FUNCTIONAL AND STRUCTURAL STUDY OF THE AHI-1 SH3 DOMAIN,
CHARACTERIZATION OF THE BCR-ABL-AHI-1-DYNAMIN-2 PROTEIN
COMPLEX AND INVESTIGATION OF ONCOGENIC ROLES OF DYNAMIN-2 IN
CHRONIC MYELOID LEUKEMIA

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in
THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)
December 2016
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Abstract

Tyrosine kinase inhibitor (TKI) therapies have been introduced into clinical practice with remarkable effects on chronic myeloid leukemia (CML). However, early relapse, acquired drug resistance and persistence of leukemic stem cells (LSCs) remain problematic. Improved treatments specifically targeting key molecular elements active in CML LSCs are needed. One candidate is the oncoprotein AHI-1 (Abelson helper integration site-1), which is highly deregulated in LSCs. It harbors two key domains, SH3 and WD40-repeat, which are known important mediators of protein-protein interactions. An AHI-1-mediated protein complex containing BCR-ABL and JAK2 has been shown to modulate transforming activity and TKI-response/resistance of CML LSCs.

In this study, I investigated the functional roles of the AHI-1 SH3 domain in regulation of cellular resistance of primitive CML cells to TKIs. I showed that deletion of the SH3 domain of Ahi-1 significantly enhanced apoptotic response of BCR-ABL+ cells to TKIs compared to cells expressing full-length Ahi-1. I solved the crystal structure of the AHI-1 SH3 domain and identified several unique features, providing potential target sites for designing specific drugs.

Using immunoprecipitation/mass spectrometry, I identified a novel protein interaction between AHI-1 and Dynamin-2 (DNM2), a GTPase, through the AHI-1 SH3 domain. I showed that DNM2 expression was significantly upregulated in CML stem/progenitor cells compared to normal bone marrow cells. I also determined that the AHI-1 SH3 domain and the proline rich
domain of DNM2 were mainly responsible for their interaction. Most importantly, I identified a novel protein complex in CML cells, containing BCR-ABL, AHI-1 and DNM2.

Furthermore, I demonstrated an oncogenic role of DNM2 in primitive CML cells by showing that knockdown of DNM2 greatly impaired the survival of CML stem/progenitor cells and sensitized them to TKI treatments. Lastly, I illustrated that DNM2 might be involved in deregulation of endocytosis, ROS production and autophagy in TKI-insensitive CML stem/progenitor cells.

This study detailed the identification and characterization of the newly-identified BCR-ABL-AHI-1-DNM2 protein complex and described the oncogenic functions of DNM2 in primitive CML cells. It further suggested that targeting DNM2 may facilitate eradication of LSCs as a new treatment option in CML.
Preface

Xiaohu Liu conducted all investigations presented in this thesis, except for the parts stated below, under the supervision of Dr. Xiaoyan Jiang at the Terry Fox Laboratory at the BC Cancer Research Centre, Vancouver, Canada. I designed and carried out experiments, analyzed and interpreted data, composed and edited the thesis. Dr. Xiaoyan Jiang contributed to the experimental design, data interpretation and editing of this thesis.

Most of the studies described in Chapter 3 and some work presented in Section 4.2.1 and 4.2.3 have been published as a first-author publication. Liu X, Chen M, Lobo P, An J, Cheng SWG, Moradian A, Morin GB, Van Petegem F and Jiang X. (2012) Molecular and structural characterization of the SH3 domain of AHI-1 in regulation of cellular resistance of BCR-ABL+ chronic myeloid leukemia cells to tyrosine kinase inhibitors. Proteomics 12: 2094-2106. Xiaohu Liu contributed above 80% of the work including performing experiments, analyzing data, generating figures and writing the manuscript. Dr. Min Chen from the Dr. Xiaoyan Jiang lab helped with data analysis and figure generation in the section of “The SH3 domain of AHI-1 is required for mediation of TKI sensitivity in BCR-ABL+CML cells”. Paolo Lobo from the Dr. Filip Van Petegem lab at UBC, helped solve the crystal structure of the AHI-1 SH3 domain. Dr. Annie Moradian from the Dr. Gregg Morin lab at the Michael Smith Genome Sciences Centre, helped with the mass spectrometry analysis. Dr. Xiaoyan Jiang, Dr. Min Chen, Dr. Gregg Morin and Dr. Filip Van Petegem contributed to the development of the concept and design of experiments and assisted with writing the manuscript.
Xiaohu Liu generated the majority of data and all the figures in Chapter 4 and 5. Dr. Katharina Rothe performed the experiments and analyzed the data for Figure 5.3 E-M and Figure 5.4. Dr. Tobias Maetzig from Dr. Keith Humphries lab at the Terry Fox Laboratory, helped design a lentiviral vector used to generate lentiviruses containing DNM2 shRNA for infecting primary CML cells. All studies performed with primary samples from CML patients or healthy donors were approved by the University of British Columbia Clinical Research Ethics Board, certificate number H12-02372.
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List of Abbreviations

ABL = Abelson tyrosine-protein kinase
AHI-1(human)/Ah1-1 (mouse) = Abelson helper integration site-1
ALL = Acute lymphoblastic leukemia
Allo-SCT = Allogeneic stem cell transplantation
AP = Accelerated phase
ATF-1 = Activating transcription factor 1
ATG = Autophagy related
BAR = Bin-Amphiphysin-Rvs
BC = Blast crisis
Bcl-2 = B-cell CLL/lymphoma 2
BCR = Breakpoint cluster region
BM = Bone marrow
BO = Bosutinib
Cbl = Cbl proto-oncogene, E3 ubiquitin protein ligase
CCyR = Complete cytogenetic response
CFC = Colony forming cell
CME = Clathrin-mediated endocytosis
CML = Chronic myeloid leukemia
CLL = Chronic lymphocytic leukemia
Co-IP = Co-immunoprecipitation
CP = Chronic phase
CREB = cAMP response element-binding protein
CTCL = Cutaneous T-cell lymphoma
CXCL12 = Chemokine (C-X-C motif) ligand 12
CXCR4 = CXC chemokine receptor 4
DA= Dasatinib
DNM2 = Dynamin-2
EE = Early endosome
EEA1 = Early endosome antigen 1
ERK = Extracellular signal-regulated kinase
FA = Focal adhesion
FISH = Fluorescence in situ hybridization
FOXO3a = Forkhead box O3
FT = Flow through
GAB2 = GRB2-associated binding protein 2
GAP = GTPase activating protein
GEF = Guanine nucleotide exchanging factor
Grb2 = Growth factor receptor-bound protein 2
GSK3β = Glycogen-Synthase-Kinase 3 Beta
HA = Human influenza hemagglutinin
HCQ = Hydroxychloroquine
HIF = Hypoxia inducible factor
HSC = Hematopoietic stem cell
Hsp90 = Heat shock protein 90
IFNα = Interferon α
IM = Imatinib
IMR = Imatinib resistant
IL-1= Interleukin 1
IP = Immunoprecipitation
JAK2 = Janus kinase 2
JSRD = Joubert syndrome related disorder
LAMP1 = Lysosomal-associated membrane protein 1
LC3 = Microtubule-associated protein light chain 3
LE = Late endosome
LSC = leukemia stem cell
LTC-IC = Long-term culture initiating cell
MAPK = Mitogen-activated protein kinase
Mcl-1 = Myeloid cell leukemia 1
MEK = MAPK/ERK kinase
mL = Milliliter
MMR = Major molecular remission
mTOR = Mammalian target of rapamycin
Myc = V-Myc avian myelocytomatosis viral oncogene homolog
NADPH = Nicotinamide adenine dinucleotide phosphate
NFκB = Nuclear factor kappa-light-chain-enhancer of activated B cells
NL = Nilotinib
nM = Nanomolar
p27Kip1 = Cyclin-dependent kinase inhibitor 1B
p62 = Sequestosome 1
PDK1 = 3-phosphoinositide dependent protein kinase-1
p-DNM2 = Phospho-Dynamin-2
Ph = Philadelphia chromosome
PH = Pleckstrin homology
PI3K = Phosphatidylinositol-4,5-Bisphosphate 3-kinase
PIP2 = Phosphatidylinositol 4,5-bisphosphate
PIP3 = Phosphatidylinositol (3,4,5)-bisphosphate
POI = Protein of interest
PRD = Proline rich domain
RAS = Rat Sarcoma Viral Oncogene
ROCK = Rho-associated, coiled-coil containing protein kinase
ROS = Reactive oxygen species
SDF-1 = Stromal-cell-derived factor 1
SEM = Standard error of the mean
SH2 domain = Src homolog 2 domain
SH3 domain = Src homolog 3 domain
SHC = non-targeting control sequence
shDNM2 = Dynamin-2 small hairpin RNA
SHP1 = SH2 domain-containing protein tyrosine phosphatase 1
SHP2 = SH2 domain-containing protein tyrosine phosphatase 2
SNP = Single nucleotide polymorphisms
SOS = Son of sevenless homolog
STAT5 = Signal transducer and activator of transcription 5
TKI = Tyrosine kinase inhibitor
TNF = Tumor necrosis factor
ULK1 = Unc-51 like autophagy activating kinase 1
USP9x = Ubiquitin specific peptidase 9, x-linked
μM = Micromolar
Acknowledgements

First, I would like to express my deepest gratitude to my supervisor Dr. Xiaoyan Jiang. To me, you are not just a scientific supervisor, but also a great mentor. Your tremendous support and guidance make this long journey much easier than it is supposed to be.

I would also like to thank my committee members Drs. Gregg Morin and Filip Van Petegem for their advice and suggestions. Your expertise and critiques are highly appreciated and I could not ask for better committee members.

Furthermore, I feel really grateful for working with the current and past members of the Jiang lab, Josephine, Kevin, Damian, Katharina, Leon, Sharmin, Kyi Min, Clark, Min, Sujie, Kelly, Ryan, Vanessa, Rachel, Jonathan, Jasmin and Akie for their love. You guys create an incredible lab atmosphere so that working in the lab is no longer dull and boring. I cherish every single second around you guys and could not imagine what my life would be in the past 6 years without you in it.

My heartfelt thanks go to my dearest family. Being a graduate student is hard, however being the wife of a graduate student can be unbearable. I thank my wife Monica for practically taking care of the family and raising my daughter Scarlett herself but hardly complaining about my constant absence during this period of time. Your love is what gets me through all the difficulties. I do not know if I can ever thank you enough. Finally, I would like to thank my parents. You are the pillars of my life and always try to support me in any possible way you could. I will forever be grateful for your unconditional love.
I dedicate this thesis to my lovely wife, adorable daughter and beloved parents for always being there for me.
Chapter 1: Introduction

1.1 Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder originating from the hematopoietic stem cell compartment (Figure 1.1) [1]. CML occurs with an incidence of 1-2 per 100,000 population each year, and accounts for 15% - 20% of all newly diagnosed adult leukemias [2-4]. This malignancy occurs with a median age of around 60 years, with a slight male to female preponderance (1.3-1.8:1) [2-4]. The common signs and clinical manifestations include anemia, splenomegaly, fatigue, weight loss and abdominal discomfort [2]. However, a significant proportion of CML patients are asymptomatic and only diagnosed by chance when their white cell counts are found elevated via unrelated medical examinations [4].

CML can be divided into three phases-chronic (CP), accelerated (AP) and blast crisis (BC)-depending on the symptomatic severity and number of blast cells in the blood/bone marrow (BM, Figure 1.1). The initial CP is a relatively indolent stage featuring an excessive proliferation of fully differentiated myeloid cells in the BM and peripheral circulation. However, without medical intervention, the disease invariably progresses through a transitional AP into the fully transformed BC. The final phase is characterized by the rapid expansion of myeloid and/or lymphoid differentiation-arrested blast cells (>20%) in the BM and peripheral blood. The patients in BC respond poorly to
any treatment and almost always die from bone marrow failure-related diseases [5-8].

The pathophysiology of CML can be traced to a genetic lesion - the Philadelphia chromosome (Ph), which is found in approximately 95% of CML patients [9, 10]. The Philadelphia chromosome is formed by the reciprocal translocation between the long arms of chromosome 22 and chromosome 9, which leads to the juxtaposition of the BCR (breakpoint cluster region) gene and the proto-oncogene ABL (Abelson tyrosine-protein kinase) gene (Figure 1.1) [11]. The resultant BCR-ABL fusion gene encodes a chimeric oncoprotein with constitutively active tyrosine kinase activity that stimulates several key signaling pathways, resulting in increased proliferation, resistance to apoptosis, loss of stromal adhesion and highly error-prone DNA repair mechanisms [12-15]. Therefore, BCR-ABL is recognized as the main driving force of CML leukemogenesis and considered as a valid therapeutic target.
Figure 1.1. Formation of the Philadelphia chromosome (Ph) and the development of chronic myeloid leukemia (CML). CML is a hematopoietic stem cell (HSC) disease, initiated by the aberrant acquisition of the Philadelphia chromosome (Ph) that is formed by the reciprocal translocation between chromosome 22 and chromosome 9, which results in a fusion gene $BCR-ABL$. The transformed HSCs take over the hierarchy of hematopoiesis and promote leukemogenesis (CMP: common myeloid progenitors, CLP: common lymphoid progenitors, GMP: granulocyte-macrophage progenitors, MEP: megakaryocyte-erythrocyte progenitors, MEG: megakaryocytes, G: granulocytes, M: macrophages, RBC: red blood cells). The chronic phase of CML is characterized by a massive expansion of granulocytes. The acquisition of additional genetic alterations causes disease progression to the lethal blast crisis, which is characterized by rapid expansion of myeloid and/or lymphoid differentiation-arrested blast cells. Reproduced and modified from Lydon, 2009 [16] and Ren, 2005 [17] (with permission through the Copyright Clearance Center).
1.1.1 Uncontrolled BCR-ABL tyrosine kinase activity

The normal kinase activity of the ABL protein is tightly regulated, while its oncogenic counterpart, BCR-ABL, escapes the regulatory mechanisms due to BCR fusion, thereby possessing aberrantly high kinase activity (Figure 1.2A) [18-21]. The inactive ABL is usually present in an autoinhibited form in which the SH2 and SH3 domains dock to the back of the kinase domain to restrict its conformational flexibility, and an N-terminal myristoyl group inserts into the pocket at the base of the kinase domain that locks it in the inactive conformation (Figure 1.2B) [19]. However, the replacement of the N-terminal myristoyl group for BCR in BCR-ABL causes the loss of the “locking” effect [22, 23]. Meanwhile, the presence of the coiled-coil domain at the N-terminus of BCR promotes oligomerization of BCR-ABL, leading to the proximity-induced activation of the BCR-ABL kinase by trans-autophosphorylation (Figure 1.2C) [24-27]. Furthermore, one of the structural consequences of the autophosphorylation is to cause the displacement of the SH2-SH3 “clamp” from the kinase domain to further release the autoinhibition (Figure 1.2C) [22, 23, 28, 29]. Hence, due to the loss of negative regulation, BCR-ABL can no longer be controlled and exhibits constitutively active kinase activity.
Figure 1.2. Schematic diagram and conformations of ABL and BCR-ABL. (A) Domain structures of ABL and BCR-ABL. (B) The ABL compacted inactive conformation. The figure is modified using PDB 2FO0 (C) The active conformation of BCR-ABL. The coiled-coil domain and BCR region are colored in red and purple, respectively. The SH2 domain, the SH3 domain and the kinase domain are colored in cyans, blue and grey, respectively. The SH2-SH3 connector and SH2-kinase linker are colored in pink, and the N-terminal myristate group and the Cap region are colored in brown. The schematic presentation is modified using PDB 1OPL.
BCR-ABL is present as a long-lived protein due to several protective mechanisms. Firstly, BCR-ABL forms a tight association with Hsp90, a chaperone that helps to stabilize the BCR-ABL protein structure [30, 31]. Secondly, the deubiquitinase Usp9x keeps BCR-ABL in the un-ubiquitinated state, preventing BCR-ABL from undergoing proteasomal degradation [32]. Lastly, downregulation of SHP1 (SH2 domain-containing protein tyrosine phosphatase 1) leaves BCR-ABL in the phosphorylated state, which keeps BCR-ABL in the active form and escapes the destiny of degradation [33-35]. Therefore, the stable presence of hyper-active BCR-ABL kinase continuously drives the disease progression by phosphorylating downstream substrates.

### 1.1.2 Networking of BCR-ABL signals

Owing to the deregulated kinase activity, BCR-ABL affects and alters almost every cellular process. BCR-ABL orchestrates a massive signaling network and therefore identification and characterization of the “hubs”/“nodes” can be extremely helpful in order to comprehend the malignant transformation by BCR-ABL (Figure 1.3). Three major cascades involving the RAS/MAPK, PI3K/AKT and JAK/STAT pathways, among others, are hijacked and perturbed by BCR-ABL and have been intensively investigated [36, 37].

RAS proteins are small GTPases involved in regulating multiple fundamental cellular functions [38], and activation point mutations and amplifications of RAS are
often observed and comprise about one third of all human cancers [39]. In CML, BCR-ABL constitutively activates RAS-associated signaling pathways [40]. The autophosphorylation at Tyr177 in the BCR region recruits an adaptor protein Grb2, which binds to SOS, a GEF (guanine nucleotide exchanging factor) of RAS, to promote the activation of RAS [41-43]. Subsequently, RAS turns on the MAPK pathway through sequential activation of Raf and MEK1/2 (Figure 1.3) [44]. Finally, the ultimate pathway effector ERK1/2 regulates gene transcription by activation of numerous transcription factors such as CREB, ATF-1, c-Fos, c-Myc and NFκB to promote cell proliferation and inhibit apoptosis [45-47].

The PI3K/AKT pathway, another essential signaling cascade, controls many biological activities such as cell growth, apoptosis, survival and motility, and is frequently deregulated in many types of cancers [48, 49]. In CML, the PI3K/AKT pathway is highly activated and, more importantly, is indispensable for BCR-ABL transformation and disease progression [50, 51]. BCR-ABL influences the PI3K/AKT signaling by forming several protein complexes including BCR-ABL/Grb2/GAB2, BCR-ABL/Shc and BCR-ABL/CrkI/c-Cbl, which can all recruit and activate PI3K (Figure 1.3) [52-54]. Subsequently, PI3K phosphorylates and converts PIP2 to PIP3, which provides the docking sites for the serine/threonine kinases AKT and PDK1 so that AKT can be activated by PDK1 phosphorylation [55-57]. The active AKT then phosphorylates downstream targets including GSK3β, FOXO3a and p27Kip1 to promote
proliferation and inhibits apoptosis. Moreover, one of the important AKT targets is the mTOR-associated signaling that is a strong pro-survival pathway and has been shown to be constitutively active in CML cells [58, 59].

The JAK/STAT axis is a critical signal mechanism that is responsive to multiple stimuli including cytokines, hormones and growth factors [60]. The JAK/STAT cascade is highly implicated in the cell proliferation, differentiation, apoptosis and migration, and plays an essential role in hematopoiesis and development of the immune system [60]. The JAK/STAT signaling is comprised of two components: the receptor-associated JAKs and the transactivator STATs. The cytokines binding to their receptors trigger the trans-autophosphorylation-induced activation of JAKs. Subsequently, the active JAKs phosphorylate the receptors, which serve as docking platforms to recruit STATs for their phosphorylation by JAKs [61]. Then, STATs undergo phosphorylation-mediated dimerization and translocate into nuclei to promote the transcription of pro-survival genes Cyclin D1, Cyclin E1 and c-Myc, and anti-apoptotic genes Bcl-2, Bcl-xl, Mcl-1 and survivin [62]. In CML, JAK2 and STAT5 (a downstream target of JAK2) have been found to possess aberrantly high activities [63, 64]. BCR-ABL phosphorylates and activates JAK2, resulting in a constitutively activated JAK2/STAT5 axis that contributes to cytokine-independent growth of leukemic cells [65, 66]. Moreover, JAK2 is able to phosphorylate BCR-ABL at Tyr177, which, as mentioned above, is critical for BCR-ABL to control RAS/MAPK and PI3K/AKT pathways [67]. In addition, STAT5 activation can
also be directly stimulated by BCR-ABL phosphorylation, independent of JAK2 [68].

More importantly, STAT5 is required for full BCR-ABL transforming ability and CML maintenance/development and high STAT5 levels correlate with drug resistance and poor prognosis [69-71].

Besides these three transduction cascades, BCR-ABL also manipulates Src kinase Lyn/Hck-, Rac-, β-integrin-, Rho-ROCK-mediated signaling pathways [72-78]. Hence, BCR-ABL is a master regulator, disturbing and altering normal cellular circuits to facilitate the proliferation and expansion of CML cells and to promote disease progression.
Figure 1.3. BCR-ABL signaling network. BCR-ABL affects multiple cellular activities to promote CML leukemogenesis through the regulation of downstream signaling transduction pathways. Three major pathways that are strongly deregulated by BCR-ABL are RAS/MAPK, PI3K/AKT and JAK2/STAT5 pathways. Reproduced and modified from O’Hare et al, 2010 [79] (with permission through the Copyright Clearance Center).

1.1.3 Current therapeutics for CML

The first CML treatment was reported in the 19th Century [80]. Since then, different
therapeutic approaches such as hydroxycarbamide, busulphan, interferon α (IFNα) and splenic irradiation have been attempted for CML treatments [80]. Among these, only IFNα induces modest complete cytogenetic response (CCyR, Ph negativity) in about 20% of patients and prolongs survival [81-83]. However, the overall clinical outcomes of IFNα treatments are not satisfactory and the associated side effects make it impossible for long-term use [81-83]. Allogeneic stem cell transplantation (allo-SCT) is considered to be the only “curative therapy” that results in long-term disease remission and survival [80, 84]. However, Allo-SCT comes with a multitude of risks and limitations, including high morbidity and mortality rate. The age requirement of the patients and the difficulty in locating matched donors do not allow this potentially curative therapy to be a feasible option for the majority of CML patients [80, 84].

Fortunately, the advent of tyrosine kinase inhibitors (TKIs) has completely revolutionized the treatment of CML. TKIs are small ATP-competitive inhibitors which occupy the active site of the BCR-ABL kinase domain and inhibit BCR-ABL activities [85]. Over the last 10 years, the application of TKI therapy has greatly hindered CML progression and has led to a major improvement in disease prognosis [86-88]. Long-term clinical outcomes are astonishingly satisfactory. Due to the high selectivity towards BCR-ABL, TKIs exhibit highly acceptable safety profiles for long-term administration [89, 90]. The achievement of up to 90% five-year survival rates with TKI treatments has allowed early phase CML to become one of the few fatal malignancies to be converted
into a manageable chronic disease. Currently, three TKIs (imatinib, dasatinib and nilotinib) are used as the first-line CML treatments and another two (bosutinib and ponatinib) are available if drug resistance and intolerance are present in patients [91].

Imatinib (IM, formerly known as STI571) is the first BCR-ABL tyrosine kinase inhibitor approved in 2001 by the US Food and Drug Administration (FDA) as a first-line treatment for CML treatments [86, 87, 92]. Structurally, this compound inserts into the entire central region of the kinase domain and effectively locks BCR-ABL in the inactive conformation to restrain BCR-ABL activities [93, 94]. From multiple clinical trials, including the 8-year follow-up of the IRIS (The International Randomized Study of IFN-α and STI571) study, CML patients in early chronic phase with IM treatments achieved and maintained impressive complete hematological remission (CHR, normal white blood cell count), complete cytogenetic remission (CCyR, Ph negativity tested by FISH) and major molecular remission (MMR, 3-5 logs of reduction in BCR-ABL transcripts). More importantly, IM treatments improved the 8-year overall survival rate (85%) and reduced progression to advanced disease rate (92%), compared to the mean expected survival of 2-3 years for CML patients in the pre-TKIs era [95, 96]. Therefore, Imatinib can be considered a paradigm for successful molecularly targeted cancer therapy. However, despite the impressive clinical outcomes of IM therapeutics, a significant proportion of patients develop drug resistance over time. One of the most common mechanisms of IM resistance is the gain of point mutations in BCR-ABL kinase domain.
To date, more than 100 kinase domain mutations have been identified, which interfere with IM targeting by directly blocking IM entry, loss of the residues required for IM interaction or alteration of the conformation of the kinase domain disfavoring IM binding [97-99].

These clinical obstacles have driven researchers to design and develop second generation of TKIs. Nilotinib (NL) and Dasatinib (DA) received FDA approval in 2007 and 2006, respectively, as second-line treatments for IM-resistant/intolerant patients in all the disease phases and now have also been approved to be used as front-line CML treatments [100-102]. NL was developed as an IM analog but its improved fit in the active site of the kinase domain confers a higher potency (30 fold) against BCR-ABL compared to IM [103]. Furthermore, NL was designed to make fewer interaction contacts with the kinase active site thus enabling it to bind to numerous BCR-ABL mutants [104-108]. Therefore, NL shows high activity towards most IM-resistant mutations. DA is a strong dual ABL/SRC kinase inhibitor and up to 250 times more potent against BCR-ABL compared to IM, due to its ability to bind to both active and inactive forms of the kinase domain [105, 109]. DA reacts with the BCR-ABL protein in a different manner from IM, therefore it is quite active against a broad spectrum of the IM-insensitive mutations [104, 106]. As a second-line therapy, DA and NL single treatments induced CCyR in around 35% of IM resistant/intolerant patients in CP and resulted in 4-year and 5-year overall survival of up to 80%, respectively [91, 95, 96, 110]. When tested in the
first-line setting in CP patients, both DA and NL show significant superiority over IM in achieving and maintaining CCyR and MMR, and limiting disease progression into AP/BC [95, 96].

