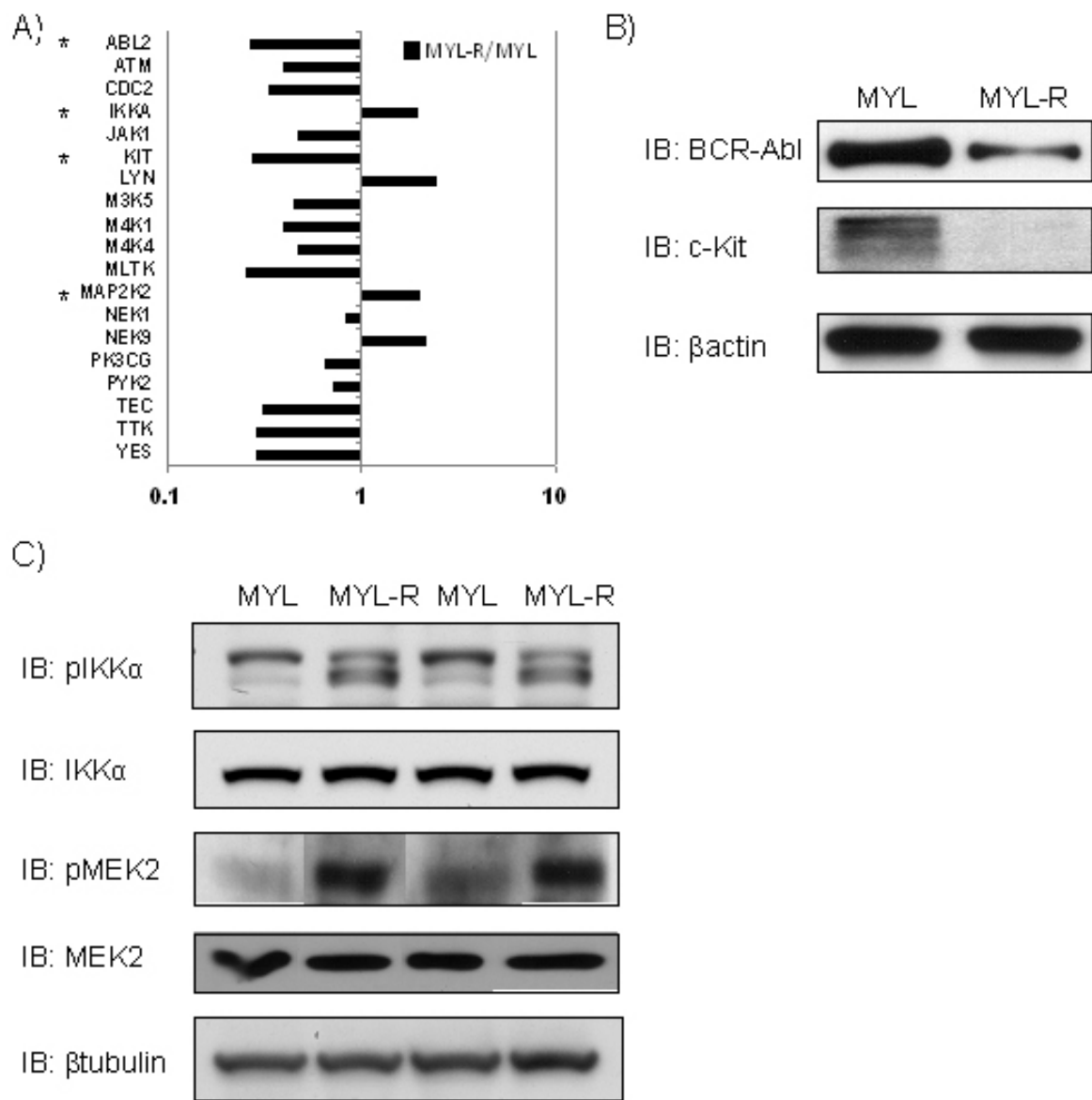
In this dissertation, I have elucidated the molecular machinery, at least in part, responsible for drug resistance in several models of drug-resistant leukemia. I initially describe the importance of ENT function for nucleoside analog treatment; however, the primary focus of my work has been adaptation of kinase signaling to

drug treatment. In this regard, identification of Lyn kinase-dependent regulation of miR181 miRNAs and Mcl-1 expression contributes to our knowledge of imatinib resistance, as well as CML and AML biology. Further study of the molecular networks affected by Lyn will shed light on the role of this pro-survival kinase in drug-resistant leukemia.