However, all three abovementioned TKIs and another ABL/SRC dual inhibitor bosutinib (BO) (second-generation TKI) show very little activity against the gate keeper mutation T315I, which creates an entry block and steric clashes in the kinase domain. The identification of this mutation prompted the discovery of ponatinib, the third generation of TKI, with 500 times more potency than IM [111]. Due to its special structural design, ponatinib is able to bind to the T315I mutant by circumventing the steric hindrance caused by the bulky Ile at the gate-keeper position [111, 112]. Ponatinib shows pan-BCR-ABL inhibitory activity against almost all the currently-known the BCR-ABL kinase mutations, and has been approved for patients who are unresponsive to all the prior TKI treatments and bear T315I mutations in all the disease phases [106, 113]. However, some patients developed arterial thrombosis in a phase 2 clinical trial, and therefore further clinical trials were suspended [114]. Interestingly, a new allosteric inhibitor, ABL001, has recently been developed and it appears to inhibit most forms of BCR-ABL including the T315I mutant by a distinct, allosteric mechanism through specifically binding to the myristoyl pocket of the BCR-ABL protein [115-117]. These studies also have shown that ABL001 selectively inhibits the proliferation of CML cells with BCR-ABL mutations in vitro and a combination of ABL001 and nilotinib treatment
produces a sustained regression of leukemic cell growth in a xenograft model of BCR-ABL+ KCL-22 induced leukemia [116]. In spite of high efficacy of TKIs in CML treatments, TKI monotherapies are not curative in most patients, as molecular remissions are rare.

1.1.4 Therapeutic challenges in CML

Due to the unprecedented success of TKI therapeutics, CML now is considered more of a chronic disorder instead of a deadly malignancy. However, a permanent cure has not been achieved. Even in the “TKI era”, approximately 20-30% of patients still have a high chance of developing TKI resistance and progressing into the advanced phase [118, 119]. A significant number of the patients ceased TKI treatments due to the unsatisfactory clinical outcomes caused by emergence of drug resistance and/or drug intolerance [80]. Moreover, most patients harbour residual leukemic cells, and over 60% of patients show evidence of molecular relapse upon IM discontinuation; thus, life-long treatment is required, burdening patients with potential side-effects and a high cost (e.g. annual price for dasatinib is >$100,000) [120, 121]. One of the largest therapeutic challenges is patients developing TKI resistance. The fact that BCR-ABL kinase domain mutations are not detected in all of the TKI-resistant patients indicates that BCR-ABL mutations do not account for all cases of reduced TKI responses [110]. In the past decade, many resistance mechanisms have been identified, which can be categorized into BCR-ABL-dependent
and BCR-ABL-independent mechanisms.

1.1.4.1 BCR-ABL-dependent mechanisms of TKI resistance

One of the most common TKI-resistant mechanisms are acquired mutations in the BCR-ABL kinase domain, which block or attenuate TKI binding (Figure 1.4A). Although the second-generation TKIs (DA, NL and BO) cover a wide spectrum of the kinase mutations as a result of enhanced structural designs and binding modes, these inhibitors are still susceptible to some mutations. NL is not very effective against T315I and kinase phosphate binding loop (P-loop) mutations, including G250E, Y253H and E255V/K, while poor response to DA and BO is associated with T315I and mutations within the kinase hinge region, such as F371L and V299L [107, 122, 123]. The emergence of the T315I-inclusive compound mutations, which might be generated by sequential TKI treatment-driven selective pressure, conveys resistance to all the available TKIs, including ponatinib, leaving very limited therapeutic options [124, 125]. Hence, in the era of TKIs, other clinical approaches are still urgently needed.

Secondly, overexpression of BCR-ABL is linked with resistance as observed from both cell line work and primary CML patients (Figure 1.4B) [99, 126]. The overproduced BCR-ABL results in the increased generation of kinase domain mutations that further confer resistance and also promotes the elevation of genomic instability by inducing overproduction of reactive oxygen species (ROS) and unfaithful DNA repairs. Therefore,
the amplification of BCR-ABL contributes to disease progression to the advanced phase during which responses to TKIs worsen [127-129].

1.1.4.2 BCR-ABL-independent mechanisms of TKI resistance

In the past 15 years, several BCR-ABL-independent TKI resistant mechanisms have been identified, which are: (1) the reduction of intracellular TKI concentration due to altered expression/activities of drug influx and efflux [118, 122, 130, 131]; (2) BCR-ABL-independent activation of compensatory signaling pathways including SRC kinase Hck/Lyn- and STAT3-mediated signaling [132-135]; (3) the persistence of the CML leukemia stem cells (LSCs, Figure 1.4C - E) [136-139].

An increasing body of evidence points to CML LSCs as the ultimate culprits responsible for BCR-ABL-independent resistance mechanisms [140]. LSCs resemble their normal hematopoietic stem cell (HSC) counterparts but are characterized by reduced quiescence and self-renewal capacities, which confer LSCs with higher proliferative potential [141-144]. The findings that LSCs are independent of BCR-ABL kinase activity for survival have changed the perspectives on TKI resistance [145, 146]. The reasons for LSCs resistance to TKIs are attributed to their unique intrinsic properties and extrinsic microenvironment (Figure 1.4E).

Inherently, a fraction of LSCs stay in the quiescent state, which provides LSCs a route to escape from TKI targeting, as TKIs primarily target dividing cells [147-149].
However, this idea has been challenged by the finding that TKIs are able to target both dividing and non-dividing CML cells [146, 150], therefore it is more likely that LSCs are resistant to TKIs due to some intrinsic BCR-ABL-independent mechanism. CML LSCs also possess unique signaling signatures, which are unrelated to direct BCR-ABL kinase activity. The aberrant activation of these signaling pathways including Wnt-β-catenin, Sonic hedgehog, TGF-β-AKT-FOXO3A-BCL6, ALOX5 metabolism and PML-related signaling [151-159], allows for maintaining LSC properties such as quiescence and self-renewal capability and sustaining LSC survival, thereby freeing LSCs from BCR-ABL addiction and in turn TKI targeting.

Extrinsically, the BM niche where LSCs reside provides a sanctuary to protect LSCs by secretory cytokines or growth factors, hypoxic environment and direct interactions. The abnormal production of MIP1α/β, IL-1α/β and TNFα from the leukemic BM niche promotes proliferation of LSCs and clonal dominance over normal HSCs [141]. The hypoxic microenvironment also favors the maintenance of the quiescent state and self-renewal potential of LSCs, creating an intrinsic protective system from TKI targeting [136, 160, 161]. Moreover, it has been shown that hypoxia induces suppression of BCR-ABL expression so that the targets of TKIs are no longer present in LSCs [160, 162, 163]. Lastly, the interactions between LSCs and BM niche ensure that LSCs stay in the “shelter” of the BM niche-mediated protection as described above [164-169]. One of the most well-studied mechanisms for retention of LSCs in the BM niche is the
CXCR4-CXCL12 axis. CXCR4 (CXC chemokine receptor 4) is a G protein coupled-chemokine receptor, which is responsive to its ligand SDF-1 (stromal-cell-derived factor 1, also known as CXCL12) that is largely secreted by the bone marrow stromal cells [170-173]. Previous studies have shown that the CXCR4-CXCL12 axis plays a central role in homing and retention of hematopoietic stem/progenitor cells in the bone marrow [174, 175]. In recent years, a growing amount of evidence has demonstrated that CXCR4 and CXCL12 interactions are exploited by various types of leukemic cells for migration to the bone marrow for niche-mediated chemoresistance and promotion of cell survival by CXCL12-triggered activation of MAPK and PI3K-AKT pathways [176, 177]. In CML, the BCR-ABL kinase activity causes downregulation of surface CXCR4 expression on CML stem/progenitor cells, which results in the adherent defection and egress of immature leukemic cells from bone marrow [178]. However, TKI treatments restore surface CXCR4 expression, thereby promoting CML cells to migrate back to the bone marrow for protection, which is one of the mechanisms of TKI resistance [167].

Although TKIs are capable of eliminating the bulk population of leukemic cells, LSCs can persist despite long-term TKI therapy due to the abovementioned properties. Therefore, LSCs serve as a reservoir for minimal residual disease and are responsible for relapse upon TKI discontinuation [140]. Hence, the eradication of CML LSCs may achieve the ultimate goal of curing CML.
Figure 1.4. Schematic representation of BCR-ABL-dependent and -independent TKI-resistant mechanisms. The BCR-ABL-dependent mechanisms include (A) acquisition of mutations in the BCR-ABL kinase domain, which affects the effective binding of TKIs, and (B) amplification of BCR-ABL that causes the overproduction of the BCR-ABL kinase. The BCR-ABL-independent mechanisms include (C) the reduced intracellular concentrations of TKIs due to upregulation of efflux transporters and/or downregulation of influx transporters, (D) activation of other compensatory pathways, and (E) the persistence of TKI-refractory CML leukemic stem cells.

1.1.5 Deregulated production of reactive oxygen species (ROS) in CML

Reactive oxygen species (ROS) are a group of natural byproducts from normal oxygen metabolism, including superoxide, hydrogen peroxide and hydroxyl radicals [179, 180]. Mitochondria are the main sources of ROS production, and utilize ROS to regulate redox signaling in cells [181]. Under normal physiological conditions, ROS, especially
hydrogen peroxide, function as a secondary messenger to mediate a range of cellular functions such as cell proliferation, motility, cell-cycle progression and antimicrobial defense [182, 183]. In contrast, under pathophysiological conditions, elevated ROS escape the surveillance of the antioxidant machinery by overwhelming the capacity or causing the excessive consumption of ROS scavengers (e.g. NADPH) [184]. Consequently, the over-produced ROS causes DNA damage, leading to genomic instability that leads to an increased frequency of DNA mutations, chromosomal abnormalities and aneuploidy (23909437, 21278445). In addition, ROS overproduction can also cause aberrant oxidation of lipids and proteins, resulting in redox imbalance and oxidative stress [182, 185-187]. In this manner, deregulated ROS production has been observed in a variety of human diseases such as rheumatoid arthritis, atherosclerosis, amyotrophic lateral sclerosis and also malignances, including CML [188-190].

In normal hematopoiesis, ROS management is crucial in the preservation of HSC functions and migration of HSCs/progenitors [191-195]. In CML, however, the BCR-ABL kinase activity leads to accumulation of high levels of ROS that cause oxidative DNA damages (e.g. double strand breaks), which, combined with an unfaithful repair system, results in an accumulation of point mutations and chromosomal aberrations [119, 196-207]. ROS deregulation in CML has been reported to contribute to disease progression to the advanced phase [202, 208, 209]. The transition from CP to BC is considered to be the consequence of a series of critical genetic alterations leading to the
selection of CML-BC clones that possess higher growth advantage, a differentiation block and altered BM adhesion [208, 209]. Genomic instability caused by high levels of ROS is likely to be one of the main drivers for these genetic events. Furthermore, it was shown that ROS overproduction leads to a higher frequency of mutations in the BCR-ABL kinase domain, which contributes to TKI resistance in CML cells [188, 198, 199]. Several recent studies have revealed that in primitive CML populations, the accumulation of high levels of ROS was not completely dependent on BCR-ABL kinase activity, and consequently TKIs were unable to reduce ROS production to the normal level [210, 211]. Therefore, the deregulated ROS in primitive CML cells continued producing BCR-ABL kinase mutations even during TKI treatments, resulting in TKI-refractory LSCs and primitive progenitors.

Due to the oncogenic roles of ROS in CML development, targeting ROS can be considered as another therapeutic option for CML treatments. Although inhibition of BCR-ABL by TKIs is able to reduce ROS levels and its related genomic instability, due to the BCR-ABL-independent ROS production, TKI monotherapy may not be sufficient to completely eliminate ROS effects. Several studies have shown that combined treatments of TKIs and antioxidants or ROS scavengers strongly inhibited growth, induced apoptosis and reduced the generation of mutations in vitro, and prolonged the survival of leukemic mice in vivo [212-216]. Hence, concurrent inhibition of BCR-ABL and ROS production could be a more effective approach in CML therapeutics.
1.1.6 Autophagy and its involvement in CML

Autophagy is an evolutionarily conserved self-preservation process that regulates cellular homeostasis by targeting long-lived proteins, intracellular aggregates and aged organelles for lysosomal degradation, and subsequently recycling essential building blocks such as amino acids, nucleic acids, sugars and ATP for generation of new biomolecules [217, 218]. Under normal physiological conditions, autophagy proceeds at a basal level and operates as a quality control system to maintain homeostatic functions [219, 220]. However, under challenging conditions, autophagic flux is upregulated in response to stimuli from nutritional deprivation, oxidative stress and targeted therapies among others to sustain cellular viability [221, 222].

Macroautophagy (hereafter referred as autophagy) is a multistep process, and each step of autophagy is under tight regulation of signaling mechanisms and numerous autophagy-related (ATG) proteins (Figure 1.5) [223, 224]. The most well characterized autophagy-repressive signaling system is the PI3K-AKT-mTOR axis [225]. Under normal conditions, mTORC1 directly inhibits ULK1 [226, 227], which is a key component of protein complex for the initiation of the autophagy process. Under starvation or stressful conditions, mTORC1 activity is suppressed by multiple signaling cascades, thereby resulting in activation of ULK1 for triggering autophagy [228, 229]. The nucleation step is dependent on the Beclin-1-mediated protein complex, whose activation requires
phosphorylation of Beclin-1 by ULK1 [229-231]. Two other complexes are necessary for the maturation of autophagosomes, double-membrane vesicles that engulf damaged or aged cytoplasmic materials. The ATG4/ATG7/ATG3 complex mediates lipid modification of LC3 (microtubule-associated protein 1 light chain, also known as Atg8), while the ATG12/ATG5/ATG16 complex regulates binding of lipidated LC3 to the autophagosome membrane [232-234]. Undergoing a series of proteolytic cleavages, pro-LC3 is processed through an intermediate LC3-I to the final PE (phosphatidylethanolamine)-conjugated form LC3-II that is associated with autophagosome membranes [235]. Currently, the LC3-I/II conversion is used in various assays to monitor autophagic flux [236]. During the maturation step, p62 (also known as SQSMT1) among others functions as an adaptor protein by recognizing and targeting ubiquitinated protein aggregates to autophagosomes through its ubiquitin-binding and LC3-interacting domains [237]. Since p62 is eventually degraded along with its cargo, the levels of p62 and autophagy are inversely correlated. Therefore, p62 is used as another autophagy indicator [238]. The fusion of autophagosomes and lysosomes to form autolysosomes is the last stage of the whole process, where the internal contents are lysed by various lysosomal hydrolyses [239-242].
Figure 1.5. **Overview of the autophagy process.** Autophagy is a multi-step process, which is initiated under stressful conditions. Autophagy starts with the formation of double-membrane structure attached with PIP3 (phosphatidylinositol triphosphate), which is regulated by ULK- and Beclin-1 complexes. The autophagosome maturation is mediated by ATG4/ATG7/ATG3 and ATG12/ATG5/ATG16 complexes that facilitate the binding of LC3-II PE (phosphatidylethanolamine) to autophagosomal membrane. Lastly, the fusion of the autophagosome and lysosome leads to the degradation of the internal materials such as damaged proteins and organelles within the autophagosome.

In the past several decades, a growing body of evidence has demonstrated that autophagy is heavily involved in a range of cellular activities including cell development, aging, homeostasis and immunity [243]. Therefore impaired autophagy has been implicated in diverse diseases, including cancers [244, 245]. With respect to malignancies, autophagy is currently viewed to function as a tumor-suppressive mechanism that prevents tumor initiation by mitigation of genetic defects and oxidative stress and removal of oncoproteins and also as a pro-survival pathway in established tumors to protect cancer cells from hypoxic stress, transformation-induced metabolic stress and
anti-cancer agent-triggered death. The dual and conflicting role of autophagy in tumorigenesis is highly dependent on the stages of cancer development [246-248].

In the hematopoietic system, autophagy has been demonstrated as a crucial mediator in HSC maintenance [249, 250]. In the case of CML cells, BCR-ABL kinase activity suppresses autophagy by increasing the activity and expression of mTORC1, a negative regulator of autophagy, through the PI3K/AKT pathway and upregulating the expression of miR-30a, which is a potent suppressor of two key autophagy genes Beclin-1 and ATG5 [251, 252]. However, the relatively “low” basal level of autophagy in CML stem/progenitor cells is still significantly higher than that in normal BM cells and plays an important role in BCR-ABL transformation [253]. More importantly, several studies indicate that autophagy is strongly induced via BCR-ABL inhibition by TKIs in CML cell lines and primary cells, and the upregulated autophagy acts as a cytoprotective mechanism against TKI-mediated apoptosis [254-256]. These findings suggest that autophagy is another pathway conferring TKI resistance.

Targeting autophagy using genetic or pharmacological approaches has been shown to enhance TKI-induced cytotoxic effects in drug-resistant CML cell lines and primitive CML cells, and the combination of autophagy inhibition and TKIs significantly prolong survival in mouse models of CML [257-259]. Based on these promising results, a phase 2 clinical trial testing the combination effects of IM and HCQ, an autophagy inhibitor, in CML patients is currently being carried out [260]. These findings underscore the
significance of autophagy in the cell survival and TKI response of primitive CML populations, and autophagy-targeted therapy could be another option to overcome disease resistance.

1.2 Abelson helper integration site 1 (Ahi-1/AHI-1)-mediated protein interaction network and disease development

It has been reported that Abelson murine leukemia virus (A-MuLV), which harbors the oncogene v-abl [261, 262], can induce pre-B-cell lymphomas. However, the expression of v-abl alone is not sufficient to induce full malignant transformation in target cells and therefore additional genetic events are needed [263, 264]. Furthermore, A-MuLV is a replication-defective retrovirus and is dependent on a non-defective helper MuLV to replicate in vivo and in vitro [265]. The helper Moloney MuLV, an insertional mutagen involved in the development of A-MuLV-induced lymphoma, was found in a specific region designated Ahi-1, in 16% of Abelson pre-B-cell lymphomas. The insertional mutagenesis frequency significantly exceeds the frequency expected for random integrations, suggesting that rearrangement of the Ahi-1 site contributes to pre-B-cell lymphomagenesis [263, 266]. In addition, the Ahi-1 locus was also identified as a common provirus integration site in c-myc-induced murine T cell leukemia, Molony MuLV-induced rat thymomas and acute myeloid leukemia in Nf1 heterozygous mice [267-269]. These findings provide evidence that the Ahi-1 locus is indeed a target of
provirus insertional mutagenesis and involved in the development of different types of murine leukemia and lymphomas.

Interestingly the Ahi-1 locus was found to be closely linked to the c-myb proto-oncogene within a 120-kbp DNA fragment, however upon A-MuLV infection, no significant change of c-myb expression was observed, implying that there might be a novel gene within the Ahi-1 locus, which was mutated and dysregulated by provirus insertions in tumor development [268, 270]. This gene was cloned, identified as Ahi-1 and mapped to mouse chromosome 10, and subsequently the human counterpart AHI-1 was found to be located in human chromosome 6 [271].

1.2.1 AHI-1 structure, regulation and expression

Human AHI-1 gene spans across a 200-kb region of chromosome 6 containing 33 exons [271]. Three AHI-1 isoforms have been identified to date, with alternative splicing occurring at the 3’-region (Figure 1.6) [271]. The 1.3kb region upstream to the AHI-1 start codon was identified as a functional promoter which contains two putative TATA boxes, a cis-acting element CCAAT box and binding sites for transcription factors such as Sp1, SRY, CTF, c-fos and Oct-1[272].

The AHI-1 mRNA encodes a 1096 amino acid protein (Isoform I) consisting of three major protein-protein interaction domains: an N-terminal coiled-coil domain, a WD40-repeat domain and an SH3 domain (Figure 1.6) [271]. The coiled-coil domain is a
structural motif composed of two or more α helices wrapping around each other in a bundle structure. Through this special structural organization, proteins can self-assemble via homo-oligomerization or form molecular complexes by hetero-oligomerization to affect multiple cellular processes [273]. The WD40-repeat domain displays a β-propeller structure, often comprising seven blades. This domain mediates molecular complexes in different binding modes through its top surface, bottom surface or side [274]. Interestingly, the WD40-repeat domain-containing proteins are involved in a wide range of cellular functions, including protein ubiquitination, signaling transduction, chromatin remodeling, transcription regulation and RNA processing [275, 276]. The SH3 domain is one of the most abundant protein-interacting domains and shows strong binding affinity to proline-rich motifs. The SH3 domain modulates cellular behaviors through facilitating the assembly of signaling protein complexes and large protein networks [277-279]. AHI-1 also harbors two PEST sequences and several PXXP motifs, which mediate protein degradation and binding to SH3 domain-containing proteins, respectively [279, 280]. Thus, AHI-1 appears to be an adaptor protein that mediates protein-protein interactions in signal transduction.
Figure 1.6. Schematic presentation of human AHI-1 isoforms. Human AHI-1 (Isoform I) contains multiple structural motifs harbouring a coiled-coil domain, a WD40-repeat domain, an SH3 domain, several PXXP motifs and two PEST sequences. Compared to isoform I, isoform II lacks a large portion of the SH3 domain and the entire C-terminal region, while isoform III contains additional coding sequences in the C-terminus. Reproduced and modified with permission from Esmailzadeh et al, 2011.

Both mouse and human Ahi-1/AHI-1 are expressed in multiple organs, particularly mouse and human brains exhibit strong Ahi-1/AHI-1 expression, implicating an essential role of Ahi-1/AHI-1 in regulating normal brain functions [281]. In the hematopoietic systems of both mice and humans, the Ahi-1/AHI-1 expression decreases as the most primitive HSCs undergo differentiation to mature cells [282]. This observation suggests that AHI-1 may play a role in regulating stem cell functions and executing specific differentiation programs.
1.2.2 AHI-1 in hematopoietic malignancies

The first piece of evidence that linked the deregulation of AHI-1 to hematopoietic malignancies came from the experimental observation that *AHI-1* expression was significantly higher in a panel of established leukemic cell lines of myeloid, B-cell and T-cell origin compared to normal BM cells [282]. The following studies have shown that *AHI-1* expression is significantly elevated at all stages of CML cell differentiation in the three disease phases. Subsequently, two reports have gone into great length elucidating the transforming potential of AHI-1 in CML leukemogenesis [283, 284]. Overexpression of *Ahi-1* alone increases proliferative activity in IL-3-dependent pro-B BaF3 cells and mouse hematopoietic stem/progenitor cells, and the effect is significantly enhanced in the cells overexpressing both Ahi-1 and BCR-ABL. Furthermore, immunodeficient mice injected with Ahi-1/BCR-ABL co-expressed cells exhibited much shorter disease latency compared to mice injected with either Ahi-1 or BCR-ABL-transduced cells [284]. Given the fact that the highest expression of AHI-1 and BCR-ABL was detected in CML stem cell-enriched population [282], these results indicate cooperative activities of AHI-1 and BCR-ABL in the transformation of hematopoietic cells and mediation of clonal expansion of leukemia stem cells. In relation to signaling regulation, protein expression and activities of JAK2 and STAT5 were elevated and sustained in cells with co-expression of BCR-ABL and *AHI-1*, suggesting that AHI-1 may play a role in facilitating BCR-ABL oncogenic activity associated with the JAK/STAT5 axis [284].
Indeed, AHI-1 physically interacts with BCR-ABL and JAK2 through its WD40-repeat domain and N-terminal region, and the protein complex BCR-ABL-AHI-1-JAK2 has been shown to contribute to BCR-ABL transforming activities, TKI resistance and disease progression [283, 284].

In addition, suppression of AHI-1 expression using shRNA targeting strategies markedly reduced colony forming ability and increased responses to TKIs in CD34⁺ CML stem/progenitor cells [284]. Combining TKI and JAK2 inhibitors caused the disruption of the BCR-ABL-AHI-1-JAK2 protein complex and degradation of all three oncoproteins, consequently inhibiting proliferation and colony forming ability and inducing apoptosis of TKI-resistant CML stem/progenitor cells [283]. Importantly, oral JAK2 inhibitors combined with a TKI (IM or DA) significantly enhanced the survival of leukemic mice and eradicated infiltrated leukemic cells in multiple hematopoietic tissues in an aggressive mouse model of BCR-ABL⁺ human leukemia [283, 285]. This combination treatment also significantly decreased long-term engraftment of CD34⁺ CML stem/progenitor cells in immunodeficient mice [283]. All of the findings demonstrate a strong oncogenic role of AHI-1 in regulating CML LSC properties and drug resistance and highlight that targeting AHI-1-mediated protein complexes is a valid option in elimination of LSC to improve treatment of CML.