6.5. Figures

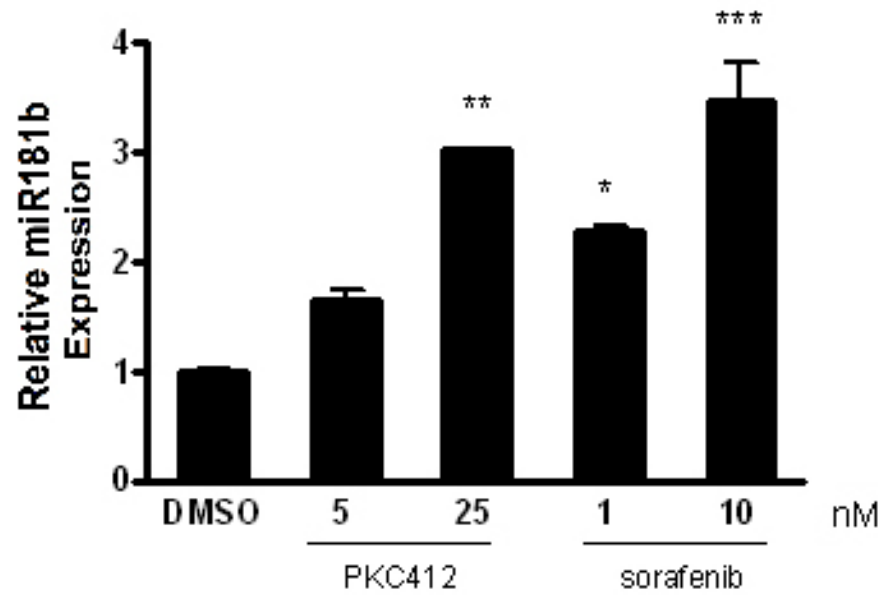


6.5.1. Analysis of kinase expression in MYL and MYL-R cells. A) Schematic depiction of mass spectrometry-based analysis of kinase expression using KIB technology. B) Kinase family tree depicting the expression of kinases in MYL and MYL-R cells (adapted from [93]). C) Percentage of kinases whose expression was >2-fold increased, >2-fold decreased, and unchanged (<2-fold) in MYL-R cells compared to MYL cells. Dotted lines indicate a 2-fold difference in expression.

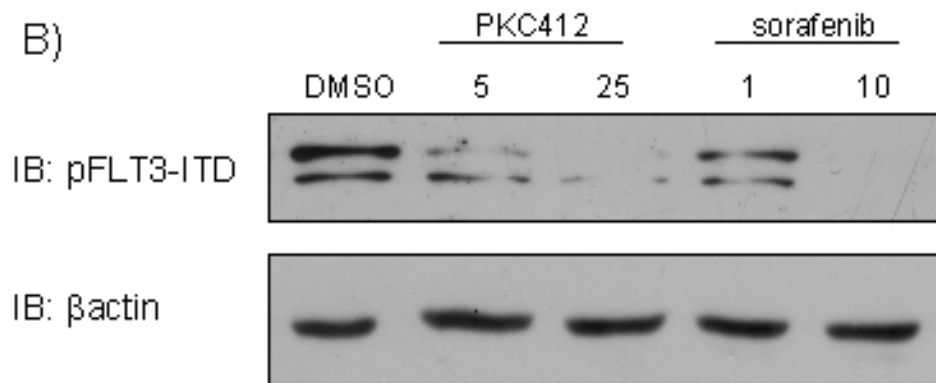


6.5.2. Confirmation of kinase expression in MYL and MYL-R cells. A) The expression of kinases in MYL-R cells in comparison to MYL cells. An asterisk denotes a kinase selected for Western blot analysis. B and C) Cells (2×10^6) were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown.

A)



B)



6.4.3. FLT3-ITD inhibition increases miR181b expression. A and B) MV4-11 cells (4×10^6) were treated with DMSO, PKC412, or sorafenib for 24 hr. A) Total RNA was extracted and miR181b expression was determined by qRT-PCR. Data represent the mean \pm S.E.M. of triplicate samples (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). B) Cells were lysed and Western blot analysis was performed using the indicated antibodies. Representative data are shown.

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