Interestingly, transcript levels of AHI-1 were found to be highly expressed in Hut78 and Hut102 cells derived from different cutaneous T-cell lymphoma (CTCL) patients.
[282], a lymphoproliferative disorder characterized by the infiltration of malignant T cells on the skin, which links the deregulation of AHI-1 with CTCL pathogenesis [286, 287]. Indeed, as compared to normal T-cell counterparts, the AHI-1 expression is significantly increased in primary patient samples of Sezary Syndrome that is the leukemic variant of CTCL [288]. Furthermore, suppression of AHI-1 in Hut78 cells significantly impairs the overproduction of autocrine cytokines and reduces growth factor independence in vitro, which are two characteristic transforming phenotypes, and causes the inability of these lymphoma cells to induce tumors in vivo [288]. These findings suggest that AHI-1 expression regulates this multi-factorial autocrine mechanism that contributes to the autonomous growth of CTCL cells. AHI-1 was also found to negatively regulate the expression of a tumor suppressor BIN1 in CTCL [288, 289]. Recent studies have demonstrated that BIN1 expression is significantly reduced in CTCL primary samples and the attenuated expression of BIN1 results in the inefficient induction of Fas/Fas ligand-mediated apoptosis, which is one of the major underlying causes of disease persistence [289, 290]. Hence, AHI-1 may also drive CTCL pathogenesis by altering the expression of signaling molecules.

**1.2.3 AHI-1 in neuronal/neuropsychiatric diseases and metabolic disorders**

As described in Section 1.2.1, AHI-1 has been reported to be highly expressed in several regions of the human brain, suggesting an important role of AHI-1 in neuronal
functions and brain development. *AHI-1* was identified as the first causative gene [281, 291] mutated in Joubert syndrome (JS), which is a rare autosomal recessive disorder characterized by abnormal brain development and mental retardation, and also associated with extra-neurological symptoms including retinal dystrophy and renal cystic disease (known as Joubert syndrome related disorders, JSRD) [292-294]. A high frequency of *AHI-1* mutations was found in patients with JSRD [281, 295, 296]. Most of the mutations are frameshift or nonsense mutations, leading to truncated AHI-1 with partial or complete loss of WD40-repeat and/or SH3 domains [281,291, 296, 297]. Some missense mutations such as R723Q, R830W and E1086G, which are located in these two critical domains, have also been associated with JSRD [297-301]. Mutational analysis suggests that AHI-1 functions to coordinate assembly of multiprotein complexes through its protein-protein interaction domains, regulating the development of brain diseases.

At the cellular level, JS and JSRD are classified as ciliopathies, a diverse group of genetic disorders characterized by the dysfunction of the primary cilium [302, 303]. The primary cilium is a conserved sensory organelle in many types of mammalian cells and is essential for modulation of a range of cell signaling pathways and development and maintenance of multiple organ systems [304, 305]. Since AHI-1 was demonstrated to be a crucial structural and functional component of the primary cilium, AHI-1 defects and deregulation have been considered to be one of the underlying causes in the pathogenesis of JSRD [306-310].
Several AHI-1 single nucleotide polymorphisms (SNPs) are also closely associated with the increased risk for schizophrenia, autism, major depressive disorder and bipolar disorder [311-314]. Mechanistically, AHI-1 deficiency causes the increased lysosomal degradation of the TrkB receptor that is critical for neuronal differentiation, maturation and mood regulation [315, 316] and reduced release of depression-related neurotransmitters (serotonin and dopamine) [317], both of which contribute to the depressive phenotype. These data suggest a functional role of AHI-1 in regulating human mood, cognition and social behavior.

Lastly, it has been reported that there is a strong association of AHI-1 variants and copy number variation of the AHI-1 gene with the risk of type 2 diabetes and obesity, respectively [318, 319]. At the molecular level, AHI-1 functions as an energy metabolism sensor and has a positive impact on food intake. In the brainstem, the AHI-1 expression is under insulin regulation. High brain insulin levels under energy-rich circumstances cause decreased AHI-1 expression, therefore decreasing food intake [320]. Hypothalamic AHI-1, on the other hand, mediates feeding behavior by interacting with and causing the lysosomal degradation of serotonin receptor 2C (5-HT2CR), which is a negative regulator of food intake [321].

Taken altogether, these findings emphasize a pivotal role of AHI-1 in brain development, from being a key element in the formation of an essential cellular organelle (primary cilium) to regulating the complex neuronal network and brain physiology.
Therefore, AHI-1 is clearly an attractive target and further delineating its biological functions in pathogenesis of brain disorders and other diseases may lead to the development of new therapeutics for treatment of these diseases.

1.3 Biological functions and regulation of Dynamin-2 (DNM2)

Dynamin, a mechanochemical enzyme, belongs to a family of proteins called the dynamin-related proteins, which are comprised of three classical dynamins and dynamin-like proteins [322, 323]. Dynamin family proteins are atypical large GTPases that possess low affinity for GTP binding but high propensity for oligomerization [324]. These family members are highly involved in a wide range of cellular processes such as the fission and fusion events of plasma and mitochondrial membranes, peroxisome and vacuolar fission, ER fusion, cytoskeleton remodeling and anti-viral protection [323].

Dynamin was first discovered as a protein that formed helices around microtubules, which triggered the formation of microtubule bundles [325, 326]. Three dynamin isoforms (DNM1, DNM2 and DNM3) that share about 80% sequence identity were identified in mammals [327]. In respect to expression pattern, DNM1 is mainly expressed in neuronal tissues and is responsible for the rapid formation of synaptic vesicles, while DNM3 shows some redundant presynaptic functions as DNM1 but is also highly expressed in testes and implicated in sperm release. DNM2, however, is ubiquitously expressed and involved in clathrin-mediated and/or -independent endocytosis in all types
of cells [328]. The deletion of DNM2, but not DNM1 or DNM3, causes early embryonic lethality in animal models and mutations in DNM2 found in two congenital neuromuscular disorders suggest pleiotropic roles of DNM2 over the other two isoforms in embryonic development and nervous/muscular systems [329-332].

1.3.1 DNM2 structure, activity and regulation

Human DNM2, one of the three classical dynamins, was identified through screening of a fibroblast library [333]. Human DNM2 is mapped on chromosome 19 (19p13.2) and consists of 22 exons in a 114-kilobase region [333]. Human DNM2 cDNA encodes an 870 amino-acid protein and harbors five functional domains (Figure 1.7A): (1) a GTPase (G) domain needed for GTP binding and hydrolysis, (2) an α-helical middle domain (MD) mediating dynamin self-assembly and oligomerization, (3) a pleckstrin homology (PH) domain that targets phosphatidylinositol 4, 5-bisphosphate (PIP2) for membrane binding and stimulates GTPase activity, (4) a GTPase-enhancing domain (GED) domain that acts as an intra-molecular GTPase activating protein (GAP) by directly binding to the GTPase domain, and (5) a proline–rich domain (PRD), which interacts with SH3 or SH3/BAR (Bin-Amphiphysin-Rvs) domain-containing proteins to direct dynamin to the endocytosis site and promote membrane fission [334, 335].

During the membrane fission process, DNM2 molecules undergo self-assembly, which leads to a drastic increase in the GTPase activity compared to the basal level, to
form helical collars encircling the neck regions of the vesicles (Figure 1.7B) [336-340]. DNM2 helical collars function as a power stroke to tighten and constrict the vesicle necks until leading eventually to membrane fission [334, 341].

Figure 1.7. The crystal structure of Dynamin (DNM) and cryo-EM images of Dynamin assembly. (A) The crystal structure of DNM reveals that in the monomeric form, dynamin folds back on itself with the GTPase (G) domain and PH domain separated by the stalk consisting of four helices from the middle domain and GTPase-enhancing domain (GED) domain. The proline-rich domain (PRD) that is located to the C-terminal of the GED is removed for the purpose of protein crystallization. (B) The end-on, side-on and cross-section views of a pseudo-atomic model of the assembled dynamin polymer. Reproduced and modified from Chappie et al, 2013 [334] (with permission through the Copyright Clearance Center).
Post-translational modification of DNM2 is one of the most important mechanisms regulating DNM2 GTPase activity. Particularly, phosphorylation of DNM2 at Tyr231 (MD) and Tyr597 (PH domain) by the Src or c-ABL kinase has been shown to be crucial to fully activate DNM2 [342-344].

1.3.2 DNM2 in endocytosis

One of the main biological roles of DNM2 is to regulate endocytosis (Figure 1.7A) [335]. Endocytosis is an evolutionarily conserved biological activity in all eukaryotic cells [345]. Traditionally, endocytosis is defined as a cellular process that mediates the uptake of nutrients and other types of molecules into the cells and regulates the composition of the molecules embedded on the plasma membrane through vesicles originated from plasma membrane invagination and budding-off [346]. However, in the past two decades, endocytosis has been heavily implicated in numerous cellular functions, including signaling cascades, cell cycle regulation, cell fate determination, apoptosis, cell polarity and transcription [347-350].

The involvement of DNM2 in every step of clathrin-mediated endocytosis (CME) has been elucidated in great detail. DNM2 was found to co-localize with clathrin before and during coated-vesicle internalization, indicating that DNM2 plays an essential role in the formation and maturation of clathrin-coated pits (CCPs) (Figure 1.8A) [351, 352]. Moreover, multiple DNM2 binding partners regulate DNM2 functions during
DNM2-mediated membrane fission [353-356]. For instance, the SH3/BAR domain-containing proteins amphiphysin and endophilin facilitate DNM2-mediated endocytosis by targeting DNM2 to CCPs through SH3-PRD interactions and generating membrane curvature for optimal DNM2 self-assembly through BAR domains. The regulation of focal adhesion (FA) dynamics is an excellent example that demonstrates how DNM2-mediated endocytosis affects cellular physiology. Focal adhesion kinase (FAK), a key regulator of focal adhesion disassembly, recruits DNM2 to FAs, where DNM2 is activated by Src phosphorylation [357-359]. The activated DNM2 then mediates clathrin-dependent endocytosis of β1-integrin, which is a major component of FA, leading to FA turnover [360, 361]. By regulating FA dynamics, DNM2 is directly involved in controlling cell adhesion and migration.

DNM2 is also heavily associated with endocytic pathways. Once primary endocytic vesicles bud off from the plasma membrane, they are first received by early endosomes (EEs), which function as sorting stations to decide the final destination of the cargos within the vesicles. There are two main routes for the cargo. The majority of them are recycled back to the plasma membrane via EEs. The rest are delivered to the lysosome for degradation through maturation of EEs to late endosomes (LEs) and, eventually, fusion of LEs with lysosomes (Figure 1.8B) [320]. DNM2 was found to be located in endosomal compartments and involved in both recycling and degradation pathways [362-365]. Furthermore, DNM2 plays a crucial role in lysosome homeostasis by directly
participating in scission and regeneration of nascent lysosomes from tubular extensions of autolysosomes [366].

1.3.3 DNM2 in other cellular activities

In addition to being involved in endocytosis processes, DNM2 also functions as an integral modulator of actin cytoskeleton dynamics and the microtubule network. DNM2 has been identified in multiple protein complexes that are involved in regulating different actin-rich structures, suggesting a link between these two proteins in cytoskeleton formation (Figure 1.8A) [327, 335]. It was also revealed that DNM2 and actin physically interact and promote polymerization of each other, which indicate a close coupling between actin dynamics and membrane remodeling [367, 368].

Pioneer studies demonstrated that DNM2 assembles around microtubules to promote microtubule bundle formation, while subsequent studies showed that DNM2 played a role in destabilizing microtubule polymers in order to sustain microtubule dynamics [326, 369]. Moreover, DNM2 is also found to participate in almost every step of mitosis in a microtubule-dependent manner [370]. Particularly, DNM2 is reported to be associated with mitotic spindles during mitosis, suggesting a key role of DNM2 in cell cycle progression [370].
Figure 1.8. Involvement of Dynamin in endocytosis and actin networks and a schematic presentation of the endocytic pathway. (A) During clathrin-dependent and –independent endocytosis, classical Dynamins assemble into ring structures at the necks of budding vesicles, and exert mechanical forces for membrane scission. Dynamins also participate in organizing actin meshworks such as membrane ruffles, podosomes /invadopodia and actin pedestals. The orange dots represent dynamin proteins. (B) Cargo within the endocytic vesicles are firstly delivered to the early endosomes (EEs). EEs can recycle the cargo back to the plasma membrane or mature into late endosomes (LEs) through transition to the multivesicular body (MVB). The fusion of LEs and lysosomes forms endolysosomes, where the internal contents are degraded. Lysosomes are recycled for future actions. Reproduced and modified from Ferguson et al, 2012 [336] (with permission through the Copyright Clearance Center).
1.3.4 DNM2 in cancers

Because of versatile roles in a variety of cellular functions mentioned above, the deregulation of DNM2 has been implicated in the oncogenesis of numerous malignancies. In recent years, a large area of research has been focused on elucidating the molecular roles of DNM2 in cancer development. In prostate cancer, upregulation of DNM2 expression has been found to cause a higher turnover rate of focal adhesions to promote cell invasion [371]. Elevated DNM2 activity was found to facilitate actin dynamics to promote migratory phenotypes (e.g. cells branching with numerous lamellipodial extensions) in pancreatic ductal carcinoma [372]. In breast cancer progression, DNM2 has been shown to regulate the trafficking of membrane-type 1-matrix metalloproteinase (MT1-MMP) from late endosomes to the plasma membrane in an atypical PKC-dependent manner for extracellular matrix degradation, thereby leading to the dissemination of carcinoma cells [373]. In the central nervous system, DNM2 participates as a component in PDGFRα-SHP2 signaling cascades to potentiate glioma tumor growth and invasion [374]. Surprisingly, in pancreatic tumor cells, DNM2 can even act as an adaptor protein to indirectly regulate the GTPase activity of the oncoprotein Rac1, a key member in actin dynamics and branching, by stabilizing Rac1 GEF Vav1 from lysosomal degradation [375]. In all the cases described above, genetic depletion and pharmacological inhibition of DNM2 or overexpression of the dominant-negative DNM2 mutant causes proliferative defects in cancer cells and impairs
tumor migration, suggesting that DNM2 can be a valid therapeutic target for cancer treatments.

Unexpectedly, DNM2 has also been identified as a tumor suppressor. Lower expression of DNM2 is linked to the later stage of invasive cervix carcinoma [376]. DNM2 and miR-119a, which is encoded from the opposite strand of DNM2, were found to cause the degradation of the oncoproteins HIF-1 and HIF2 and suppress their expression, respectively. The overexpression of miR-119a also attenuates tumor growth and metastasis of ovarian cancer cells under hypoxic conditions [377]. Both examples suggest that DNM2 functions as a negative regulator in disease progression. In addition, DNM2 mutations and downregulation have been detected in T-cell acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL), respectively [378, 379]. However, the underlying mechanisms of how DNM2 is involved in leukemogenesis are yet to be determined. In normal T cell development, DNM2 facilitates actin organization at immunological synapses and modulates multiple signaling pathways controlling TCR-mediated T cell activation [380]. Therefore, it has been proposed that loss-of-function of DNM2 due to mutations or reduced DNM2 expression leads to impaired immune synapses so that leukemic cells escape antitumor immune responses, which leads to the accumulation of leukemic cells and neoplastic progression.

Taken together, whether DNM2 acts as an oncogene or tumor suppressor seems to depend on the specific cancer type, and this further demonstrates the multifaceted
functions of DNM2 in cellular physiology and its different roles in various types of cells.

1.4 Thesis objectives

The hypothesis of this study is that targeting critical domains (SH3 and WD40-repeat) of AHI-1 and its interacting sites to destabilize the AHI-1-mediated molecular complexes can be a more effective therapeutic approach for CML treatment. The overall objective of my thesis is to characterize the AHI-1 SH3 and WD40-repeat domains by solving their crystal structures and identifying their binding partners, and subsequently investigate the functional roles of the interactions between AHI-1 and the newly-identified interacting proteins in CML cells. Unfortunately, the AHI-1 WD40-repeat domain was not successfully purified and crystallized (Appendix A1 – A3); therefore, this thesis is primarily focused on the AHI-1 SH3 domain and the interactions mediated by this domain. Three projects were carried out:

1. Molecular and Structural Characterization of the SH3 Domain of AHI-1 in Regulation of Cellular Resistance of BCR-ABL\(^+\) Cells to Tyrosine Kinase Inhibitors.

2. Investigation of a Newly-Identified Interaction between AHI-1 and DNM2 and Characterization of the BCR-ABL-AHI-1-DNM2 Protein Complex in CML Cells.

3. Investigation of the Functional Roles of DNM2 in BCR-ABL\(^+\) Cells and
TKI-insensitive Stem/progenitor Cells obtained from CML Patients.

Previous studies have demonstrated that AHI-1 functions as an adaptor protein, coordinating the formation of protein complexes through its N-terminal region, SH3 domain, and WD40-repeat domain [271]. In CML, AHI-1 bridges BCR-ABL and JAK2 via its WD40-repeat domain and N-terminal region, and the protein complex has been shown to contribute to BCR-ABL transforming activity and disease development [283, 284]. However, the SH3 domain of AHI-1 has not been well characterized. The work presented in Chapter 3 details the biological and structural investigation of the AHI-1 SH3 domain. By comparing the biological effects of wildtype and SH3-deleted Ahi-1 in BCR-ABL+ cells, I demonstrate that the AHI-1 SH3 domain contributes to TKI resistance in CML cells. This finding indicates a regulatory role for AHI-1 in the resistance of CML cells to TKIs, with the SH3 domain being directly involved in mediation of TKI-induced response/resistance, which provides a rationale to target the AHI-1 SH3 domain. I then solve the crystal structure of the AHI-1 SH3 domain and studied its structural details. Several unique structural features identified in this study may be utilized to design or screen for high-affinity inhibitors that specifically target the AHI-1 SH3 domain and its interacting proteins.

Chapter 4 starts with the identification and verification of DNM2 as a new interacting protein of the AHI-1 SH3 domain in CML cells. DNM2 is a large GTPase, mainly involved in endocytosis/intracellular trafficking and actin/microtubule
organization. I further dissect the interaction between AHI-1 and DNM2, and demonstrate that this interaction is mainly attributed to the binding between the SH3 domain of AHI-1 and the proline rich domain of DNM2, and takes place in endosomal compartments. Subsequently, I identify a novel protein complex containing BCR-ABL, AHI-1 and DNM2 in CML cells, and showed that DNM2 was a BCR-ABL downstream target within this protein complex.

In Chapter 5, I establish an oncogenic role of DNM2 in CML cell line model systems and TKI-insensitive stem/progenitor cells from CML patients in vitro. I report that suppression of DNM2 led to reduced survival, increased apoptosis, and the impaired ability to produce colonies and higher sensitivity to TKI treatments in BCR-ABL+ and CD34+ CML cells. Lastly, I demonstrate that DNM2 is implicated in the deregulation of endocytosis, excessive ROS production and abnormal activation of autophagy in BCR-ABL+ and CD34+ CML stem/progenitor cells. Based on these findings, I propose that DNM2 could be considered as a new therapeutic target to eliminate TKI-insensitive CML stem and progenitor cells.
Chapter 2: Materials and Methods

2.1 Cell lines and cell culture

The human BCR-ABL+ cell lines K562, IM-resistant K562 (K562 IMR), BV173, BCR-ABL-transduced BaF3 (BaF3 B/A), BCR-ABL/Ahi-1 co-transduced BaF3 (BaF3 B/A Ahi-1), BCR-ABL/SH3 domain-deleted Ahi-1 co-transduced BaF3 (BaF3 B/A Ahi-1 SH3Δ), BCR-ABL-transduced UT7 (UT7 B/A) and BCR-ABL T315I mutant-transduced UT7 (UT7 T315I) were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA), 0.1 mg/mL streptomycin (Thermo Scientific, Waltham, MA), 100 U/L penicillin (Thermo Scientific) and $10^{-4}$ M β-mercaptoethanol (STEMCELL Technologies, Vancouver, BC). This RPMI 1640 medium with all these supplements will be referred to as complete RPMI medium thereafter. Parental BaF3 cells were maintained in complete RPMI medium with additional 5 ng/mL murine IL-3 (STEMCELL Technologies). K562 SHC, K562 shDNM2b, K562 shDNM2d, BV173 SHC and BV173 shDNM2 were maintained in complete RPMI medium containing puromycin at concentration of 2 μg/mL, 0.25 μg/mL, 2 μg/mL, 1 μg/mL and 0.5 μg/mL, respectively. Human 293T cells were cultured in Dulbecco’s modified Eagle’s media (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum, 0.1 mg/mL streptomycin, 100 U/L penicillin and L-glutamine (STEMCELL Technologies). All the cell lines were maintained in 10 cm
Falcon® tissue culture dishes (Corning Inc., Lowell, MA).

2.2 Human cells

Primary CML samples were obtained from newly-diagnosed CP-CML patients before any TKI intervention, and BM cells were obtained from health donors (ALLCELLS, Alameda, CA). The subsequent IM-responders were classified based on the European Leukemia Net treatment guidelines, whereas the IM-nonresponders were defined as the patients who did not meet the criteria from the guidelines [106, 381]. EasySep CD34 positive selection kits (STEMCELL Technologies) were utilized to enrich for CD34+ cells, and the purity was verified by flow cytometry using a FITC (fluorescein-isothiocyanate- labelled anti-human CD34 antibody (BD Biosciences, San Jose, CA). CD34+ cells were maintained in Iscove’s medium (STEMCELL Technologies) supplemented with bovine serum albumin (BSA), insulin, transferrin (all three additives were obtained from STEMCELL Technologies) and 10^{-4} M 2-mercaptopethanol (Sigma-Aldrich, St. Louis, MO), and recombinant human growth factor cocktail (20 ng/mL IL-3, 20 ng/mL IL-6, 100 ng/mL Flt3-ligand, and 20 ng/mL G-CSF, STEMCELL Technologies). Consent forms to use the human samples were granted according to the Declaration of Helsinki, and all the procedures were approved by the Research Ethics Board at the University of British Columbia.
2.3 Inhibitors

Imatinib and Nilotinib were provided by Novartis (Basel, Switzerland), and Dasatinib and MitMAB were obtained from Bristol-Myers Squibb (New York, NY) and Abcam (Cambridge, UK), respectively.

2.4 Cloning

2.4.1 Cloning of the AHI-1 SH3 domain into the pET28HT vector

A vector containing full-length human AHI-1 was used as the template to amplify the sequence encoding the SH3 domain by PCR using the forward primer 5'-tacttcaatccaatgcacatcaggtagatacagcacc-3' and the reverse primer of 5'-ttatccacttcatagtactgtcagttc-3'. The pET28HT vector was digested by SspI (NEB, Ipswich, MA) for 3 hours at 37°C. Both the PCR product and the SspI-cut vector were subjected to T4 polymerase (Fermentas, Waltham, MA) treatments with dCTP (NEB) and dGTP (NEB), respectively for 40 minutes at 23°C, to create the long overhangs, followed by 20-minute heat inactivation of the polymerase at 75°C. The T4-treated insert and vector were mixed together, and the overhangs were annealed for 10 minutes at 23°C, followed by transformation into E. coli DH5α under kanamycin selection.

2.4.2 Cloning of full-length DNM2 and DNM2 PRΔ into the KA391 vector

A vector that contains full-length human DNM2 was obtained from Thermo Fisher Scientific (Hampton, NH) and primers with ASCI and PACI hangers were used for
amplification of the full-length DNM2 (Forward primer: 5’-atcgggcgccatggcaacccgggg-3’; Reverse primer: 5’-atcgttaattagcagcaggtggattgge-3’) and DNM2 PRDΔ (Forward primer: 5’-atcgggcgccatggcaaccgggg-3’; Reverse primer: 5’-atcgttaattagcagcaggtggacacagtgggag-3’). PCR products and the KA391 vector (kindly provided by Gregg Morin Lab, Genome Science Centre, Vancouver) were sequentially digested by ASI and PAC1 restriction enzymes, followed by ligation using T4 ligase (NEB). Finally, the ligation products were transformed into the one shot® MAX efficiency® DH5α™-T1R competent cells (Invitrogen, Carlsbad, CA).

2.4.3 Cloning of DNM2 shRNA into the pRRL-PPT-SF-GFP-pre vector

The pGFP-C-lenti vector (OriGene, Rockville, MD) that contains the non-targeting sequence or DNM2 shRNA constructs and the pRRL-PPT-SF-GFP-pre vector (kindly provided by Keith Humphries lab, Terry Fox Laboratory, Vancouver, BC) were used as templates to amplify the U6 promoter-shRNAs (Forward primer: 5’- tagGctagccccaggtgaagacgccgagccga-3’; Reverse primer: 5’-catgccttgcaaaatggcgtactgcaaccctactgacacatatccacagg-3’) and the SFFV promoter (Forward primer: 5’-tgcaagtacgcccattttcgcaaggcctagc-3’; Reverse primer: 5’-ccacggtctgacggtcagctgtaagttgattc-3’). The two PCR products were then mixed and used as the template for the third overlapping PCR. The TOPO vector (Invitrogen) that the final PCR product was cloned into and the pRRL-PPT-SF-GFP-pre vector were digested with NdeI (NEB) and AgeI (NEB) restriction enzymes, followed by the ligation reaction using T4 ligase. Finally, ligation products were transformed into the
one shot® MAX efficiency® DH5α™-TIR competent cells.

2.5 Viability assay

The total counts of viable cells were assessed on a Neubauer hemacytometer (Hausser Scientific, Horsham, PA) using the trypan blue (Life Technologies) exclusion method. For cell lines, aliquots of 1x10^5 cells were seeded in 12-well Falcon plates (Corning Inc.) in 1 mL complete RPMI medium for each test condition and incubated in a 37°C, 5% CO₂, humidified cell culture incubator. For primary CML cells, 1x10^4 cells were seeded in 96-well Falcon plates (Corning Inc.) in 100 μL ISCOVE medium with all the supplements as described in Section 2.2 in the presence or absence of inhibitors.

2.6 Apoptosis assay

Apoptosis analysis was performed using an Apoptosis Detection kit (eBioscience, San Diego, CA). After being treated with inhibitors for 24 or 48 hours, the cells were pelleted and washed twice with Hanks buffer (STEMCELL Technologies) with 2% FBS. Subsequently, the cells were resuspended in 100 μl binding buffer, and incubated with 2.5 μl of PI and 2.5 μl of APC-conjugated Annexin V at 23°C in the dark for 15 minutes. 400 μl binding buffer was added and cells were analyzed using a FACS Calibur (BD Bioscience). Total apoptotic cell numbers were calculated as the sum of “early” apoptotic cells (Annexin V⁺ only) and “late” apoptotic cells (Annexin V⁺/PI⁺).
2.7 Colony-forming cell (CFC) assay

The colony-forming cell assay is an *in vitro* assay to test the potential of hematopoietic or leukemic progenitor cells to proliferate or to give rise to colonies of mature cells in semisolid media [382]. Briefly, 1000 primary CD34+ CML cells were mixed with 3mL MethoCult™ H4230 containing 20 ng/mL IL-3, 20ng/mL IL-6, 20ng/mL G-CSF, and 20ng/mL GM-CSF (all obtained from STEMCELL Technologies) in the presence or absence of the desired inhibitors. The mixture was evenly added into CFC plates (STEMCELL Technologies) in duplicate. The colonies produced were counted after 12 to 14-day incubation.

2.8 Long-term culture-initiating cell assay (LTC-IC)

The LTC-IC assay is an *in vitro* assay to assess the ability of primitive hematopoietic or leukemic cells to produce colonies after being cultured for 5-6 weeks [383]. Briefly, primary CD34+ CML cells were plated onto humanized feeders (kindly provided by the Eaves lab, Terry Fox Laboratory) engineered to produce human IL-3, G-CSF and stem cell factor in myelocult H5100 medium (STEMCELL Technologies) containing 10⁻⁶ M solucortef (STEMCELL Technologies) for 6 weeks. Half-medium change was performed every week. Inhibitors were added on the first day when the assays were set up. After 6 weeks, all the cells were collected, and 1x10⁴ viable cells were plated for CFC assays.
2.9 RNA extraction and quantitative real-time PCR (Q-RT-PCR)

Total RNA was extracted using TRIzol reagent (Life Technologies) according to manufacturer’s instructions [384]. During the RNA precipitation step, glycogen (Life Technologies) was used to help visualize the RNA pellet. RNA pellet was then dissolved in RNase-free water (Life Technologies), and the concentration was measured using a nanodrop ND-100 spectrophotometer (Life Technologies). Subsequently, cDNA was synthesized from 500 ng RNA with SuperScript® VILO™ Master Mix (Life Technologies). The PCR mixture was made up of 1 µL of 20 µM primer pairs, 6 µL SYBR® Green PCR Master Mix (Life Technologies), 1 µL of cDNA and 4 µL of RNase-free water, and quantitative real-time PCR was performed on the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Data analysis was performed with the ∆∆Ct method with GAPDH or β2-microglobulin (β2M) as control. The primers for human DNM2 used in this study are 5’-ggtgctggagaacttcgtg-3’ (forward primer) and 5’-tcggcatgttctgtttttga-3’ (reverse primer).

2.10 Protein extraction and quantification

Cells were pelleted, washed with Dulbecco’s Phosphate Buffer Saline (PBS) (Life Technologies) and lysed in the lysis buffer that contained 900 µl phosphorylation solubilization buffer (PSB), 100 µL 10% NP-40 Alternative Protein Grade Detergent (Calbiochem, San Diego, CA) solution, 5 µL phenylmethylsulfonyl fluoride
(Sigma-Aldrich), and 5 μL protease inhibitor cocktail (Sigma-Aldrich) and 10 μl 10% sodium dodecyl sulfate (SDS) solution for 20 minutes. Then, the lysate was centrifuged at 13,000 rpm for 20 minutes at 4°C, and the supernatant was collected. The protein concentration was measured using the Bradford assay. 20 μL of 1:10 diluted protein lysate and 200 μL of 1:5 diluted Bio-Rad Protein Assay Dye (Bio-Rad, Hercules, CA) were mixed in triplicates to a 96-well Falcon plate, and the absorbance of proteins samples were measured using the Elx808TM Absorbance Microplate Reader (BioTek Instruments, Winooski, VT).

2.11 Western blotting

A 20-50 μL of protein lysate (cell lines) or the whole protein lysate from 1.5x10^5 CD34+ CML cells were mixed with 4x SDS loading dye, followed by heating at 95°C for 10 minutes. Samples then were loaded and separated in 8%-15% SDS-PAGE, followed by transfer to a PVDF membrane (Millipore, Billerica, MA). The membrane was subsequently blocked in Tris-buffered saline Tween 20 (TBST) with 5% BSA (Sigma-Aldrich) for 1 hour at 23°C, followed by incubation overnight with a primary antibody at 4°C. One the second day, the membrane was washed with TBST for 3 x 5 minutes, and then incubated with a corresponding secondary antibody for 1 hour at 23°C. After 3 x 10-minute wash of TBST, the membrane was incubated with enhanced chemiluminescence (ECL) reagents for 2 mins and then exposed on a CLINICSELECT
Blue X-ray film (Carestream, Rochester, NY).

The primary antibodies used for western blotting were anti-HA (abm, Richmond, BC), anti-Myc (CST, Danvers, MA), anti-His (abm), anti-DNM2 (Abcam, Cambridge, UK) anti-N-ter-AHI-1 (abm), murine anti-C-ter-Ahi-1 (abm), anti-ABL (8E9: BD Biosciences), anti-phospho-tyrosine (4G10: Millipore), anti-JAK2 (CST), anti-phospho-JAK2 (CST), anti-STAT5 (CST), anti-phospho-STAT5 (CST), anti-AKT (CST), anti-phospho-AKT (CST), anti-ERK (Santa Cruz), anti-phospho-ERK (Santa Cruz), anti-β-actin (abm), anti-GAPDH, anti-ULK-1 (CST), anti-Beclin-1 (CST), anti-LC3B (CST) and anti-p62 (Sigma-Aldrich).

2.12 Co-immunoprecipitation (co-IP) assay

Cell lysates (0.5-1 mg of protein) were incubated overnight with a specific primary antibody or IgG (Santa Cruz Biotechnology, Dallas, TX) at 4°C, and the immune complexes were incubated with the protein A or protein G bead flurry (Santa Cruz Biotechnology) for another two hours at 4°C on the second day. Beads were washed in a sequential order with PSB/NP40/SDS, PSB/NP40 and PSB buffers and then resuspended in 4x SDS-loading buffer, followed by heating at 95°C for 10 minutes. After removal of beads by centrifugation, supernatants were used for western blotting.
2.13 293T cell transfection

For the use in co-IP assays, 16-24 hours before transfection, 7-8x10^6 293T cells were plated in a 10-cm Falcon ® tissue culture dish in DMEM media supplemented with 10% FBS and L-glutamine. 20 µg of DNA plasmids and 60 µg of polyethylenimine (PEI) (Polysciences Inc, Warrington, PA) were mixed in 1 mL DMEM without phenol red (Life Technologies) and incubated at 23°C for 20 minutes. Then the mixture was added to the cells, followed by incubation at 37°C for 48 hours. Subsequently, cells were washed by cold PBS, scraped off the culture dish and pelleted for cell lysis. For the use in immunostaining, 0.9x10^6 293T cells were added into a 6-well plate (Corning Inc.) containing small coverslips (Thermo Fisher Scientific) coated with poly-L-lysine (R&D SYSTEMS, Minneapolis, MN). A mixture of 3 µg of plasmids and 9 µg of PEI was used for the 24-hour transfection. All the other steps were the same.

2.14 Proximity ligation assay

Plating and transfection of 293T cells in 6-well plates were performed as described in Section 2.13. Cells were fixed with 3.7% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for 15 minutes at 23°C, followed by permeabilization with PBS containing 0.1% Triton-100 (Sigma Aldrich) for 10 minutes at 23°C. The cells were then blocked with Blocking Solution (Sigma-Aldrich) in a 37°C incubator for 30 minutes, and incubated overnight with primary antibodies diluted with Antibody Diluent
(Sigma-Aldrich) at 4°C. One the second day, cells were washed twice with Wash Buffer A (Sigma-Aldrich) for 5 minutes and incubated with PLA probe PLUS and MINUS (Sigma-Aldrich) that was diluted 5x with Antibody Diluent at 37°C for 1 hour. Cells then washed with Wash Buffer A for 2x5 minutes under gentle agitation, followed by incubation with the Ligation-Ligase solution at 37°C for 30 minutes. Cells then washed with Wash Buffer A for 2x2 minutes under gentle agitation, followed by incubation with the Amplification-Polymerase solution at 37°C for 100 minutes. Subsequently, cells were washed with Wash Buffer B for 2x10 minutes under gentle agitation, followed by 1-minute wash with 0.01x Wash Buffer B. Finally, the slides were mounted in Prolong Gold antifade reagent with DAPI, and the fluorescent signals were detected using a confocal Nikon X1 microscope. Primary antibodies used in this study were anti-HA and anti-Myc antibodies with 1:750 dilution.

2.15 Immunostaining and confocal analysis

For immunostaining of Ahi-1 and DNM2 in BCR-ABL/Ahi-1 co-transduced BaF3 cells, cells were harvested and washed three times with cold PBS. Cells were plated at a density of 2x10^5 cells/mL on poly-L-lysine coated slides (Electron Microscopy Sciences) for 20 minutes. The cells were then fixed and permeabilized in ice-cold methanol for 20 minutes in the freezer. The cells were then blocked with PBS containing 3% BSA at 23°C for 1 hour, followed by incubation overnight with primary antibodies at 4°C. On the
second day, the cells were incubated with secondary antibodies at 23°C in the dark for one hour. Finally, the slides were mounted in Prolong Gold antifadre reagent with DAPI. The cells were washed three times with PBS between each step. The conditions of primary/secondary antibodies for Ahi-1 and DNM2 (abcam) were 1:250/1:500 and 1:400/1:750, respectively, diluted in PBS containing 0.2% fish gelaton (Sigma Aldrich) and 0.1% goat serum (Sigma Aldrich).

For immunostaining in 293T cells, the coverslips with adherent cells were transferred into the wells of a 6-well plate where all the procedures were performed. Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at 23°C, followed by permeablization with PBS containing 0.1% Triton-100 (Sigma Aldrich) for 5 minutes at 23°C. The blocking and primary incubation steps for 293T cells were the same as the suspension cells. The conditions of primary/secondary antibodies for immunostaining of Myc tag, HA tag, ABL(8E9), EEA1 (CST) and LAMP-1 (CST) were 1:400/1:750, 1:400/1:750, 1:250/1:500, 1:200/1:500, 1:400/1:750, respectively, diluted in PBS containing 0.2% fish gelaton and 0.1% goat serum. Imaging was acquired with a Nikon X1 confocal microscope.

2.16 Staining of surface CXCR4

5x10^5 BV173 cells or 1x10^5 CD34^+ CML cells were harvested and washed by cold PBS. The cells then incubated with APC-conjugated anti-CXCR4 antibody (BioLegend,
San Diego, CA) (1:1000 diluted in PBS) at 4°C for 1 hour. After PBS wash and addition of PI, the cells were subjected to FACS analysis.

2.17 Cell migration assay

The migration assays were performed in 5 μm pore size Transwells (Corning Inc.). 9x10^5 BV173 cells in 200 μL RPMI containing 1% FBS were added to a transwell insert. The bottom chamber was filled with 600 μl RPMI containing 1% FBS and 500 nM CXCL12 (Thermo Fisher Scientific). After 4-hour incubation at 37°C, the number of cells that migrated to the bottom chamber was determined using the trypan blue exclusion method.

2.18 Transferrin uptake assay

For cell lines, cells were transferred to a 15 mL Falcon tube and kept on ice for 10 minutes to stop endocytosis. The cells then washed twice with a HEPES-based buffer at pH7.4 containing 20 mM glucose (Bdh Inc., Conestoga, PA) and 1% BSA (referred as wash buffer thereafter). Subsequently, the cells were placed in a 24-well plates in 500 μL wash buffer containing fluorescent diferric (Fe^{3+}) human Transferrin conjugates (Life Technologies) and incubated for 30 minutes at 37°C. The final concentrations of Transferrin conjugates for BaF3, K562 and BV173 cells were 12, 15 and 25 μg/mL, respectively. Subsequently, the cells were kept on ice for 30 minutes to terminate the
reaction. After two washes of PBS, cells were plated on poly-L-lysine coated slides for 20 minutes, followed by fixation with 4% paraformaldehyde in PBS for 10 minutes at 23°C. The slides were then washed twice with PBS and mounted in Prolong Gold antifade reagent with DAPI. For CD34+ CML cells, cells were placed in a FACS tube in 100 μL medium with Transferrin conjugates at 25 μg/mL, and all the subsequent procedures were performed in the FACS tube. Slides were viewed using a Nikon C1 confocal microscope. Results obtained from at least two independent experiments were used in signaling quantification with ImageJ.

2.19 ROS staining

Cells were harvested and washed twice with PBS. For cell lines, cells were plated in a 24-well plate in 500 μL complete RPMI medium with the CellROX Reagent (Life Technologies) that recognizes various types of ROS and incubated for 30 minutes at 37°C. The final concentrations of the CellROX reagent for BaF3, K562 and BV173 cells were 4, 3 and 4 μM, respectively. After incubation and two washes of PBS, cells were plated on poly-L-lysine coated slides for 20 minutes, followed by fixation with 4% paraformaldehyde in PBS for 10 minutes at 23°C. The slides were then washed twice with PBS and mounted in Prolong Gold antifade reagent with DAPI. For CD34+ CML cells, cells were placed in a FACS tube in 100 μL medium with the CellROX reagent at 5 μM, and all the subsequent procedures were performed in the FACS tube. Slides were
viewed using a Nikon C1 confocal microscope. Results obtained from at least two independent experiments were used in signaling quantification with ImageJ.

### 2.20 Lentivirus production and infection

18-20 hours before transfection, $6 \times 10^6$ 293T cells were plated in a 10-cm Falcon® tissue culture dish in 10 mL DMEM supplemented with 10% FBS and L-glutamine and incubated in the 37°C, 5% CO₂ incubator. In total, 7 dishes were needed for one construct. 4 hours prior to transfection, the medium was replaced and reduced to 4.5 mL. The following procedures took place in the Level 3 laboratory. A 250 μL DNA mixture containing 6 μg of shRNA plasmids, packaging vectors (1.5 μg of REV and 3.9 μg of ΔR), 2.1 μg of vesicular stomatitis virus glycoprotein (VSV-G) envelope and Opti-MEM® medium (Thermo Fisher Scientific) and a 250 μL PEI solution containing 40 μL of PEI and 210 μL Opti-MEM® medium (Life Technologies) were prepared in two separate tubes, and the mixed and incubated for 20 mins at 23°C. Subsequently, the 500 μL mixture of transfection reagents was added to 293T cell in one culture dish in a dropwise fashion. After 48-hour incubation, the viral supernatant was collected and passed through a 0.45 μm filter (Pall Corporation, New York, NY) and then subjected to 1.5-hour ultracentrifugation. The resultant virus pellet was resuspended in serum free medium supplemented with 5% DNase, and then aliquoted and stored at -80°C for future use.

For stable knockdown of DNM2 in K562 and BV173 cells, 10 μL of concentrated
viruses generated from the pGFP-C-shLenti viral vectors containing a non-targeting sequence or shRNA constructs targeting human DNM2 were added to 4x10^5 cells in a 24-well plate in 400 μL RPMI 1640 in the presence of protamine sulfate for 18 hours. After 18-hour incubation, infected cells were washed with PBS and cultured in complete RPMI medium. Finally, the sorted GFP^+ K562 or BV173 cells were maintained in complete RPMI medium containing puromycin. For primary CD34^+ CML cells, the cells were thawed and pre-stimulated with serum free medium with the growth factor cocktail for 16 hours, followed by 6-hour infection with 5 μL of concentrated viruses in the presence of protamine sulfate. Cells were then washed and cultured for three days. The sorted GFP^+ cells were used for the future studies.

2.2.1 The purification of the AHI-1 SH3 domain

Proteins were expressed in *E. coli* Rosetta (DE3) pLac I (Novagen, Merck KGaA, Darmstadt, Germany) incubated at 37°C for three hours, induced with 0.1 M IPTG at O.D. of around 0.6, and incubated for another 20 hours at 16°C. Cells were lysed using sonication in 20 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% glycerol, 25 μg/ml DNaseI, 25 μg/ml lysozyme and 100 mM PMSF. After centrifugation to remove cell debris, the supernatant was loaded onto a Talon affinity column (Clontech, Mountain View, CA), and eluted in buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl and 500 mM imidazole 8.0). The protein elute was dialyzed against 20 mM Tris-HCl pH 8.0, 10 mM NaCl and
simultaneously cleaved with recombinant TEV protease overnight at 4°C. The flow-through of a second Talon column was collected and dialyzed against 20 mM Tris-HCl pH 8.0, 10 mM NaCl for 1 hour at 23°C. The protein was loaded onto a MonoQ column (GE Healthcare, Mississauga, ON), and eluted in a linear gradient (10%-40%) of 0.01-1 M NaCl in 20 mM Tris-HCl pH 8.0 over 40 column volumes. The elute was collected, concentrated to around 2 ml, and applied onto Superdex 200 gel filtration column (GE Healthcare) in buffer of 20 mM Tris-HCl pH 8.0, 50 mM NaCl. The peak fractions were collected, concentrated to around 9 mg/ml and stored at -80°C.

2.22 Crystallization and data collection

Crystals were obtained using hanging drop vapour diffusion. The best crystal was obtained in 0.2 M ammonium sulphate, 10 mM sodium bromide, 0.1 M sodium acetate (pH 3.0) and 7% PEG200 MME. Crystals were cryo-protected under the same conditions, supplemented with 20% glycerol, and flash-cooled in liquid nitrogen. Data were collected using the Canadian Light Source (CLS) 08ID-1 beamline at a wavelength of 0.9806 Å and a temperature of 100 K. The best data used to solve and refine the structure provided a resolution of 1.53 Å and were processed using XDS [385].

2.23 Structure solution, refinement and analysis

To solve the phase problem, a molecular replacement model (1ABQ) was used [386].
Molecular replacement was performed with Phaser [387] using PDB1ABQ [14] as a search model. The model was refined via autobuilding procedures implemented in Arp/Warp [388], followed by successive rounds of manual model building in Coot [389] and refinement with Refmac5 [390]. The PROCHECK [391] program was used to validate the geometry of the model.

### 2.24 Affinity purification and mass spectrometry analysis

His-tagged SH3 domain was expressed and purified using the strategy described in Section 2.1, except that the His tags were not cleaved. Protein lysate was extracted from K562 cells (2x10^7), which were lysed in PSB buffer (150 mM NaCl, 1.5 mM MgCl, 10 mM Tris-HCl 8.0, 0.5% NP-40 and a complete protease inhibitor cocktail). His-tagged SH3 domain (10 µg) was incubated with anti-His antibodies (10 µg, Applied Biological Materials Inc, Vancouver, BC) and the protein lysate at 4°C overnight. 40 µL of protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the mixture and incubated for two hours at 4°C. PBS was used to wash the beads five times, followed by 250 mM and 500 mM NaCl washes. The samples were then heated at 70°C boiled for 10 minutes, and the supernatant subjected to SDS-PAGE using a 4-12% gradient NuPAGE gel (Invitrogen, Burlington, Ontario). The SDS-PAGE gel was stained and visualized with Coomassie blue stain. For control experiments, the K562 lysate was incubated as above but the AHI-1 SH3 protein was omitted. The prominent bands and their cognate
slices from the control lane were excised and processed for tandem mass spectrometry analysis using in-gel dehydration, alkylation, trypsin digestion, and extraction. The samples were analyzed on a 4000 QTrap mass spectrometer (Applied Biosystems/Sciex, Foster City, CA) using standard protocols, as described [392]. The MS/MS spectra were searched against the Uniprot human database using the Mascot (Matrix Science, Boston, MA) search engine. The proteins identified were not observed in the control samples.

2.25 Statistical analysis

Results are shown as the mean ± SEM of values obtained in independent experiments. Differences between two groups were assessed using the two-tail Student’s t-test for paired samples. Differences between three or more than three groups of samples were assessed using Analysis of Variance (ANOVA) test. A p value <0.05 was considered significant.
Chapter 3: Molecular and Structural Characterization of the SH3 Domain of AHI-1 in Regulation of Cellular Resistance of BCR-ABL+ Cells to Tyrosine Kinase Inhibitors

3.1 Introduction

Chronic myeloid leukemia (CML) is a multistep, multilineage hematopoietic disease that originates in hematopoietic stem cells (HSC) and is characterized by the fusion oncogene BCR-ABL [393, 394], encoding a BCR-ABL oncoprotein (p210BCR-ABL) with constitutively elevated tyrosine kinase activity that develops and maintains leukemogenesis and drives disease progression. BCR-ABL can phosphorylate key components in intracellular signalling pathways, including the RAS, PI3K, JAK-STAT and NF-κB pathways, to promote proliferation and inhibit apoptosis [395]. The tyrosine kinase inhibitors (TKIs) Imatinib (IM), Dasatinib (DA) and Nilotinib (NL) have been introduced as effective therapeutics for CML [396, 397]. However, early relapse and TKI-resistant disease have emerged as significant clinical setbacks [92, 398]. Resistance is largely due to BCR-ABL tyrosine kinase domain mutations that prevent TKI binding and properties of CML stem/progenitor cells that make them less responsive to TKIs [92, 394, 399]. Therefore, it is imperative to improve treatment by targeting other key molecular elements which are also active in CML stem/progenitor cells.

One candidate is Ahi-1/AHI-1 (Abelson helper integration site 1), which was identified as a cooperating oncogene in a v-abl-induced murine lymphoma model [400].
Human AHI-1 consists of an N-terminal coiled-coil domain, a WD40-repeat domain and a SH3 domain, which are all known to mediate protein-protein interaction, indicating that AHI-1 serves as an adaptor protein, regulating signalling pathways [400]. Elevated expression of AHI-1 occurs in several human leukemic cell lines, particularly in a CML cell line (K562) and in BCR-ABL+ primary CML stem cells, where BCR-ABL transcripts are also highly elevated [282, 401]. Recently, we have demonstrated that AHI-1 can physically interact with BCR-ABL and Janus kinase 2 (JAK2); this complex contributes to the transforming activities of BCR-ABL in vitro and in vivo and plays a key role in the IM response/resistance of primary CML stem/progenitor cells [402]. These results suggest that AHI-1 could be targeted with molecular therapies to destabilize this protein-protein interaction complex in CML.

Here in this thesis, I investigated the biological functions and structural features of the human AHI-1 SH3 domain. Upon TKI treatment, BCR-ABL+ cells transduced with an AHI-1 SH3 deletion mutant underwent apoptosis at a much higher frequency than BCR-ABL+ cells expressing full-length AHI-1, suggesting that the AHI-1 SH3 domain might play a role in mediating TKI resistance of CML cells. Following these findings, the AHI-1 SH3 domain was successfully purified and crystallized, and its structure was determined using X-ray crystallography. Several structural features of the AHI-1 SH3 domain were uncovered, and a binding pattern between the AHI-1 SH3 domain and its ligands was proposed. Hence, these studies provided valuable insight into the
identification of possible key interaction sites in regulation of drug resistance and may ultimately lead to the identification and development of novel small molecule inhibitors for CML.

3.2 Results

3.2.1 The SH3 domain of AHI-1 is required for mediation of TKI sensitivity in BCR-ABL+ CML cells

To investigate the role of the AHI-1 SH3 domain in regulation of cell proliferation and TKI response/resistance, several mutant forms of murine Ahi-1, including a SH3 domain deletion (SH3Δ) and a N-terminal deletion (N-terΔ, containing SH3 and WD40-repeat domains) were generated and stably transduced into parental BCR-ABL inducible BaF3 cells, in which the level of expression of BCR-ABL can be down-regulated by exposure to doxycycline (Figure 3.1A, B & E). Overexpression of full-length Ahi-1 resulted in a significantly lower frequency of Annexin V+ apoptotic cells with doxycyclin (suppression of BCR-ABL protein expression) compared to BCR-ABL inducible cells after 48 hours (Figure 3.1C & D). Interestingly, cells expressing the SH3Δ mutant displayed significantly increased Annexin V+ cells in comparison to Ahi-1-overexpressed cells; this was also seen, to a much lesser extent, in cells expressing the N-terΔ mutant (Figure 3.1C & D).

Furthermore, cells with overexpression of full-length Ahi-1 displayed significantly
decreased apoptotic cells compared to parental BCR-ABL inducible cells in the presence of 2 µM IM, 2 µM NL or 50 nM DA after 48 hours of treatment, while in cells containing the SH3Δ mutant, a significantly increased number of apoptotic cells was observed upon the same TKI treatment, although TKIs caused a similar reduction of BCR-ABL phosphorylation in these cells (Figure 3.2 A, B & C). BCR-ABL+ cells transduced with the N-terΔ mutant were found to be significantly more sensitive to the drug treatments compared to cells transduced with full-length Ahi-1, but with lower sensitivity than cells carrying the Ahi-1 SH3 domain deletion mutant (Figure 3.2 A & B). These results indicated that the SH3 domain of Ahi-1 plays an important role in mediating cellular apoptosis and TKI response/resistance in BCR-ABL+ cells.
Figure 3.1. Detection of increased apoptotic cells by suppression of BCR-ABL expression in BCR-ABL inducible cells co-transduced with an Ahi-1 SH3 domain deletion mutant. (A) Schematic diagram of functional domains of full-length Ahi-1 and its mutants, including one mutant lacking the SH3 domain (SH3Δ) and one mutant without the N-terminus of Ahi-1(N-terΔ). These mutants were cloned into a MIY vector (MSCV-IRES-YFP) and transduced into BaF3 cells containing BCR-ABL by retroviral transduction. (B) The levels of protein expression of different mutants were detected by specific anti-Ahi-1 antibodies either detecting the N-terminus or C-terminus of Ahi-1 as indicated. (C) BCR-ABL inducible cells co-expressing full-length Ahi-1 and its mutants were cultured with or without doxycyclin (suppression of BCR-ABL expression) for 24 and 48 hours and the treated cells were stained with Annexin V/PI to detect apoptotic cells. Representative FACS plots for detection of Annexin V/PI after 48 hours are shown. (D) Percentage of total apoptotic cells after 48 hours of doxycycline treatment of BCR-ABL inducible cells co-expressing full-length Ahi-1 and its mutants as determined by Annexin V/PI staining. Values shown are the mean ± SEM of triplicate measurements. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.001 (ANOVA). (E) The levels of BCR-ABL expression were suppressed in BCR-ABL inducible cells co-expressing full-length Ahi-1 and its mutants in the presence of doxycyclin for 24 and 48 hours, as detected by an anti-ABL antibody (8E9). GAPDH was utilized as a loading control.
Figure 3.2. Detection of increased apoptotic cells as a result of tyrosine kinase inhibitors in BCR-ABL inducible cells co-transduced with an Ahi-1 SH3 domain deletion mutant. (A) BCR-ABL inducible cells co-expressing full-length Ahi-1 and its mutants were cultured with Imatinib (IM), Nilotinib (NL) and Dasatinib (DA) and the treated cells were stained with Annexin V/PI to detect apoptotic cells after 24 or 48 hours. Representative FACS plots for detection of Annexin V/PI cells 48 hours after IM, NL and DA treatments are shown. (B) Percentage of total apoptotic cells after 48 hours of drug treatment of BCR-ABL inducible cells co-expressing full-length Ahi-1 and its mutants as determined by Annexin V/PI staining. Values shown are the mean ± SEM of triplicate measurements. ** = p<0.01, *** = p<0.001 (ANOVA). (C) The levels of phosphorylation of BCR-ABL were detected using an anti-tyrosine phosphorylation antibody (4G10) in BCR-ABL inducible cells co-expressing full-length Ahi-1 and its mutants in the presence of Imatinib (IM 2.0 µM) after 48 hours. GAPDH was utilized as a loading control.
3.2.2 Expression and purification of the AHI-1 SH3 domain

To understand the molecular and structural functions of the AHI-1 SH3 domain, purification of the AHI-1 SH3 domain was performed for further structural determination. The human AHI-1 SH3 domain was fused with an N-terminal 6xHis tag and a TEV (tobacco etch virus) cleavage site (Figure 3.3A). This fusion protein was successfully overexpressed in *E. coli* (Figure 3.3B). The protein lysate containing the tagged-AHI-1 SH3 domain from 2 L of *E. coli* culture was loaded onto a cobalt affinity column that binds to the 6xHis tag (Figure 3.3C). The eluate from this column exhibited the enriched tagged-AHI-1 SH3 domain with greatly reduced protein contaminants as compared to the total protein lysate (Lane 1, Figure 3.3D, as compared to Figure 3.3B). Subsequently, the eluate was subjected to overnight proteolytic cleavage using 6xHis-tagged TEV protease and loaded onto a second cobalt affinity column (Figure 3.3E). On this column, the cleaved 6xHis tags from the fusion proteins and 6xHis-tagged TEV proteases were retained on the column, while the flow through from this column contained the tag-free AHI-1 SH3 domain with smaller molecular weight (Lane 3, Figure 3.3D).

The collected flow through was then loaded onto an anion exchange column, which separates proteins based on their surface charges. The fractions corresponding to the peak in the chromatography (bottom panel, Figure 3.3F) were tested on a Coomassie blue-stained gel (top panel, Figure 3.3F), which showed a significant removal of protein contaminants. Finally, the collected fractions were pooled and loaded onto a gel filtration

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column that separates proteins based on their shapes and sizes equilibrated in 20 mM Tris 8.0 and 50 mM NaCl. Only one peak was observed in the chromatograph (bottom panel, Figure 3G), and the corresponding fractions contained the highly-purified AHI-1 SH3 domain proteins (top panel, Figure 3.3G). The purified AHI-1 SH3 domain was concentrated to 9 mg/mL for crystallization trials.
Figure 3.3. The sequence and protein purification procedure of the AHI-1 SH3 domain. (A) Cartoon representation of the His-tagged AHI-1 SH3 domain. The full-length His-tagged AHI-1 SH3 domain consists of a 6xHis tag, a TEV cleavage site and the AHI-1 SH3 domain (residue 1048-1116). (B) The His-tagged AHI-1 SH3 domain was successfully overexpressed in E. coli, as indicated on a Coomassie gel. (C) – (E) The purification procedure started with two cobalt affinity columns (C) and (E). The protein bands indicated in the red and green boxes represent the eluted His-tagged SH3 domain from the 1\textsuperscript{st} cobalt column and the tag-free SH3 domain from the 2\textsuperscript{nd} cobalt column, respectively. (F) and (G) The coomassie blue gels and the corresponding chromatographs showed the eluted proteins from a MonoQ column and a Superdex200 gel filtration column, respectively.

3.2.3 Crystallization of the AHI-1 SH3 domain

Crystallization experiments were performed using hanging drop vapour diffusion. 1 \textmu L of purified protein (9 mg/mL) was mixed with 1 \textmu L of reservoir solution on a coverslip, equilibrating over 500 \textmu L of reservoir solution. Initial crystallization trials were set up in the conditions of 0.2 M ammonium sulfate with various concentrations of polyethylene glycol monomethyl ether 2000 (PEG2000 MME, 0\%, 10\%, 20\%, 30\% or 40\%) and 0.1 M buffer of different pHs (pH 3.0 sodium acetate, pH 4.0 sodium acetate, pH 5.0 sodium acetate, pH 6.0 Hepes, pH 7.0 Hepes, pH 8.0 Tris or pH 9.0 Tris). Small crystals appeared within 5 days in the conditions containing 10\% or 20\% PEG2000 MME at pH 3.0 or 4.0 (Figure 3.4A). Subsequently, fine crystallization screening was carried out in the conditions of 0.2 M ammonium sulfate with various concentrations of PEG2000 MME (0\%, 2\%, 4\%, 6\%, 8\%, 10\%, 12\%, 14\%, 16\%, 18\% or 20\%) and 0.1 M buffer of different pHs (pH 3.0, pH 3.2, pH 3.4, pH 3.6, pH 3.8, pH 4.0, pH 4.2, pH 4.4 sodium acetate). At pH 3.0 and 3.2, crystals were obtained in the conditions with 6\%, 8\%
and 10% PEG2000 MME (Figure 3.4B), while at pH 4.0 and 4.2, crystals were observed in the conditions with 18% and 20% PEG2000 MME (Figure 3.4C). Furthermore, various concentrations of ammonium sulfate (0.1 M, 0.2 M, 0.3 M and 0.4 M) were tested in the conditions of pH 3.0/3.2, PEG2000 MME 6%, 8% and 10%; pH 4.0/4.2, PEG2000 18% and 20%). Within these conditions, 0.2 M ammonium sulfate was still found to be the best condition for crystal growth. Lastly, fine crystallization screening was carried out on these conditions, including 0.2 M ammonium sulfate with 5%, 6%, 7%, 8%, 9% or 10% PEG2000 MME at pH 3.0 or 3.2, or 15%, 16%, 17%, 18%, 19% or 20% PEG2000 MME at pH 4.0 or 4.2. The best crystals were observed in the condition of 0.2 M ammonium sulphate, 0.1 M sodium acetate (pH 3.0), 7% PEG200 MME with 10 mM sodium bromide to aid in the phase problem; these crystals were used for X ray diffraction experiments (Figure 3.4D).
Figure 3.4. The examples of protein crystals of the AH1-1 SH3 domain obtained from crystallization trials. (A) Small crystals were obtained in the conditions of 0.2 M ammonium sulfate, 0.1 M pH 4.0 sodium acetate and 20% PEG2000 MME from initial screening. (B) Crystals were obtained in the condition of 0.2 M ammonium sulfate, 0.1 M pH 3.0 sodium acetate and 6% PEG2000 MME. (C) Crystals were obtained in the condition of 0.2 M ammonium sulfate, 0.1 M pH 3.0 sodium acetate and 18% PEG2000 MME. (D) The crystals that were used for X-ray diffraction were obtained in the condition of 0.2 M ammonium sulphate, 10 mM sodium bromide, 0.1 M sodium acetate (pH 3.0) and 7% PEG200 MME.
3.2.4 The AHI-1 SH3 domain adopts a canonical SH3 folding except for an unusually C-terminal α helix

To understand the molecular and structural functions of the AHI-1 SH3 domain, the crystal structure was further investigated. The crystal structure of human AHI-1 SH3 domain was solved at 1.53-Å resolution (Table 3.1), encompassing the region from residues Thr-1052 to Pro-1116. The overall topology of the AHI-1 SH3 domain is similar to those of other SH3 domains, which consists of five anti-parallel β strands (β1=Thr1055-Ala1058, β2=Ile1077-Lys1083, β3=Trp1088-Gly1094, β4=Gln1097-Pro1102 and β5=Val1106-Ser1108, Figure 3.4A & B) arranged as two orthogonal β sheets, forming a compact β barrel. One of the β sheets is formed by β1, β5 and the first half of β2, while the other is formed by β3, β4 and the second half of β2 (Figure 3.4B).

Strands β1 and β2 are joined by a long hairpin loop (RT loop=Leu1059-Asp1076), which flips onto the β barrel. Strands β2 and β3, β3 and β4, β4 and β5 are connected by an n-Src loop (Asp1084-Asp1087), a distal loop (Lys1095-Gly1096) and a 3_10 helix (Ala1103-His1105), respectively. In addition, there is one short α helix at the C-terminus, comprising six residues (Glu1109-Glu1114), which is not seen in other SH3 domains (Figure 3.4B). The hydrophobic core that holds the structure of the SH3 domain is formed by Val1056, Ala1058, Leu1070, Ile1072, Ile1078, Val1080, Phe1101 and Val1106 (Figure 3.4C).

Interestingly, I found that the AHI-1 SH3 domain possesses several structural
characteristics. Firstly, there is one classic β bulge in strand β2, formed through hydrogen bonding between the carbonyl group of Tyr1090 and the amide nitrogens of Phe1081 and Phe1082, providing a necessary kink in strand β2 in order for the second half of β2 to form a β sheet with β3 and β4 [403, 404] (Figure. 3.4D). Secondly, there are four β turns, which are responsible for keeping the RT loop in a “hairpin” configuration and bringing together strands β1 to β4 to form the core of the AHI-1 SH3 domain [405]. Thirdly, the RT loop, which is highly involved in binding to proline rich motifs [406], is stabilized by extensive intra-loop hydrogen bonds, as well as the H-bonds between the loop and the protein core residues. In the AHI-1 SH3 domain, Glu1069 is one of these residues which play a central role in the intricate network of hydrogen bonds. This residue forms four hydrogen bonds, via its side-chain carboxylate group and amide nitrogen, with Tyr1062, Asn1065 and Arg1066 (Figure. 3.4E).
Figure 3.5. The overall structure of the AHI-1 SH3 domain and two structural features. (A) Sequence and secondary alignment of several SH3 domains with the AHI-1 SH3 domain. The residues that form the hydrophobic core are highlighted in yellow, and the residues that are generally conserved are colored in red. The amino acid numbers correspond to the AHI-1 sequence. The sequence labeled with a blue box is a unique sequence insertion in the PI3K SH3 domain. (B) Schematic diagram of the folding of the AHI-1 SH3 domain. Five β-strands are labeled in blue, the 3_10 helix and C-terminal helix are colored in red, and three loops are colored in grey. (C) Surface representation of the AHI-1 SH3 domain. The residues that form the hydrophobic core of the domain are
indicated. (D) View of the β bulge found in the β2 strand. Hydrogen bonds are denoted by dashed lines. Only main chain atoms are shown for clarity. (E) The network of hydrogen bonds centered around Glu1069. Hydrogen bonds are denoted by dashed lines. Figures were generated using the PyMOL program.

Table 3.1. Data Collection and Refinement Statistics

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| Refinement                   |          |
| Resolution (Å)               | 56.04-1.53 |
| No. reflections              | 33024    |
| R<sub>work</sub> / R<sub>free</sub> | 12.43/13.91 |
| No. atoms                    | 1310     |
| Protein                      | 1123     |
| Water                        | 166      |
| Other                        | 21       |
| B-factors                    | 24.69    |
| Protein                      | 20.56    |
| Water                        | 50.88    |
| Other                        | 38.49    |
| R.m.s. deviations            |          |
| Bond lengths (Å)             | 0.03     |
| Bond angles (°)              | 2.10     |
| Ramachandran                 | 98.4/1.6 |
| (core/allowed %)             |          |

*Values in parentheses are for highest-resolution shell.
3.2.5 Identification of an additional ligand binding site in the AHI-1 SH3 domain

The ligand binding area of a SH3 domain is characterized as a hydrophobic patch surrounded by several charged/polar residues [407]. In the AHI-1 SH3 domain, residues Tyr1060, Tyr1062, Asn1065, Arg1066, Glu1069, Asp1087, Trp1088, Pro1102, Asn1104 and His1105 generate three general clefts named pocket 1 (P1), pocket 2 (P2), and pocket 3 (P3), arranged linearly from right to left (Figure 3.6A). Pocket 1 is composed of one highly-conserved aromatic residue, Tyr1060, and two polar residues, Asn1104 and His1105. In most SH3 domains, instead of a His residue, there is either a Tyr or Phe residue that forms pocket 1 (Figure 3.6B). The His residue, compared to Tyr or Phe, may not provide as much of a hydrophobic surface for interacting with proline-rich motifs. However, the nitrogen in the imidazole ring can form a hydrogen bond with the carbonyl group of Asp1061 on the RT loop, which may stabilize the RT loop (Figure 3.6B). The largest pocket, pocket 2, is constructed with Tyr1062, Asp1087, Trp1088, Pro1102 and His1105. Pocket 3 consists of Tyr1062, Asn1065, Arg1066, Glu1069 and Trp1088, and is inside the valley formed by the RT loop and n-Src loop. P3 is usually named as the “specificity” pocket because it contributes to the ligand binding affinity and specificity [408]. Unlike P1 and P2, which hold the signature proline residues in the ligand “PXXP” motif, P3 interacts with residues outside the “PXXP” motif in the ligand. More interestingly, due to the unusual C-terminal α helix, there might be a fourth binding site, next to pocket 1, created by residues Tyr1060, Arg1074, Leu1111, Leu1115 and Pro1116,
which has a positively charged surface (Figure 3.6A & C). This binding site may increase ligand binding specificity and affinity through interaction with residues other than the “PXXP” motif in AHI-1 ligands.

One of the special features of the AHI-1 SH3 domain is three large negatively charged patches scattered on its surface (Figure 3.6C). One of them is formed by the n-Src loop, which creates a negatively charged surface, facilitating the binding of the positively charged residue, such as Lys or Arg, adjacent to the “PXXP” within the proline rich domain (Figure 3.6C, left). The other two patches are formed by the unusual C-terminal helix and the region at the end of the RT loop (Figure 3.6C, right). These two negatively charged patches are distant from the binding site of the proline rich domain and unlikely to participate in the ligand-SH3 domain interaction.

3.2.6 Mapping the binding interface of the AHI-1 SH3 domain

The majority of ligands interacting with SH3 domains adopt a left-handed type II polyproline (PPII) helix, which contains a core element, “PXXP”. SH3 domain ligands are pseudosymmetrical and therefore can bind in two opposite orientations: Class I and Class II. The orientation of ligand binding is determined largely by the presence of a positively charged “compass” residue (Lys or Arg), either N-terminal (Class I orientation) or C-terminal (Class II orientation) to the PXXP motif [409, 410]. Since the AHI-1 SH3 domain displays canonical SH3 domain folding, it is reasonable to predict that its ligand
also adopts a PPII helix conformation and contains a PXXP motif.

In this study, I characterized the binding interface of the AHI-1 SH3 domain using *in silico* modeling, a valuable method that allows for future prediction and design of small inhibitors of this domain. The PPII PD1R peptide (KRPLPPLPS) was used [411], which interacts with the phosphoinositide 3-kinase (PI3K) SH3 domain (pdb code 3l5R), to map the binding interface of the AHI-1 SH3 domain and study the potential binding pattern. Using the CCP4 suite with 54 C\(_\alpha\) atoms, the AHI-1 SH3 domain was superimposed with the SH3 domain of PI3K; the root mean square deviation of C\(_\alpha\) atoms between the PI3K SH3 domain and the AHI-1 SH3 domain was found to be 1.30Å (Figure 3.6D). The major structural deviation arises from the regions of the n-Src loop and the C-terminus, with an unusual 15-residue insertion in the PI3K n-Src loop and an uncommon C-terminal helix in AHI-1. However, in the conventional “PXXP” binding site, most conformations of the conserved hydrophobic residues resemble each other, indicating that P1 and P2 pockets, which accommodate the two signature proline residues, are similar in these two SH3 domains (Figure 3.6E). This structural arrangement of P1 and P2 holding the “PXXP” motif is in agreement with other SH3 domains [386, 412, 413].

Although the overall structure among most SH3 domains is similar, significant differences in structural properties are observed in pocket 3, due to high sequence deviation of the RT loop and the n-Src loop, which construct P3. Therefore, P3 is
implicated in binding affinity and specificity [414]. However, despite the structural
difference, pocket 3 of most SH3 domains is organized to hold the basic “compass”
residue. The interaction between this positively charged residue and P3 dictates the
binding orientation and is crucial for binding affinity. Figure 3.5F shows the interaction
between the Arg residue in the PD1R peptide and P3 of the PI3K SH3 domain (green),
which is superimposed with the residues responsible for forming P3 of the AHI-1 SH3
domain (red). This Arg residue can fit into the binding cleft, and the negative charges of
the cleft provide a suitable environment for accommodating this positively charged
residue (Figure 3.6F). One of the features of binding between P3 and the basic residue is
a highly conserved acidic residue, either Glu or Asp, residing at the bottom of pocket 3.
This negatively charged residue facilitates the insertion of the “compass” residue into
pocket 3 by forming either hydrogen bonds or by ionic interaction with the basic residue.
The Arg residue in the PD1R peptide has been shown to be able to interact ionically with
Asp21 of the PI3K SH3 domain [411]. Although Glu1069 in the AHI-1 SH3 domain
(corresponding to Asp21) bends outward, not the proper position to form an ionic
interaction with the Arg residue (Figure 3.6F), it is possible due to “induced fit” upon
ligand binding, Glu1069 might be able to ionically interact with the basic residue.
Another interesting aspect of the interaction between the Arg residue of PD1R peptide
and P3 of the PI3K SH3 domain is that Arg18 and Trp55 of the PI3K SH3 domain and
the Arg residue of PD1R peptide are parallel to each other, and form an “Arg-Arg-Trp”
stack; this hydrophobic complex can enhance the ligand-SH3 domain interaction for PI3K. Moreover, unlike strict conservation of Trp55 among SH3 domains, much structural variation is seen at the position of Arg18 (Fig. 3.4A). Therefore, it is plausible to predict that this “Arg-Arg-Trp” stack might play a role in defining binding specificity. In the AHI-1 SH3 domain, there is also an Arg residue (Arg1066) at the position for Arg18 of PI3K, and Arg1066 and Trp1088 (corresponding to Trp55 in the PI3K SH3 domain) also appear to be parallel (Figure 3.6F). It is likely that there might also be formation of an “Arg-Arg-Trp” complex in the AHI-1 SH3 domain based on this molecular modeling analysis.
Figure 3.6. Binding sites, surface hydrophobicity and electrostatic potential of the AHI-1 SH3 domain. (A) Surface representation of the AHI-1 SH3 domain, indicating the residues forming three regular binding pockets and an additional potential pocket. P1, P2, P3 and P4 are shown using red circles. (B) The surface hydrophobicity of the AHI-1 SH3 domain. As the residue hydrophobicity scale increases, the color gradually changes from white to green. A hydrogen bond, shown in a red dashed line, is formed by the imidazole group of His1105 and the carbonyl group of Asp1061. (C) The surface electrostatic potential of the AHI-1 SH3 domain. Red corresponds to negative potentials and blue to positive potentials. Three large negative patches are indicated, which are the n-Src loop, the end of RT loop and the C-ter helix. The scale used to represent the surface potentials is provided. (D) Superimposition of structures of the AHI-1 and PI3K SH3 domains. The AHI-1 SH3 domain is shown in red, and the PI3K SH3 domain (PDB code 1GBQ) is colored in green. The conserved hydrophobic residues forming the ligand binding pockets are shown in a ball-and-stick representation (numbered according to the AHI-1 SH3 sequence). (E) Space-filled model illustrating the potential interaction interface of the AHI-1 SH3 domain (skyblue) using the proline-rich peptide PD1R (yellow, PDB code 1GBQ) known to bind to the SH3 domain of PI3K. Three binding pockets (P1, P2 and P3) and two proline residues within PD1R peptide are indicated. (F) Superimposition of the PD1R-bound PI3K SH3 domain with the AHI-1 SH3 domain illustrating the potential binding pattern of P3 of the AHI-1 SH3 domain and the basic “compass” residue within the proline rich domain. Only the residues constructing P3 of the AHI-1 SH3 domain (red) and PI3K SH3 domain (green) are shown. A partial peptide sequence containing the positively charged Arg “compass” residue is shown in yellow. The residues that are responsible for the formation of “Arg-Arg-Trp” stack are indicated (red for the AHI-1 SH3 domain and green for the PI3K SH3 domain). Figures were generated using the PyMOL program.

3.3 Discussion

In this study, I investigated the biological functions of the oncoprotein AHI-1 SH3 domain in mediating TKI response/resistance in CML cells. I also demonstrated new structural features of this functional domain.

Our lab recently demonstrated that AHI-1 physically interacts with BCR-ABL and JAK2 kinases to mediate transforming activity and TKI response/resistance of CML
stem/progenitor cells, and that the N-terminal region and WD40-repeat domain of AHI-1 are responsible for interacting with JAK2 and BCR-ABL, respectively [283, 284]. However, the SH3 domain, an important signal transduction regulator, does not seem to be required for binding JAK2 and BCR-ABL in CML cells. Furthermore, the ligands of the AHI-1 SH3 domain have not been identified thus far. Therefore, I was prompted to investigate the biological and structural functions of the AHI-1 SH3 domain.

In this study, it was shown that BCR-ABL+ cells expressing full-length Ahi-1 exhibited significantly decreased apoptosis as compared to parental BCR-ABL inducible cells in the presence of TKIs, demonstrating that Ahi-1 played a role in protecting CML cells against TKIs (Figure 3.2). Interestingly, under the same conditions, deletion of the SH3 domain significantly enhanced the apoptotic response of BCR-ABL+ cells upon exposure to TKIs in comparison to BCR-ABL+ cells expressing full-length Ahi-1, indicating that without the SH3 domain, AHI-1 lost its oncogenic ability in mediating TKI resistance of BCR-ABL+ cells (Figure 3.2). Moreover, cells expressing the N-terΔ mutant that still carried the SH3 domain showed significantly decreased apoptotic cells, as compared to cells containing the SH3Δ mutant, further confirming the functional role of the AHI-1 SH3 domain in mediating TKI response in BCR-ABL+ cells; whereas these cells were significantly more sensitive to TKIs relative to cell overexpressing full-length Ahi-1, indicating that TKI resistance was also contributed by the N-terminal region of Ahi-1, which was shown to mediate the interaction between AHI-1 and JAK2 (Figure
Notably, more cellular apoptosis was observed in BCR-ABL-transduced cells in the presence or absence of Ahi-1 and its SH3Δ mutant with doxycycline treatment as compared to the same cells treated with TKIs (Figure 3.1 & 3.2). The possible explanation might be that doxycycline caused a complete suppression of both phosphorylation and protein expression of BCR-ABL (Figure 3.1E), while TKIs only partially suppressed phosphorylation of BCR-ABL, in particular under IM treatment (Figure 3.2C). In summary, these results suggested that the SH3 domain of AHI-1 is involved in regulation of TKI sensitivity/resistance in CML cells, possibly through interacting with other proteins, yet to be identified.

Determination of protein structures using X-ray crystallography provides a powerful tool to investigate protein interactions in enormous detail, delineates structure-function relationships at the atomic level and facilitates drug discovery [415-418]. In this study, close inspection of the crystal structure of the AHI-1 SH3 domain identified several special features, which might help to elucidate how the AHI-1 SH3 domain mediates protein interactions and predict potential interacting ligands. Moreover, these structural features could also be used to design inhibitors specifically targeting AHI-1 SH3 domain interactions, which might potentially mitigate the effects of the AHI-1 SH3 domain-mediated TKI resistance in CML cells.

Firstly, due to the unusual C-terminal α helix, there might be a fourth binding site,
which may increase ligand binding specificity and affinity through interaction with residues other than the “PXXP” motif and the basic “compass” residue. A similar situation has been observed in the interaction between the C-terminal Src kinase (CSK) SH3 domain and the PEP peptide containing “PXXP” motif. The strength of ligand-SH3 binding is enhanced by the intensive hydrophobic interactions formed by the C-terminal 310 helix of the PEP peptide and the residues outside the conventional binding grooves of the CSK SH3 domain. Secondly, like the PI3K SH3 domain, the two important residues, Arg1066 and Trp1088, which build pocket 3, may also form an “Arg1066-Arg-Trp1088” stack upon binding to a “compass” residue Arg within the proline rich domain, further increasing the ligand-SH3 domain binding affinity (Figure 3.6F). Although I found that Arg1066 (from AHI-1) and Arg18 (from PI3K) do not overlap well, and that the Arg residue does not quite fit into the “Arg1066-Arg-Trp1088” stack, it is possible that binding to the actual ligand may result in the “induced fit” of the AHI-1 SH3 domain so that the Arg residue of the ligand might be inserted into a proper position for formation of the “Arg1066-Arg-Trp1088” stack. This binding-induced structural change has been seen in the interaction between NEF protein and FYN SH3 domain. Moreover, due to the low occurrence of the residue Arg1066 among other SH3 domains (Figure 3.5A), the potential existence of the “Arg1066-Arg-Trp1088” might add another layer of ligand binding affinity. Lastly, the His1105 replacement of the conserved Tyr or Phe, which sets up the boundary between P1 and P2, may reduce the hydrophobic surface area, and create a
slightly hydrophilic spot in the hydrophobic binding patch (Figure 3.6B). In other SH3 domains, a Tyr or Phe residue at this position tends to form hydrophobic interactions with the X residues (preferably aliphatic) within the “PXXP” motif. However, the His residue may prefer polar residues that can form hydrogen bonding with its imidazole group. This unique His residue could facilitate ligand selection. All these structural properties distinguish the AHI-1 SH3 domain from other SH3 domains and make it possible to develop specific inhibitors for the AHI-1 SH3 domain.

However, it needs to be noted that though there are many available crystal structures of SH3 domains, to my knowledge, so far there has not been any SH3 domain inhibitors that are widely accepted and used. It is not due to lack of trying, but because there are several major obstacles to overcome. Firstly, despite the low sequence conservation, most SH3 domains share a similar overall structural folding and “PXXP” motif binding site, which makes generating inhibitors targeting specific SH3 domains quite challenging. Secondly, due to the relatively flat/shallow binding interface, the binding affinity between SH3 domains and their ligands is not very strong (Kd: ~ 10-100 μM) [277-279]. This transient weak binding is suitable for efficient signaling transduction, but makes it hard to design/generate high-affinity inhibitory ligands. Therefore, directly targeting SH3 domains has remained a rather difficult task.
Chapter 4: Identification and Functional Characterization of a New BCR-ABL-AHI-1-DNM2 Protein Complex in Regulation of Leukemic Properties of Primitive CML Cells

4.1 Introduction

AHI-1, functioning as an adaptor protein, has been well documented to modulate normal organogenesis and also disease development through coordinating protein networks [306, 307, 309, 310]. As described in Section 1.2.2 and 3.1, AHI-1 plays a cooperative role with BCR-ABL in CML cells, and the AHI-1-mediated protein complex containing BCR-ABL and JAK2 via its WD40-repeat domain and the N-terminal region has been demonstrated to contribute to CML leukemogenesis and TKI resistance [283, 284]. In Chapter 3, I investigated the functional importance of the AHI-1 SH3 domain for AHI-1 oncogenic activity in BCR-ABL+ cells and discussed the possibility of using it as a therapeutic target. However, how the SH3 domain of AHI-1 participates in CML pathogenesis and TKI resistance still remains unknown. Research has shown that the SH3 domain is one of the most important regulators of protein interactions. Through interacting with PXXP motif-containing proteins, the SH3 domain mediates formation of protein complexes that participate in numerous molecular and cellular activities [419, 420]. Given its biological evidence in CML cells, I hypothesized that the AHI-1 SH3 domain interacts with key components in BCR-ABL-mediated pathways, which enhances
the transforming activity of BCR-ABL and TKI resistance. This chapter presents the identification of interacting proteins of the AHI-1 SH3 domain in BCR-ABL+ cells and demonstrates their functional relevance in the context of CML leukemogenesis and drug resistance.

By using affinity purification/mass spectrometry, a list of potential interacting partners of the AHI-1 SH3 domain was identified. Among these proteins, Dynamin-2 (DNM2) showed the highest identification score and peptide coverage. Furthermore, DNM2 transcripts were found to be significantly increased in primary CML cells compared to normal bone marrow (BM), particularly in stem/progenitor populations. The interaction between DNM2 and AHI-1 was demonstrated to be mainly dependent on the binding between the SH3 domain of AHI-1 and the proline rich domain (PRD) of DNM2, and one of the 14 PXXP motifs within the PRD of DNM2 was predicted to be the binding site of the AHI-1 SH3 domain in silico. Lastly, I showed that in BCR-ABL+ cells, AHI-1 mediates the formation of a protein complex comprising BCR-ABL, AHI-1 and DNM2, within which BCR-ABL phosphorylates and presumably aberrantly activates DNM2.

4.2 Results

4.2.1 Identification of potential interacting proteins of the AHI-1 SH3 domain in BCR-ABL+ CML cells

To investigate the underlying mechanisms of the AHI-1 SH3 domain in mediation of
abnormal cell growth and TKI response/resistance, I screened for binding partners of the human AHI-1 SH3 domain using mass spectrometry (MS). An N-terminal 6xHis-tagged AHI-1 SH3 domain was purified and incubated with the protein lysate from K562 cells, which is a CML cell line derived from a blast crisis patient. AHI-1 SH3 domain protein complexes, and a control where the AHI-1 SH3 domain was omitted, were immunoprecipitated with anti-His antibodies, then washed with PBS, 250 mM NaCl and 500 mM NaCl in sequence and finally eluted with SDS sample buffer (Figure 4.1A & B). The samples corresponding to the two salt washes and elution were separated using SDS-PAGE (Figure 4.1B). The high salt washes were used to remove non-specific binding, which was evident in a Coomassie-stained SDS-PAGE (Figure 4.1B, Lane 2-5). Two elution lanes (Lane 6 and 7) were cut into 10 gel pieces with 2 pieces containing the two prominent bands in Lane 7 (indicated by boxes) and subjected to analysis by tandem MS.

The two prominent bands described above were identified as Collagen Type XVIII Alpha 1 (top) and Dynamin-2 (bottom), which were confirmed in another IP/mass spectrometry replicate. Along with these two proteins, a list of candidate interacting proteins was revealed and is summarized in Table 4.1. All these proteins fulfilled the following criteria: only present in the IP experiment but not in the control sample, identification of at least two unique peptides for each protein and a Mascot score > 50 (Table 4.1). Among all the potential AHI-1 SH3 domain interacting proteins, Dynamin-2
(DNM2) exhibited the highest identification score (2,134) and peptide coverage (43). Interestingly, DNM2 is a GTPase that is involved in numerous biological functions such as endocytosis, intracellular trafficking and actin and microtubule networking [335]. Moreover, DNM2 contains several proline rich motifs (PRD) in its C-terminus, which have been shown to interact with SH3 domains of several proteins [421-424]; therefore, it is likely that DNM2 could be one of the ligands of the AHI-1 SH3 domain.
Figure 4.1. Identification of interacting proteins of the AHI-1 SH3 domain using immunoprecipitation/mass spectrometry. (A) Cartoon representation of immunoprecipitation (IP) using anti-His antibodies. In the IP experiment, the purified His-tagged AHI-1 SH3 domain was mixed with protein lysates from K562 cells and anti-His antibodies, followed by pulldown with protein A/G beads. In the control experiments, the purified SH3 domain was omitted. (B) The IP complexes were washed with 250 mM NaCl and 500 mM NaCl, and then eluted using the SDS sample buffer. Samples from washes and elution were separated in SDS-PAGE, followed by staining with coomassie blue. Two prominent bands that were present in Lane 7 but absent in Lane 6 (shown in boxes) were identified as Collagen XVIII and Dynamin-2.
Table 4.1. Tandem MS identification of potential interacting proteins of the AHI-1 SH3 domain

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<td>43</td>
<td>14</td>
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<td><strong>Collagen Type XVIII α1</strong></td>
<td>572</td>
<td>12</td>
<td>74</td>
<td>● An extracellular matrix protein&lt;br&gt;● Endogenous cleavage in the C-terminal region results in production of endostatin, which inhibits endothelial cell proliferation and angiogenesis&lt;br&gt;● Involved in regulation of retinal and neural structures</td>
</tr>
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<td>10</td>
<td>6</td>
<td>● A transmembrane glycoprotein&lt;br&gt;● A main component of the nuclear pore complex&lt;br&gt;● Plays an essential role in nuclear pore assembly, cargo trafficking and structural integrity</td>
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<td><strong>Dynamin-1</strong></td>
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<td>● A large GTPase&lt;br&gt;● Mainly expressed in neuronal cells&lt;br&gt;● Clathrin-mediated endocytosis; formation of synaptic vesicles</td>
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<td><strong>Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase 3</strong></td>
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<td>2</td>
<td>● A membrane-bound homodimeric enzyme, located in rough ER&lt;br&gt;● intermolecular collagen cross-linking</td>
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<td># PXXP motifs</td>
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<td>• A molecular chaperone that has ATPase activity</td>
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<td></td>
<td></td>
<td></td>
<td>• Promotes protein folding and maturation</td>
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<td></td>
<td></td>
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<td>• Involved in signalling transduction and cell cycle control</td>
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<tr>
<td>WAS/WASL Interacting WAS/WASL Interacting</td>
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<td>52</td>
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<td>• Plays a role in actin cytoskeleton</td>
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<td></td>
<td>• An adaptor protein</td>
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<td></td>
<td></td>
<td>• Plays a role in actin cytoskeleton reorganization</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Participates in recruitment and activation of WASL</td>
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<tr>
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<td>• A transcriptional suppressor of Smad-mediated transcription</td>
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<tr>
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<td></td>
<td></td>
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<td>• A key component of mRNA spliceosome</td>
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<td></td>
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4.2.2 The transcript levels of DNM2 are increased in CD34+ CML stem/progenitor cells

To determine if DNM2 may be implicated in the pathogenesis of CML, the transcript levels of DNM2 were examined in CD34+ normal and CML cells. It was shown that the transcript levels of DNM2 were significantly increased in treatment-naive CD34+ stem/progenitor cells from CML patients who were classified retrospectively, after IM therapy, as IM-responders (n=11) and IM-nonresponders (n=15) in comparison to CD34+ normal BM cells (n=7, p=0.013 and 0.037 Figure 4.2 A). In particular, the highest expression of DNM2 was observed in some IM-nonresponders (Figure 4.2 A). Moreover, DNM2 exhibited significantly higher expression in CML stem-enriched cell populations (lin−CD34+CD38−) and progenitor cells (lin−CD34+CD38+) compared to more mature cells obtained from IM-nonresponders (lin+CD34+, 2-fold, Figure 4.2 B). These results suggested that DNM2 may play an important role in CML, in particular in TKI-resistant CML stem/progenitor cells, and further investigations are warranted.
**Figure 4.2. Comparison of DNM2 expression in CD34+ cells from normal and CML patient samples.** (A) Q-RT-PCR was used to examine the expression of DNM2 in cells from normal BM, IM-responders (IM R) and IM-nonresponders (IM NR). The DNM2 transcripts were normalized to the control gene β2M, and bars represent the mean of data for each group. (B) Comparison of the transcript levels of DNM2 in the stem cell-enriched population (CD34+CD38-), progenitors (CD34+CD38+) and mature (CD34-) cells from IM-nonresponders. * = p<0.05, ** = p<0.01 (student t test).

**4.2.3 Validation of the interaction between DNM2 and AHI-1**

DNM2 has been identified as the top interacting candidate of the AHI-1 SH3 domain from IP/mass spectrometry (Section 4.2.1). In order to eliminate possible false positive results from the IP/mass spectrometry experiment, co-immunoprecipitation (co-IP) assays and co-localization analysis were performed to test the interaction between DNM2 and full-length Ahi-1 in Ahi-1 or/and BCR-ABL-transduced BaF3 cells. The co-IP results revealed that DNM2 was stably associated with Ahi-1 in Ahi-1-transduced BaF3 cells and in BCR-ABL inducible cells co-expressing full-length Ahi-1, indicating that there is an interaction between Ahi-1/AHI-1 and DNM2 (Figure 4.3A). Moreover, confocal analysis showed that DNM2 (red) and Ahi-1 (green) co-localized (yellow) in the cytoplasm of BCR-ABL/Ahi-1 co-transduced BaF3 cells, further suggesting that there is indeed binding between AHI-1 and DNM2 (Fig. 4.3B).
Figure 4.3. Verification of the interaction between Ahi-1/AHI-1 and DNM2. (A) DNM2 was immunoprecipitated from lysates of BaF3 cells, Ahi-1-transduced BaF3 cells, BCR-ABL inducible cells, and BCR-ABL inducible cells co-expressing full-length Ahi-1, and then probed with specific antibodies as indicated. (B) BCR-ABL/Ahi-1 co-transduced BaF3 cells were fixed, permeabilized and stained with anti-DNM2 (red) and anti-Ahi-1 (green) antibodies. DAPI was used to stain nuclei. Images were acquired with a Nikon X1 confocal microscope using a magnification of 60X. The white scale bar represents 50 μm.
4.2.4 The SH3 domain of Ahi-1 and the DNM2 PRD are mainly responsible for the interaction between Ahi-1/AHI-1 and DNM2

To further dissect the interaction between Ahi-1/AHI-1 and DNM2, four Ahi-1 and DNM2 constructs were generated, including HA-tagged full-length Ahi-1 (HA-Ahi-1), HA-tagged SH3 domain-deleted Ahi-1 (HA-Ahi-1 SH3Δ), Myc-tagged full-length DNM2 (Myc-DNM2) and Myc-tagged PRD-deleted DNM2 (Myc-DNM2 PRDΔ) (Figure 4.4A). 293T cells were used to co-express different combinations of these four constructs, and 3 different assays were employed to detect potential interactions. Co-IP assays revealed that strong binding was found only between full-length Ahi-1 and DNM2, whereas the combinations of the mutant forms showed much weaker protein interactions (Figure 4.4B & C). These results suggested that the SH3 domain of Ahi-1/AHI-1 and the PRD of DNM2 are the major contributors in this interaction, although some other domains might also be involved, since in the absence of a SH3-PRD interaction, a weak AHI-1/DNM2 interaction is still detectable.

The interaction between AHI-1 and DNM2 was further investigated using the in situ proximity ligation assay (PLA), which detects protein interactions at a single-molecule level. When two proteins interact, oligonucleotide-conjugated antibodies that bind directly to target proteins or recognize the primary antibodies of the target proteins will bring the oligonucleotide pair into proximity to hybridize and form circular DNA strands. In turn, the DNA circles serve as templates, which allow polymerases to add
fluorescently-labelled nucleotides to generate concatemeric products [425, 426]. In this way, protein interactions can be visualized as individual fluorescent spots by immunofluorescence microscopy. In this study, oligonucleotide-conjugated antibodies that recognized the primary antibodies of HA and Myc tags were used for the in situ PLA. Confocal microscopy analysis revealed the strong red fluorescent signals only in 293T cells co-transfected with full-length Ahi-1 and DNM2, but not in cells co-expressing Ahi-1 and DNM2 mutants (Figure 4.5). These PLA results demonstrated that the SH3-PRD recognition is essential for the interaction between Ahi-1/AHI-1 and DNM2.

In addition, confocal images showed that co-localization signals (yellow) were observed in cells expressing full-length Ahi-1 (green) and DNM2 (red) in the cytoplasm, but barely detected in the Ahi-1/DNM2 PRDΔ co-transfected cells, which further demonstrated that the interaction between AHI-1 and DNM2 is mainly dependent on the binding between the SH3 domain of Ahi-1 and the PRD of DNM2 (Figure 4.6). Unexpectedly, in Ahi-1 SH3Δ mutant-transfected cells, Ahi-1 SH3Δ (green) was found localized in the nuclei (blue), while full-length DNM2 and DNM2 PRDΔ were cytoplasmic and thereby no co-localization signals detected (Figure 4.6). Thus, three different approaches demonstrated that the SH3 domain of AH1-1 and the PRD of DNM2 are mainly responsible for the interaction between Ahi-1/AHI-1 and DNM2.
Figure 4.4. Molecular evidence for the interaction between Ahi-1 and DNM2. (A) Four Ahi-1 and DNM2 constructs were generated, including HA-tagged full-length Ahi-1 (HA-Ahi-1), HA-tagged SH3 domain-deleted Ahi-1 (HA-Ahi-1 SH3Δ), Myc-tagged full-length DNM2 (Myc-DNM2) and Myc-tagged proline rich domain-deleted DNM2 (Myc-DNM2 PRDΔ). (B) 293T cells were co-transfected with different combinations of the Ahi-1 and DNM2 constructs. Protein extracts were subjected to anti-HA antibody immunoprecipitation and then immunoblotted with an anti-Myc antibody to detect Myc-tagged DNM2 protein constructs. (C) Protein extracts were subjected to anti-Myc antibody immunoprecipitation and then immunoblotted with an anti-HA antibody to detect HA-tagged Ahi-1 protein constructs.
Figure 4.5. Proximity ligation assay (PLA) in 293T cells co-transfected with different combinations of Ahi-1 and DNM2 constructs. 293T cells were co-transfected with different combinations of the Ahi-1 and DNM2 constructs including HA-tagged full-length Ahi-1 (HA-Ahi-1), HA-tagged SH3 domain-deleted Ahi-1 (HA-Ahi-1 SH3Δ), Myc-tagged full-length DNM2 (Myc-DNM2) and Myc-tagged proline rich domain-deleted DNM2 (Myc-DNM2 PRDΔ). These cells were fixed, permeabilized, blocked and incubated with anti-HA and anti-Myc antibodies for overnight. Then, cells were added with the PLA probe, Ligation-Ligase and Amplification-Polymerase solutions in sequence. DAPI was used to stain the nuclei. Images were acquired using a confocal Nikon X1 microscope at a magnification of 60X. The white scale bar represents 5 μm.
Figure 4.6. Co-localization studies of Ahi-1 and DNM2 mutants using confocal microscopy. 293T cells co-transfected with different combinations of Ahi-1 and DNM2 constructs, including HA-tagged full-length Ahi-1 (HA-Ahi-1), HA-tagged SH3 domain-deleted Ahi-1 (HA-Ahi-1 SH3Δ), Myc-tagged full-length DNM2 (Myc-DNM2) and Myc-tagged proline rich domain-deleted DNM2 (Myc-DNM2 PRDΔ). These cells were fixed, permeabilized and stained with anti-HA (green) and anti-Myc (red) antibodies. DAPI was used to stain the nuclei. Images were acquired using a confocal Nikon X1 microscope at a magnification of 60X. The white scale bar represents 5 μm.
4.2.5  *In silico* identification of the PXXP motif within the DNM2 PRD that is responsible for interacting with the AHI-1 SH3 domain

To determine which PXXP motif within the PRD of DNM2 was most likely responsible for binding the AHI-1 SH3 domain, *in silico* screening and modeling were performed using a sequence conservation search (Expasy ClustalW) and the crystal structure of the AHI-1 SH3 domain (Pymol). Given the previous results in Section 3.2.6, the sequence of the binding ligand of the AHI-1 SH3 domain should contain a core “PXXP” motif and a “compass” Arg residue. In total 6 out of the 14 PXXP motifs within the PRD of DNM2 met the sequence requirements for potentially binding to the AHI-1 SH3 domain (Figure 4.7A). Two (P10 and P12) out of these 6 PXXP motifs belong to the Class I ligands, which have the general consensus sequence (K/R)XXPXXP, whereas the remaining 4 (P5, P8, P11 and P12) belong to the Class II ligands, which have the general consensus sequence XPXXP(X/K/R) (Figure 4.7B) [419, 420].

Next, I looked at the 22 available structures of ligand-bound SH3 domains from different proteins (e.g. Fyn, Src, Hck, etc.) in the Protein Data Bank and divided them into two groups based on the class of ligands they bind. Subsequently, sequence alignments were run between these SH3 domains and the AHI-1 SH3 domain, and the results were sorted in a descending manner based on the sequence identity with the AHI-1 SH3 domain in Figure 4.7C & E. The Fyn SH3 domain was found to be the top candidate in both sequence alignments. Superimposition of the structures of the AHI-1 and Fyn...
SH3 domains demonstrated that these two proteins exhibited strong similarity with respect to protein folding (the root mean square deviation of Cα atoms was calculated to be 0.93Å) and the residues that make up their binding interfaces were well-overlapped (Figure 4.8A). The space-filling models revealed that these two SH3 domains also shared similar overall structures, except that the AHI-1 SH3 domain had a potential fourth binding pocket (Figure 4.8B & C).

Furthermore, I aligned the 6 PXXP motifs in the DNM2 PRD (Figure 4.7B) with the sequences of the binding ligands of the SH3 domains listed in Figure 4.7 C & E. The sequence comparison between P10 or P12 and the Class I ligands mostly exhibited sequence conservation with 3 out of 7 residues (e.g. RIPPGLP (P10) vs. RPAPKPP (Nbp2p); RRPPAAP (P12) vs. RPLPPLP (Src)) (Figure 4.7D). As compared to P5, P8, P11 or P12, many of the Class II ligands showed sequence identity in 4 of 7 residues (e.g. APPIPS (P5) vs. PPPPPHR (BBC1); PPQPIS (P8) vs. PPVPPPR (Grb2N); PPQVPSP (P11) vs. QPAVPPP (p40phox); PPAAPP (P12) vs. PPVPQPR (Plcr)), and several of them do not possess the consensus sequence (e.g. PPALPSSAP (Tuba) and APPKPPLP (Ponsin)) (Figure 4.7F). Importantly, it was observed that APPIPS (P5) showed the highest sequence similarity (5/7) with APPLP with APPLP that is the ligand of the Fyn and Src SH3 domains in all sequence comparisons between the 6 PXXP motifs from the DNM2 PRD and the ligand sequences of the listed SH3 domains (Figure 4.7D & F). Therefore, given the highest sequence identity (38%) between the AHI-1 and Fyn SH3
domains and the highest sequence similarity (5/7) between P5 within the DNM2 PRD and APPLPPR, the ligand of the Fyn SH3 domain, I postulated that it was most likely that the APPIPSR (P5) sequence within the PRD in DNM2 might provide the binding site for the AHI-1 SH3 domain.

Finally, I predicted how the AHI-1 SH3 domain bound to the potential ligand APPIPSR (P5) using molecular modeling. I converted Leu (the 4th residue) and Pro (the 6th residue) in APPLPPR, the binding ligand of the Fyn SH3 domain, to the Ile and Ser residues to generate APPIPSR (P5) and modeled P5 on the AHI-1 SH3 domain in silico. Multiple conformations of Ile and Ser were generated (Figure 4.9 & 4.10). The conformation of the Ile residue indicated in Figure 4.9B was selected as the most likely structure. As compared to the conformations of Ile residues in Figure 4.9C & D, the Ile residue in Figure 4.9B displays the least conformational discrepancy with the Leu residue in Figure 4.9A. Furthermore, the Ile residue in Figure 4.9B forms strong hydrophobic contacts with the binding surface, made up of three hydrophobic residues (Tyr1062, Phe1082 and Pro1102), of the AHI-1 SH3 domain, which is similar to what is observed for the Leu residue in Figure 4.9A; whereas the conformations of Ile residues in Figure 4.9C & D result in increased distance between these Ile residues and binding surface of the AHI-1 SH3 domain (indicated by arrows), which could lead to reduced hydrophobic interactions. Thus, having an Ile with the selected conformation at this position would be permissive for the P5 ligand binding to the AHI-1 SH3 domain.
The conformation of the Ser residue indicated in Figure 4.10C was selected as the most likely structure as this Ser residue points in the opposite direction from the hydrophobic binding surface of the AHI-1 SH3 domain and seemingly creates no steric hindrance. However, the side chain of the Ser residue in Figure 4.10B is facing the carbonyl group of the adjacent Arg (distance=3.43 Å), which might cause charge repulsion; therefore, this conformation of the Ser residue is not favorable. In Figure 4.10D, the Ser residue creates not only charge repulsion with Asp1087 in the AHI-1 SH3 domain, but also strong steric hindrance; therefore, this conformation of the Ser residue is also not acceptable. Thus, having a Ser with the selected conformation at this position would be permissive for the P5 ligand binding to the AHI-1 SH3 domain.

Finally, this APPIPSR peptide with Ile and Ser replacing Leu and Pro from APPLPPR was modelled onto the AHI-1 SH3 domain (Figure 4.11). This approach revealed that the dipeptide “AP” fits into the P1 pocket, the dipeptide “IP” was accommodated in the P2 pocket and the Arg residue inserted into the P3 pocket in the AHI-1 SH3 domain (Figure 4.11B). Hence, this model illustrated how the APPIPSR ligand in the DNM2 PRD was permissive to bind to the AHI-1 SH3 domain.
### Identification of PXXP motifs in the DNM2 PRD

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<tr>
<th>Class I ligand sequence</th>
<th>Class II ligand sequence</th>
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<td>KXXP or RXXP</td>
<td>XPXXPXK or XPXXPXK</td>
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<tr>
<td>TSTVPYPPVDDTLQSSHSSTPTQRP</td>
<td>RIPGPQP (P10) or APPIPS (P5)</td>
</tr>
<tr>
<td>VSSIHPGRRPAPRGPPTPGPPLIPPVPGAAASF</td>
<td>RRPPAAP (P12) or PGVPSP (P11)</td>
</tr>
<tr>
<td>SAPPIPSRRPAPSRPVRP</td>
<td>PPAAPSP (P12)</td>
</tr>
<tr>
<td>P5 P6 P7 P8 P9</td>
<td>P10 P11 P12 P13 P14</td>
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### The PXPP motifs within the DNM2 PRD as potential AHI-1 SH3 interacting sites

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**Sequence alignment**

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</tr>
<tr>
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<td>3/7 RPLPPLP /</td>
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<tr>
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<td>3/7 YSPPPPP /</td>
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<tr>
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<tr>
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<tr>
<td>Protein</td>
<td>% Identity</td>
<td>Class II ligand</td>
<td># residues match P5</td>
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<td>VPVAPPR</td>
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<td>APPKPPLP</td>
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Figure 4.7. Identification and classification of the PXXP motifs in the PRD of DNM2 and sequence alignments between the AHI-1 SH3 domain and SH3 domains from various proteins. (A) 14 PXXP motifs were identified in the PRD of DNM2, labelled from P1 to P14. (B) Based on the consensus sequence, 6 PXXP-contained sequences from DNM2 PRD were divided into Class I or Class II ligands. (C) The sequence alignment between the AHI-1 SH3 domain and SH3 domains from other proteins, whose complex structures with Class I ligands are available in Protein Data Bank. The percentage of the sequence identity relative to the sequence of the AHI-1 SH3 domain is shown. The highly conserved residues are highlighted in yellow. (D) The sequence alignment of the Class I ligands of the SH3 domains listed in (C) with P10 or P12 within the DNM2 PRD and the percentage of sequence identity relative to P10 or P12. The conserved proline residues within the PXXP motif are labelled in bold, and the compass residues R/K are colored in red. (E) The sequence alignment between the AHI-1 SH3 domain and SH3 domains from other proteins, whose complex structures with Class II ligands are available in Protein Data Bank. The percentage of the sequence identity relative to the sequence of the AHI-1 SH3 domain is shown. The highly conserved residues are highlighted in yellow. (F) The sequence alignment of the Class II ligands of the SH3 domains listed in (E) with P5, P8, P11 or P12 within the DNM2 PRD and the percentage of sequence identity relative to P5, P8, P11 or P12. The conserved proline residues within the PXXP motif are indicated in bold, and the compass residues R/K are colored in red.
Figure 4.8. Superimposition and space-filling models of the AHI-1 and Fyn SH3 domains. (A) Superimposition between the AHI-1 and Fyn SH3 domains. The AHI-1 SH3 domain is shown in the salmon color (PDB: 4ESR), while the Fyn SH3 domain is colored in blue (PDB: 4ZNX). (B) + (C) Space-filling models of the AHI-1 SH3 domain (salmon) with the indication of the P4 pocket and the Fyn SH3 domain (blue).
Figure 4.9. Conversion of Leu in the peptide sequence N-APPLPPR-C to Ile to generate N-APPIPPR-C. (A) The in silico model of the AHI-1 SH3 domain bound with the APPLPPR peptide (PDB: 4ZNX) that is a known binding ligand of the Fyn SH3 domain. (B) – (D) The Leu residue was converted to Ile using PyMOL and three different conformations of Ile were illustrated. Figures on the right indicate the zoomed binding interface. The surface hydrophobicity of the AHI-1 SH3 domain is indicated. As the residue hydrophobicity scale increases, the color gradually changes from white to green. The molecular surface of the indicated residues is shown. Carbon atoms are colored in green, oxygen atoms are colored in red and nitrogen atoms are colored in blue.
Figure 4.10. Conversion of Pro at position 6 in the peptide sequence N-APPLPPR-C to Ser to generate N-APPLPSR-C. (A) The *in silico* model of the AHI-1 SH3 domain bound with the APPLPPR peptide (PDB: 4ZNX) that is a known binding ligand of the Fyn SH3 domain. (B) – (D) The Pro residue was converted to Ser using PyMOL and three different conformations of Ser were illustrated. Figures on the right indicate the zoomed binding interface. The surface hydrophobicity of the AHI-1 SH3 domain is indicated. As the residue hydrophobicity scale increases, the color gradually changes from white to green. The distance between the hydroxyl oxygen of the Ser residue and the carbonyl oxygen of the adjacent Lys residue is indicated in a yellow dash line (B). The residue Asp1087 in the AHI-1 SH3 domain is indicated in (D). The molecular surface of the indicated residues is shown. Carbon atoms are colored in green, oxygen atoms are colored in red and nitrogen atoms are colored in blue.

Figure 4.11. Molecular modeling of the AHI-1 SH3 domain bound to the APPIPSR peptide within the PRD of DNM2. (A) The *in silico* model of the AHI-1 SH3 domain bound with the APPLPPR peptide. (B) The APPIPSR peptide *in silico* converted from the APPLPPR peptide was modeled on the AHI-1 SH3 domain. Three binding pockets (P1, P2 and P3) and the sequences of the ligands are indicated.
4.2.6 The interaction between Ahi-1 and DNM2 occurs in the endocytic compartments

After observing the specific co-localization signals of Ahi-1 and DNM2 in a “punctate” pattern throughout the cytoplasm (Figure 4.6), and given the fact that DNM2 is a major component in the endocytic pathway (Section 1.3.2), I hypothesized that the interaction between Ahi-1/AHI-1 and DNM2 occurred in endocytic compartments. To investigate this hypothesis, 293T cells co-expressing different combinations of the Ahi-1 and DNM2 mutants were co-immunostained with anti-HA/anti-EEA1 antibodies or anti-HA/anti-LAMP1 antibodies, followed by co-localization analysis. EEA1 and LAMP1 were used as protein markers to indicate early endosomes and late endosomes/lysosomes, respectively [427]. In full-length Ahi-1 and DNM2 co-expressed cells, Ahi-1 (green) was found to be co-localized (yellow) with EEA1 or LAMP1 (red) in the cytoplasm. In contrast, the co-localization signals were significantly disrupted in cells co-transfected with full-length Ahi-1 and the DNM2 PRDΔ mutant (Figure 4.12A & B). In cells with Ahi-1 SH3Δ, regardless of co-transfection with either full-length DNM2 or DNM2 PRDΔ, no co-localization signals were detected since the Ahi-1 SH3Δ mutant resided in the nucleus (green overlaps with blue), but EEA1 or LAMP1 (red) was localized in the cytoplasm (Figure 4.12A & B). These results suggested that Ahi-1 may reside in endosomes, and this localization may be dependent on its interaction with DNM2.
A

<table>
<thead>
<tr>
<th>HA-Ahi-1 + Myc-DNM2</th>
<th>HA-Ahi-1 + Myc-DNM2 PRΔ</th>
<th>HA-Ahi-1 SH3Δ + Myc-DNM2</th>
<th>HA-Ahi-1 SH3Δ + Myc-DNM2 PRΔ</th>
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</table>
Figure 4.12. Ahi-1 resides in the endosomal compartments in a DNM2-dependent manner. 293T cells were co-transfected with different combinations of Ahi-1 and DNM2 constructs, including HA-tagged full-length Ahi-1 (HA-Ahi-1), HA-tagged SH3 domain-deleted Ahi-1 (HA-Ahi-1 SH3Δ), Myc-tagged full-length DNM2 (Myc-DNM2) and Myc-tagged proline rich domain-deleted DNM2 (Myc-DNM2 PRΔ). These cells were fixed, permeabilized and stained with anti-HA (green) and anti-EEA1 (red, A) or anti-LAMP-1 (red, B) antibodies. DAPI was used to stain the nuclei. Images were acquired using a confocal Nikon X1 microscope at a magnification of 60X. The white scale bar represents 5 μm.
4.2.7 Identification of a new protein complex containing BCR-ABL, AHI-1 and DNM2 in BCR-ABL+ cells

As an adaptor protein, one of the major roles of AHI-1 in CML cells is to mediate the formation of protein complexes containing BCR-ABL [283, 284]. Since AHI-1 interacts with both BCR-ABL and DNM2, it raises the question whether these three proteins can form a protein complex. Co-IP experiments revealed that BCR-ABL was detected in both K562 and K562 IMR cells after immunoprecipitation with an anti-DNM2 antibody (Figure 4.13A). Furthermore, the interaction between BCR-ABL and DNM2 was also confirmed in BCR-ABL-transduced BaF3 cells (Figure 4.13B). Strikingly, the interaction was enhanced in BCR-ABL-transduced cells co-expressing full-length Ahi-1; in contrast, this enhanced interaction was not observed in cells co-transduced with BCR-ABL and the AHI-1 SH3Δ mutant (Figure 4.13B). Moreover, co-localization analysis was utilized to confirm the existence of this protein complex in 293T cells co-transfected with HA-Ahi-1, BCR-ABL and Myc-DNM2 or Myc-DNM2 PRDΔ. Strong co-localization signals were found in cells co-expressing all three full-length proteins HA-Ahi-1, BCR-ABL and Myc-DNM2 (indicated by the white arrows in Figure 4.13C). However, co-localization between Ahi-1, BCR-ABL and DNM2 PRDΔ was hardly detected. From these results, I concluded that AHI-1 may bridge BCR-ABL and DNM2 together to form a protein complex, likely through its SH3 and WD40-repeat domains (a previous study showed that AHI-1 interacts with BCR-ABL through its WD40-repeat domain; [283]), respectively, and it is named as the “BAD” (BCR-ABL-AHI-1-DNM2) complex.
Figure 4.13. Identification of the protein complex formed by BCR-ABL, AHI-1 and DNM2. (A) Co-immunoprecipitation assays in K562 and K562 IMR (IM-resistant K562) cells. Protein extracts were subjected to anti-DNM2 immunoprecipitation (IP) and then immunoblotted with anti-c-Abl antibody or anti-DNM2 antibody. (B) Co-immunoprecipitation experiments in BCR-ABL-transduced, BCR-ABL-Ahi-1 co-transduced and BCR-ABL-AHI-1 SH3ΔBaF3 cells. Protein extracts were immunoprecipitated with an anti-DNM2 antibody and then immunoblotted with an anti-c-Abl antibody or anti-DNM2 antibody. (C) 293T cells were co-transfected with HA-Ahi-1, BCR-ABL and Myc-DNM2 or Myc-DNM2 PRDΔ. Cells were fixed, permeabilized and stained with anti-Myc (red) and anti-HA (purple), anti-c-Abl (green) antibodies. DAPI was used to stain the nuclei. Images were acquired using a confocal Nikon X1 microscope at a magnification of 60X. The white scale bar represents 5 μm.
4.2.8  BCR-ABL phosphorylates DNM2 in BCR-ABL+ CML cells

Previous studies have shown that the tyrosine phosphorylation at Tyr231 and Tyr597 by SRC and/or ABL is required for full activation of DNM2 [342-344]. In CML, with the presence of the constitutively active BCR-ABL kinase and the confirmed interaction between BCR-ABL and DNM2, it is plausible to assume that DNM2 is another BCR-ABL downstream target. In order to test this assumption, co-IP experiments were carried out in several BCR-ABL+ CML cell lines. Since there are no phospho-DNM2 antibodies commercially available, my experimental approach deviated from the conventional co-IP setting. Briefly, BCR-ABL+ cells were treated with or without IM, followed by immunoprecipitation with an anti-DNM2 antibody, and then blotted with 4G10, a pan-phospho-tyrosine (p-Tyr) antibody. In this way, the modified co-IP setting may be able to demonstrate the effects of IM on the phospho-tyrosine levels of DNM2 (p-DNM2), which reflects if BCR-ABL phosphorylates DNM2. In K562 and K562 IMR cells, IM treatment greatly reduced endogenous p-DNM2 levels compared to the same cells without IM treatment, suggesting that BCR-ABL phosphorylated DNM2 in these cells (Figure 4.14A). The reduction of DNM2 phosphorylation was also confirmed in BCR-ABL-transduced, BCR-ABL/Ahi-1 co-transduced and BCR-ABL/Ahi-1 SH3Δ co-transduced BaF3 cells when these cells were treated with IM (Figure 4.14B). However, in BCR-ABL-negative parental BaF3 cells, tyrosine phosphorylation of DNM2 was not altered with or without IM treatment (Figure 4.14B). Consistent with BCR-ABL+ K562 and BaF3 cells, BCR-ABL-transduced UT7 cells also exhibited significantly reduced DNM2 phosphorylation upon IM treatment (Figure 4.14C).
However, in UT7 cells expressing a BCR-ABL-T315I mutant that prevents TKI binding, IM treatment did not decrease, but rather enhanced DNM2 phosphorylation (Figure 4.14C). In 293T cells co-expressing BCR-ABL and Myc-DNM2, IM was also able to reduce p-DNM2 levels as compared to untreated cells (Figure 4.14D). These results demonstrated that IM treatment reduced tyrosine phosphorylation of DNM2 in several BCR-ABL+ cell lines, but not in BCR-ABL- or IM-resistant BCR-ABL T315I mutant cells, providing molecular evidence that BCR-ABL phosphorylates DNM2 in CML cells.

**Figure 4.14.** BCR-ABL phosphorylates DNM2 in BCR-ABL+ CML cells. (A) Co-immunoprecipitation assays in K562 and K562 IMR cells with or with 24-hr IM treatment. K562 cells were treated with 0.5 µM IM, while K562 IMR cells were exposed to 2 µM IM. (B) Co-immunoprecipitation in BCR-ABL-transduced, BCR-ABL-Ahi-1 co-transduced, BCR-ABL-AHI-1 SH3Δ and parental BaF3 cells with or without 24-hr 2 µM IM treatment. All the protein extracts were immunoprecipitated with an anti-DNM2 antibody and then immunoblotted with a pan-anti-p-Tyr antibody (4G10) or anti-DNM2 antibody. (C) Co-immunoprecipitation assays in BCR-ABL-transduced and BCR-ABL T315I-transduced UT7 cells with or without
24-hr 2 µM IM treatment. (D) Co-immunoprecipitation assays in 293T cells co-transfected with BCR-ABL and Myc-DNM2 with or without 24-hr 5 µM IM treatment.

4.3 Discussion and future directions

The SH3 domain is one of the most important protein interaction domains. To date, more than 16,000 SH3 domains in over 12,500 different proteins have been described in the SMART database [428]. Importantly, the SH3 domain can modulate signal transduction by mediating formation of protein complexes to regulate numerous cellular events [277, 429]. In Chapter 3, I have demonstrated a functional role of the AHI-1 SH3 domain in contributing to TKI response/resistance in CML cells. In this chapter, I first elucidated how the AHI-1 SH3 domain exerts its effects in CML cells by searching for interacting partners of this domain. By using affinity purification/mass spectrometry, I identified a list of potential interacting proteins of the AHI-1 SH3 domain (Table 4.1). Among these candidates, DNM2 exhibited the highest identification score and peptide coverage. Structurally, DNM2 has a proline rich domain (PRD), which could potentially provide the binding site for the AHI-1 SH3 domain. Functionally, both AHI-1 and DNM2 have been strongly implicated in the endocytic pathway and intracellular trafficking [309, 315, 321, 335]. In addition, I have shown that DNM2 expression was significantly increased in CML cells in comparison to normal BM cells, suggesting that DNM2 may be critical in CML (Figure 4.2). Based on these reports and observations, it was reasonable to speculate that DNM2 is likely to interact with the SH3 domain of AHI-1.

The interaction between Ahi-1 and endogenous DNM2 was detected in BCR-ABL-transduced cells co-expressing Ahi-1 by both co-IP assays and co-localization
analysis, revealing that indeed DNM2 is an AHI-1 interacting protein (Figure 4.3). Moreover, it was shown that the interaction was mainly ascribed to the binding between the SH3 domain of AHI-1 and the PRD of DNM2, supported by results from co-IP assays (Figure 4.4). However, it is possible that other regions/domains of these two proteins might contribute to their interaction. Both AHI-1 and DNM2 are multi-domain proteins and the co-IP experiments showed weak interactions even in Ahi-1/DNM2 mutant-expressing cells (Figure 4.4B & C). This assumption was however not supported by other experimental approaches. In the PLA assay, only cells co-expressing full-length Ahi-1 and DNM2, but not mutant-expressed cells, exhibited fluorescent signals, suggesting that the interaction between AHI-1 and DNM2 might be solely dependent on Ahi-1 SH3-DNM2 PRD recognition (Figure 4.5). In the co-localization studies, it was observed that, unlike the cytoplasmic full-length Ahi-1, the Ahi-1 SH3Δ mutant was only visible in the nucleus, suggesting that the AHI-1 SH3 domain may act as an anchor to retain AHI-1 in the cytoplasm, possibly by interacting with other cytoplasmic proteins such as DNM2. Since Ahi-1 SH3Δ and cytoplasmic DNM2/DNM2 PRDΔ did not share the same cellular space, as evident from the co-localization studies, there should not have been any detectable interaction at all, which contradicts the detection of weak interactions between Ahi-1 SH3Δ and DNM2/DNM2 PRDΔ in the co-IP assay (Figure 4.4 & 4.6).

Although co-IP assays are still considered to be the gold standard for detection of protein interactions, there are some limitations regarding this in vitro technique. Firstly, non-specific binding of proteins to the beads or antibodies used in the IP, use of excessive antibodies and/or
protein lysates and incomplete washes could all lead to false positive results. Secondly, proteins that are normally compartmentalized in different regions of cells are now mixed together, which may result in non-physiological interactions between these proteins in IP experiments. In this study, I would consider that the weak interactions observed between Ahi-1 and DNM2 mutants were non-specific protein binding to the antibodies used for my IP, possibly caused by use of an excessive amount of protein lysates (500 μg) and antibodies (1 μg). Ectopic introduction of the different HA-tagged or Myc-tagged Ahi-1 and DNM2 constructs may have resulted in massive protein production. Therefore, mixing of the over-expressed proteins and relatively high amounts of antibodies (1 μg) used for the IP may have created high chances for non-specific binding (e.g. Myc-tagged DNM2 PRD bound to anti-HA antibodies used in IP), leading to the false positive results. If this hypothesis valid, it would be easier to explain the conundrum that in Ahi-1 SH3Δ cells, weak interactions could still be detected by co-IP experiments even when the proteins were separated in discrete cellular compartments shown in confocal images: the interaction between Ahi-1 SH3Δ (nuclear) and DNM2/DNM2 PRDΔ (cytoplasmic) may have been artificially introduced due to non-specific binding in the co-IP although it does not occur in living cells, which was supported by the results of the PLA and co-localization analyses. In the future, adjusting the amount of protein lysates and antibodies as well as using more stringent wash conditions (e.g. increasing salt concentrations) could possibly minimize the non-specific binding effects and help to obtain more reliable results in co-IP assays.

To further dissect the interaction between the SH3 domain of AHI-1 and the PRD of
DNM2, *in silico* screening and modeling were performed. Through sequence alignment and comparison, it was observed that the SH3 domains of AHI-1 and Fyn exhibited the highest sequence conservation, and APPIPSR (P5) from the DNM2 PRD displayed the highest (5/7) sequence similarity with APPLPPR, the ligand of the Fyn SH3 domain. Therefore, it was proposed that this APPIPSR, out of the 14 predicted PXXP motif-contained sequences within the DNM2 PRD, was most likely the binding site of the AHI-1 SH3 domain. However, it was worth noting that although APPIPSR (P5) and APPLPPR (the ligand of the Fyn SH3 domain) exhibit high sequence identity, these two peptides differ at position 6: P5 possesses a hydrophilic Ser residue while APPLPPR has a hydrophobic Pro residue. The residue at this position is adjacent to the “compass” Arg residue. The Pro residue in APPLPPR could provide a “kink”, a unique property of proline, which might help to position the “compass” Arg to the P3 pocket of the Fyn SH3 domain. The Ser residue in APPIPSR, on the other hand, is not able to function the same way as proline, but instead may create a hydrophilic environment that is not compatible with the hydrophobic binding interface of the AHI-1 SH3 domain. Of note, in total 6 of 10 Class II ligands shown in Figure 4.7F possess a Pro residue at position 6 (APPLPPR, QPAVPPR, PPVPPQR, PALPPK, RPQVPLR, PPPTLPK, PPTLPHR, PPVPMPR, PPPPPHR and VPVAPPR). Therefore, this might raise a question whether the *in silico* screening performed in this study was the best strategy to predict the binding site of the AHI-1 SH3 domain within the DNM2 PRD.

Despite this concern, I still consider this sequence conservation comparison a valid
approach to search for the interaction site of the AHI-1 SH3 domain in DNM2 for the following reasons. Firstly, I performed *in silico* modeling to identify the chemically-permissive conformation of the serine residue in APPIPS (P5) that could bind to the AHI-1 SH3 domain. I purposely selected one conformation of the serine residue, which points away from the hydrophobic binding surface of the AHI-1 SH3 domain (Figure 4.10C); therefore, this serine residue would not interfere with the interaction between APPIPS (P5) in the DNM2 PRD and the AHI-1 SH3 domain. Secondly, there are still 4/10 Class II ligands listed above possessing a non-proline residue at position 6, including Gln (hydrophilic), Leu (hydrophobic) and His (hydrophilic). None of these residues are capable of creating a “kink”, but the adjacent “compass” residue Arg or Lys in these ligands can still insert into the P3 pockets of their cognate SH3 domains. Thus, these observations further mitigate the concern that the polar serine residue at position 6 of APPIPS may be incompatible with binding to the AHI-1 SH3 domain. Lastly, for the consensus sequence of the Class II ligands -X(1)P(2)X(3)X(4)P(5)X(6)R/K(7), the first and fourth X residues are preferably hydrophobic residues because they participate in interacting with the hydrophobic surface of SH3 domains. However, such preference does not apply to the residue at position 6. The primary role of the residue at this position is to help the Class II ligands adopt the polyproline-2 helix, which is the conformation needed to interact with SH3 domains [419, 420]. Hence, the Ser residue at the non-conserved position 6 in APPIPS should be acceptable. Based on these analyses, I think this *in silico* approach was still a useful way for predicting the binding site within the DNM2 PRD for the AHI-1 SH3 domain.
Additionally, one observation made in this *in silico* screening is that $PPGVPSR$ (P11) within the DNM2 PRD also showed as high as 5/7 sequence similarity with $PPPVPPR$, the ligand of the Grb2 N-terminal (Grb2N) SH3 domain (Figure 4.7F). However, since the sequence identity between these two SH3 domains is only 24%, P11 was not considered as the most possible binding site of the AHI-1 SH3 domain.

*In silico* approach in this study was performed to predict the potential binding sequence within the DNM2 PRD and model the predicted binding ligand interacting with the three binding pockets (P1, P2 and P3) of the AHI-1 SH3 domain. However, the P4 binding pocket of the AHI-1 SH3 domain was not used in the ligand-search process. The reason was that in my opinion, the P4 binding pocket would not be useful for identifying the binding sequences of the AHI-1 SH3 domain by this *in silico* approach. Searching for the ligand of the AHI-1 SH3 domain in this study was based on selecting the SH3 domain that showed high sequence conservation with the AHI-1 SH3 domain and identifying which PXXP motif within the DNM2 PRD showed a high sequence match with the ligands of other SH3 domains (Figure 4.7C-F). However, the P4 binding pocket is unique for the AHI-1 SH3 domain, and absent in most other SH3 domains; therefore, it is reasonable to speculate that the sequences to the C-terminus of P10 and P12 (Class I ligands) or N-terminus of P5, P8, P11 and P12 (Class II ligands) in the DNM2 PRD, which are the potential binding sites for the P4 pocket of the AHI-1 SH3 domain, would not share high sequence similarity with the flanking sequences of the binding ligands of other SH3 domains. As expected, for example (Figure A.15), the sequences to the N-terminus of the
binding ligands of 7 SH3 domains listed in Figure 4.7F exhibited low sequence similarity with
\textit{FSAAPPIPSR(P5)} from the DNM2 PRD (Figure 4.7A). Therefore, since the sequence identity
between the flanking sequences of the PXXP motifs from the DNM2 PRD and the binding
ligands of other SH3 domains is likely to be quite low, use of the P4 binding pocket to predict
the binding sequence of the AHI-1 SH3 domain does not seem applicable by this \textit{in silico}
sequence conservation search and comparison method.

One potential way of using the P4 pocket is to \textit{in silico} generate peptides containing
sequences listed in Figure 4.7B with flanking sequences (e.g. \textit{FSAAPPIPSR (DNM2 PRD P5)})
using Pymol and then dock these peptides onto the crystal structure of the AHI-1 SH3 domain
by online molecular docking servers (CABS-dock, GalaxyWEB, etc.). The peptide that fits best
in all four binding pockets of the AHI-1 SH3 domain would be considered as the most likely
binding ligand. However, most of the molecular docking programs are highly error-prone
(personal communication with Dr. Filip Van Petegem); therefore, the data obtained from these
docking models need to be interpreted with extra caution.

Nowadays, molecular modelling has been widely used as a powerful tool for structural
analysis, simulations of molecular interactions and mapping of the binding interfaces within
protein interactions [430, 431]. However, it can still only be considered as a molecular
prediction, unless it is supported by biochemical/biophysical experimental evidence. Therefore,
in the future, biochemical binding assays need to be used to validate the \textit{in silico} prediction that
was made in this study. For instance, 14 peptides containing each PXXP motif within the PRD
of DNM2 could be synthesized in vitro. Then, ITC (isothermal titration calorimetry; [432]) or SPR (surface plasmon resonance; [433]) could be used to test which of these synthesized peptides shows high binding affinity with the AHI-1 SH3 domain. Peptide sequences containing the PXXP motifs identified as the putative binding ligands of the AHI-1 SH3 domain would then be used as targets for site-directed mutagenesis (two proline residues in the PXXP motif would be converted into alanine residues) in full length DMN2. Subsequently, the results of co-IP experiments between the full-length AHI-1 and DNM2 with different PXXP mutations (AXXA) would illustrate how these DNM2 mutants affect the binding between AHI-1 and DNM2, leading to final determination as to which PXXP site is mainly responsible for binding to the AHI-1 SH3 domain.

It is worth noting that in BCR-ABL-negative Ahi-1-overexpressing BaF3 cells (Figure 4.3A) and BCR-ABL-negative 293T cells co-transfected with full-length Ahi-1 and DNM2 (Figure 4.4B & C, 4.5 & 4.6), the interaction between AHI-1 and DNM2 could still be detected, implying that the interaction between AHI-1 and DNM2 is not BCR-ABL-dependent and solely CML-specific. Additionally, Ahi-1 was found to reside in the early and late endosomes in a DNM2-dependent manner in 293T cells, suggesting that the interaction between AHI-1 and DNM2 takes place in endosomal compartments (Figure 4.9). Therefore, it is plausible to speculate that the interaction between AHI-1 and DNM2 could also occur in other cell types (e.g. in neuronal cells where AHI-1 is highly expressed, Section 1.2.3), possibly participating in the regulation of vesicle trafficking processes.
One of the most important findings of this study was the identification of a new AHI-1-mediated protein complex containing BCR-ABL and DNM2. The co-IP results showed that BCR-ABL was stably associated with endogenous DNM2 in parental BCR-ABL inducible BaF3 cells, indicating that BCR-ABL and DNM2 interact in BCR-ABL+ cells (Figure 4.13B). Interestingly, the interaction between BCR-ABL and DNM2 was strongly enhanced when Ahi-1 was co-expressed, whereas in cells expressing Ahi-1 SH3Δ, the enhanced interaction was not detected (Figure 4.13B). These results suggested that AHI-1 as an adaptor protein bridges DNM2 and BCR-ABL through its SH3 and WD40-repeat domains, respectively, to form a protein complex in BCR-ABL+ cells; however, without the DNM-2-binding SH3 domain, AHI-1 is no longer able to mediate the interaction between BCR-ABL and DNM2. In addition, the co-localization between BCR-ABL, Ahi-1 and DNM2 was observed in cells co-expressing all three full-length proteins, but absent in cells containing the DNM2 PRDΔ protein. These findings further supported the presence of a BAD protein complex, and also confirmed that the interaction between Ahi-1 and DNM2 via the SH3-PRD recognition is important for the complex formation.

Since both BCR-ABL and DNM2 are contained in the BAD protein complex and it has been well-described that tyrosine phosphorylation is crucial for DNM2 activation [343, 344], co-IP assays were performed to test whether the BCR-ABL tyrosine kinase would phosphorylate DNM2 in CML cells. In four BCR-ABL+ cell lines (K562, BCR-ABL-transduced BaF3, BCR-ABL-transduced UT7 and BCR-ABL-transfected 293T cells), IM treatment resulted in
decreased levels of p-DNM2, suggesting that BCR-ABL phosphorylates DNM2 in CML cells (Figure 4.14). However, in BCR-ABL-negative BaF3 cells, tyrosine phosphorylation of DNM2 remained constant in the presence or absence of IM (Figure 4.14B). This eliminated the possibility that IM targeted other kinases that were responsible for DNM2 phosphorylation and further supported the idea that it was BCR-ABL that mainly phosphorylated DNM2 in BCR-ABL+ cells. Interestingly, in UT7 cells containing the BCR-ABL T315I mutant, IM treatment increased DNM2 phosphorylation. Since IM cannot inhibit the kinase activity of BCR-ABL bearing the T315I mutation, it was expected that p-DNM2 levels would stay the same upon IM treatment (Figure 4.14C). One possible reason for observing higher p-DNM2 levels is that IM treatment further enhanced the kinase activity of BCR-ABL in BCR-ABL T315I mutant cells, leading to more DNM2 phosphorylation (Figure A.16). This result also suggested that the phosphorylation level of DNM2 is correlated with the level of the BCR-ABL kinase activity, thereby once again indicating that BCR-ABL phosphorylates DNM2 in CML cells.

In this study, the biological consequences of DNM2 phosphorylation by BCR-ABL in CML cells have not been determined. Based on the previous findings that BCR-ABL, as a constitutively active tyrosine kinase, drives CML development through phosphorylating downstream targets, and tyrosine phosphorylation dictates DNM2 activation, it is reasonable to postulate that BCR-ABL could affect DNM2 activity by phosphorylation. Additionally, the presence of the highly-expressed adaptor protein AHI-1 in CML cells can strongly increase the
frequency of BCR-ABL-mediated phosphorylation events on DNM2, which presumably leads to aberrant activation of DNM2. To test this hypothesis, the GTPase activity of DNM2 [434] could be compared (1) in BCR-ABL+ cells in the presence or absence of TKIs, or (2) in 293T cells transfected with DNM2 alone, co-transfected with BCR-ABL and DNM2 or co-transfected with BCR-ABL, AHI-1 and DNM2 to confirm whether BCR-ABL has strong influence on DNM2 activity.

Finally, besides DNM2, other identified interacting candidates of the AHI-1 SH3 domain might also deserve attention, in particular Nucleoporin 210KDa (GP210) (Table 4.1). GP210 anchors the nuclear pore complex to the nuclear membrane and functions as an essential regulator for trafficking of molecules across the nuclear envelope [435, 436]. AHI-1 has been shown to serve as shuffle to translocate β-catenin into nuclei in kidney cells [307]. Therefore, it is possible that the interaction between AHI-1 and GP210 via the AHI-1 SH3 domain might facilitate the transport of cargoes from the cytoplasm to the nucleus. Future studies could be conducted to verify and investigate a potential interaction between AHI-1 and GP210. In addition, two Collagen-related proteins Collagen Type XVIII α1 and Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase 3, and two chaperone proteins HSP90-Beta and HSP73 were also identified as potential interactors of the AHI-1 SH3 domain. However, these proteins are of high avidity and frequently present in proteomic studies, therefore they are not of particular interest.

The in vitro affinity purification/mass spectrometry assay led me to the identification of a
list of AHI-1’s potential interacting proteins, particularly DNM2. However, it is worth noting that some caveats related to the \textit{in vitro} affinity purification might result in false positive and negative results. Firstly, the mixing of the purified AHI-1 SH3 domain with the protein lysate from K562 cells might artificially create chances for the AHI-1 SH3 domain to meet with certain proteins that are not normally available for AHI-1 to interact within cells due to temporal and spatial restrictions. Therefore, false positive results may be a concern. Ectopic expression of the 6xHis-tagged AHI-1 SH3 domain in leukemic cells followed by IP could be a valid alternative to minimize this concern. Secondly, a high salt wash was an excellent way to remove non-specific bound proteins. However, given the fact that the interactions between SH3 domains and their ligands are transient and in the low micromolar range \cite{277-279}, this stringent wash condition might also remove proteins that bind weaker to the AHI-1 SH3 domain, leading to the non-identification of novel binding partners of the AHI-1 SH3 domain. Therefore, attention should be paid to bands appearing only in the wash lanes of IP experiments. Thirdly, DNM2 is found abundantly in cells and this may lead to false negative results. The DNM2 molecules might occupy a large portion of the AHI-1 SH3 domain proteins, which could reduce the chances of the protein “bait” to interact with other binding partners. In order to minimize the “dominant” DNM2 effect, similar \textit{in vitro} or \textit{in vivo} affinity purification experiments using the AHI-1 SH3 domain as protein “bait” could be performed in DNM2-knockdown leukemic cell lines.

In summary, I have identified DNM2 as another AHI-1 interacting protein and studied the
molecular determinants of the interaction between AHI-1 and DNM2. Moreover, I have characterized a newly-identified “BAD” protein complex in BCR-ABL+ cells and demonstrated that BCR-ABL phosphorylates DNM2 in these cells. In the future, the biological significance of BCR-ABL-mediated DNM2 phosphorylation in CML cells needs to be investigated.
Chapter 5: Investigation of the Functional Roles of DNM2 in BCR-ABL+ cells and TKI-insensitive stem/progenitor cells from CML Patients

5.1 Introduction

In the last chapter, it was shown that the transcript levels of DNM2 were significantly increased in CD34+ CML cells as compared to normal BM cells, suggesting that DNM2 might be deregulated in CML. Furthermore, I demonstrated that BCR-ABL phosphorylated DNM2 in BCR-ABL+ cells, implying that the activity of DNM2 might be downstream of BCR-ABL signaling in CML cells. However, how the increased expression and activity of DNM2 is involved in CML leukemogenesis and drug response/resistance is unknown.

In this chapter, I demonstrated an oncogenic role for DNM2 in CML by showing that stable suppression of DNM2 causes a growth disadvantage, increases apoptosis, impairs colony-forming abilities and increases sensitivity to TKI treatments in BCR-ABL+ cell lines and primitive patient-derived CML cells. Subsequently, I provided evidence that DNM2 is strongly associated with the deregulation of three essential cellular activities including endocytosis, ROS production and autophagy in these cells. Hence, this study highlights a critical role for DNM2 in the pathogenesis of CML, and suggests that DNM2 may be a potential therapeutic target for improved treatment strategies in CML.
5.2 Results

5.2.1 Lentiviral-mediated suppression of DNM2 strongly affects survival, apoptosis, and phosphorylation of BCR-ABL and JAK2/STAT5 in BCR-ABL+ cells

To investigate the biological significance of DNM2 suppression in CML cells, I performed lentiviral-mediated DNM2 knockdown in K562 cells (Figure 5.1A). The infected cells with high GFP expression were purified by FACS and then maintained under puromycin selection in culture. Two out of four different constructs (shDNM2b and shDNM2d) achieved a very good suppression of the DNM2 RNA level (66% and 44% suppression, respectively), while transductions with the other two targeting sequences against DNM2 (constructs shDNM2a and shDNM2c) did not knockdown DNM2 in K562 cells (Figure 5.1B). Growth monitoring of these cells illustrated a decrease in viability of K562 cells correlating with the level of DNM2 protein suppression (shDNM2b vs. shDNM2d, 32% vs. 79%, Figure 5.1D). Furthermore, viability assessments after 48-hour IM treatment revealed a significant reduction of viable K562 cells, which was most obvious for cells transduced with shDNM2b and to a lesser extent for shDNM2d, as compared to SHC (non-targeting control sequence) control cells (13% and 60% vs. 73% Figure 5.1D). Moreover, K562 cells transduced with shDNM2b exhibited 64% apoptotic cells, whereas shDNM2b and control cells showed 24% and 19% apoptotic cells in the presence of IM, respectively (Figure 5.1E). Interestingly, SHC control cells treated with MitMAB [437], an inhibitor targeting the PH domain of DNM2, exhibited reduced viability (50%) and increased apoptosis compared to control cells (18% vs. 8%), indicating that pharmacological inhibition of
DNM2 resembled genetic suppression of DNM2 in K562 cells (Figure 5.1D & E). Moreover, MitMAB treatments in DNM2-knockdown cells further inhibited the DNM2 activity, which resulted in even greater biological consequences (Figure 5.1D & E).

At the molecular level, DNM2 suppression led to significant reduction of phosphorylation of BCR-ABL, JAK2 and STAT5 that corresponded to DNM2 suppressed levels in shDNM2b and shDNM2d transduced K562 cells, but spared p-AKT and p-ERK as compared to SHC controls (Figure 5.1C). These results suggested that DNM2 depletion may attenuate the kinase activity of BCR-ABL, which in turn resulted in suppression of the JAK2/STAT5 signaling cascade [64].

To determine if suppression of DNM2 could also eradicate aggressive BCR-ABL+ blast cells from late stage disease, I performed a similar DNM2 knockdown experiment in blast crisis patient-derived BV173 cells. I observed that only one construct (shDNM2b, hereby referred to as shDNM2) out of the four DNM2 lentiviral shRNAs (data not shown) exhibited 50% reduction of DNM2 expression in BV173 cells (Figure 5.2A). DNM2-knockdown BV173 cells displayed a significant growth disadvantage compared to SHC control cells (Figure 5.2C). These effects were further enhanced upon IM or DA treatments for 48 hours relative to control cells (viable cells 40 vs. 73% and 38 vs. 68%, Figure 5.2C). Moreover, IM or DA exposure induced a significant increase in apoptosis of BV173 cells infected with shDNM2 than SHC control cells (36 vs. 10% and 35 vs. 10%, Figure 5.2D). MitMAB treatments also resembled the biological effects of DNM2 suppression in BV173 cells. In addition, BV173 cells transduced with
shDNM2 exhibited lower levels of phosphorylation of BCR-ABL, JAK2 and STAT5, but unchanged p-AKT and p-ERK, which was also observed in DNM2-knockdown K562 cells (Figure 5.1C & 5.2B). Taken together, DNM2 suppression significantly reduced proliferation and increased apoptosis of BCR-ABL+ CML cells and sensitized them to TKI treatments. Hence, DNM2 may play an important role in sustaining the survival of CML cells, possibly through the BCR-ABL-mediated JAK2/STAT5 pathway.
Figure 5.1. The effects of DNM2 knockdown on cell viability, apoptosis and signaling cascades in K562 cells. (A) Schematic of four DNM2 shRNAs targeting the coding region of human DNM2, each of which is incorporated into the pGFP-C-shLenti vector. (B) Determination of DNM2 knockdown in K562 cells by four DNM2 shRNAs using q-RT-PCR and western blotting analysis. (C) The effects of DNM2 suppression on phosphorylation and protein levels of BCR-ABL, JAK2, STAT5, AKT and ERK. (D) 48-hour cell viability assays in SHC or DNM2-knockdown K562 cells treated with 0.25 μM IM or 4 μM MitMAB alone or in combination. (E) 48-hour cell apoptosis assays in SHC or DNM2-knockdown K562 cells treated with 0.25 μM IM or 4 μM MitMAB alone or in combination. Values shown are the mean ± SEM. *=p<0.05, **=P<0.01, ***= P<0.001 (student t test).
Figure 5.2. The effects of DNM2 suppression on cell viability, apoptosis and signaling proteins in BV173 cells. (A) Suppression of the transcript levels of DNM2 in BV173 cells. Only one of four DNM2 shRNAs (shDNM2) suppressed DNM2 expression in BV173 cells. (B) The effects of DNM2 suppression on phosphorylation and protein levels of BCR-ABL, JAK2, STAT5, AKT and ERK. (C) and (D) 48-hour cell viability and apoptosis assays in SHC or DNM2-knockdown BV173 cells treated with 1 μM IM, 100 nM DA or 0.5 μM MitMAB alone or in combination. Values shown are the mean ± SEM of triplicate measurements. *=p<0.05, **=p<0.01, ***= P<0.001 (student t test).
5.2.2 Stable suppression of DNM2 impairs the survival of CML stem and progenitor cells and sensitizes these cells to TKI treatments

To investigate the biological effects of DNM2 suppression in primary CD34+ CML stem/progenitor cells from IM-nonresponders, a newly developed lentiviral vector that contains a GFP marker under the control of the SFFV promoter was used, since the lentiviral constructs used for K562 and BV173 cells were not able to transduce primary CML cells. Two new viral constructs containing the same sequence of SHC or shDNM2b as was used for DNM2 knockdown in CML cell lines (referred to as shDNM2 thereafter) were cloned and then used for producing new lentiviruses (Figure 5.3A). Using this system, suppression of DNM2 was found to be around 90% in CD34+ cells obtained from three IM-nonresponders, which was verified by western blotting analysis (Figure 5.3B-D). Compared to SHC controls, DNM2-suppressed CD34+ CML cells cultured for a period of 7 days showed limited proliferative potentials both in the presence or absence of IM (Figure 5.3E-G). DNM2 knockdown severely decreased the viability of CD34+ CML cells (54%, averaged from three CML samples) compared to SHC control cells, and the effects were further enhanced upon exposure to IM (32% vs. 70%) or DA (38% vs. 74%, Figure 5.3H-J). CD34+ CML cells with DNM2 depletion also exhibited higher apoptosis than SHC control cells (63% vs. 32%), and these effects were further enhanced in these cells with IM (70% vs. 43%) or DA treatments (65% vs. 44%, Figure 5.3K-M).

Furthermore, DNM2 suppression strongly reduced the colony-forming ability of CD34+ CML cells by 38% on average relative to SHC control cells, assessed by CFC assays, and the
effects were more prominent with IM or DA treatments (Figure 5.4A-C). More importantly, the results from LTC-IC assays demonstrated that DNM2 depletion severely impaired the long-term colony growth of more primitive CML stem/progenitor cells (63%, averaged from two primary CML samples) relative to control cells, and further sensitized them to IM (28% vs. 49%) or DA (32% vs. 55%, Figure 5.4D-E). Consistent with the cell line work, MitMAB treatments in primary CML samples mimicked the biological consequences caused by DNM2 knockdown. Growth monitoring of CD34+ CML cells transduced with a SHC control vector demonstrated that MitMAB-mediated proliferative inhibition occurred in a dose-dependent manner (Figure 5.4F). Single MitMAB treatments decreased cell viability and impaired colony-forming ability of CD34+ CML cells, and the effects were further amplified upon combined treatments of MitMAB and IM (Figure 5.4G & H). Taken together, genetic and pharmacological suppression of DNM2 had a detrimental effect on the survival of CD34+ CML cells in in vitro cultures, suggesting that concurrent inhibition of DNM2 and BCR-ABL might be a more effective measure to target rare leukemic stem cell populations.
Figure 5.3. Biological effects of DNM2 knockdown in CD34+ CML cells from IM-nonresponders. (A) Schematic of the same targeting sequences of SHC or shDNM2b with a U6 promoter were cloned into a new viral vector containing the SFFV promoter. (B) - (D) Western blotting analysis confirmed that DNM2 knockdown was successful in CD34+ CML cells from three patient samples. (E) - (G) Seven-day monitoring of cell proliferation of SHC or DNM2-knockdown CD34+ CML cells in the absence or presence of 5 μM IM. (H) - (J) 72-hour cell viability assays in these cells in the absence or presence of 5 μM IM or 150 nM DA. (K) -
(M) 72-hour cell apoptosis assays in the same cells in the absence or presence of 5 µM IM or 150 nM DA.

Figure 5.4. Lentiviral-mediated knockdown of DNM2 impairs the survival of CD34+ CML stem and progenitor cells and sensitizes these cells to TKIs. (A) - (C) Numbers and types of colonies produced by SHC or DNM2-knockdown CD34+ CML cells in semi-solid culture medium in the absence or presence of 5 µM IM or 150 nM DA. (D) - (E) CFC outputs of SHC or DNM2-knockdown CD34+ CML cells cultured for 6 weeks in the presence of stromal cells with or without 5 µM IM or 150 nM DA treatments. (F) Seven-day monitoring of cell proliferation of SHC CD34+ CML cells in the absence or presence of MitMAB with indicated doses. (G) 48-hour cell viability assays in SHC CD34+ CML cells treated with indicated inhibitors, either alone or in combination. (H) Numbers and types of colonies produced by SHC CD34+ CML cells in semi-solid culture medium with treatments of indicated inhibitors, either alone or in combination. Values shown are the mean ± SEM. * = p<0.05 (student t test).
5.2.3 Inhibition of DNM2 or BCR-ABL affects transferrin uptake in BCR-ABL⁺ and primitive CML cells

Previous studies have demonstrated DNM2 as a key component of endocytosis and the endocytic pathway [335, 362, 364, 365]. Therefore, I sought to investigate whether DNM2 may also regulate endocytosis in CML cells. Endocytosis of BCR-ABL⁺ cells was evaluated using the transferrin uptake assay. Transferrin is an extracellular protein that transports iron into cells. When loaded with iron, transferrin undergoes receptor-mediated endocytosis to bring iron inside cells [438]. The transferrin uptake assay is often used to assess the efficiency of endocytosis based on the amount of transferrin that accumulates inside the cells [439, 440]. Compared to SHC controls, DNM2 knockdown significantly reduced the accumulation of transferrin in K562 cells by 40%, suggesting that DNM2 participates in the regulation of endocytosis in CML cells (Figure 5.5A & B). Similarly, BV173 or CD34⁺ CML cells with reduced DNM2 expression exhibited significantly impaired transferrin uptake abilities compared to control cells (43% or 76%, respectively, Figure 5.5C - F). Interestingly, addition of IM was also able to diminish transferrin uptake in BV173 and CD34⁺ CML cells by 56% and 62%, respectively, suggesting that the endocytic process may be controlled by the BCR-ABL kinase activity. Moreover, BV173 and CD34⁺ CML cells with DNM2 knockdown displayed the greatest reduction of intracellular transferrin signals (Figure 5.5C - F). These findings suggested that DNM2 may be directly involved in modulating endocytosis in CML stem/progenitor cells. More importantly, this study provided the first evidence that the BCR-ABL kinase activity is implicated in the
endocytic process in CML cells.

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% Transferrin uptake (relative to K562 SHC)

K562 SHC  K562 shDNM2b  K562 shDNM2d

N=3

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D

% Transferrin uptake (relative to BV173 SHC)

![Graph showing % Transferrin uptake](image)

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Figure 5.5. Suppression of DNM2 or/and inhibition of BCR-ABL affects transferrin uptake in BCR-ABL+ cells. (A) SHC control and two DNM2-knockdown K562 cell lines were stained with 15 μg/ml Alexa Fluor 647-conjugated transferrin at 37°C for 30 minutes. (B) Intracellular transferrin signals were quantified from three independent experiments, which were normalized to the signals detected in K562 SHC cells. (C) SHC control and DNM2-knockdown BV173 cells were treated with or without 1 μM IM for 24 hours. Cells then stained with 25 μg/ml Alexa Fluor 647-conjugated transferrin at 37°C for 30 minutes. (D) Intracellular transferrin signals were quantified from three independent experiments, which were normalized to the signals detected in BV173 SHC cells. (E) SHC control and DNM2-knockdown CD34+CML cells were treated with or without 5 μM IM for 24 hours. Cells were then stained with 25 μg/ml Alexa Fluor 647-conjugated transferrin at 37°C for 30 minutes. (F) Intracellular transferrin signals were quantified from two independent experiments, which were normalized to the signals detected in CD34+CML SHC cells. The white scale bar represents 50 μm. Values shown are the mean ± SEM. * = p<0.05 and ** = p<0.01 (ANOVA)

5.2.4 DNM2 suppression in CML cells results in restoration of CXCR4 expression and enhances cell migration

It has been well established that decreased surface levels of CXCR4 in CML stem/progenitor cells result in defective adhesion and egress of immature cells from the BM niche [178]. The BCR-ABL kinase activity has been linked to downregulation of CXCR4 transcription in CML cells [178]. However, whether there are other mechanisms that could affect surface CXCR4 expression at the protein level is unknown. Previous studies have shown that endocytosis of CXCR4 for lysosomal degradation is one way of regulating the surface expression of CXCR4 [441, 442]. Since it was demonstrated the DNM2 modulates endocytosis in CML cells in Section 5.2.3, I hypothesized that highly-expressed DNM2 in CML cells could cause a reduction of surface CXCR4 by mediating internalization of this receptor.

To test this hypothesis, BV173 and CD34+CML cells with DNM2 knockdown were used to assess the surface levels of CXCR4 by flow cytometry. Compared to SHC controls, knockdown
of DNM2 in BV173 cells significantly increased CXCR4 surface expression by 3 fold (Figure 5.6A & C). BV173 SHC cells exposed to IM also exhibited 3.5 times higher surface expression of CXCR4, which agreed with previous findings that IM enhances the surface levels of CXCR4 [167, 178]. Moreover, the CXCR4 surface level was augmented by about 4 fold in DNM2-knockdown cells with IM treatment (Figure 5.6A & B). Additionally, in transwell/migration assays, BV173 cells with DNM2 knockdown or IM treatment showed significantly elevated migratory tendency to media containing CXCL12, the cognate ligand of CXCR4, in comparison to SHC controls (3.3 or 2.7 fold, respectively), and the cells with both DNM2 knockdown and IM treatment exhibited the highest mobility towards CXCL12 (4.2 fold, Figure 5.6E). These experimental observations correlated well with the differential surface levels of CXCR4 expression as measured by flow cytometry in Figure 5.6A and B. Furthermore, with respect to SHC controls, DNM2 suppression in CD34+ CML cells also resulted in enhanced CXCR4 surface expression (1.2 fold, Figure 5.6C & D). These results were consistent with previous reports that BCR-ABL suppresses CXCR4 expression in CML cells and also suggested that DNM2 may be another regulator of surface CXCR4 in CML cells.
Figure 5.6. Upregulation of the CXCR4 surface levels by suppression of DNM2 or IM treatment in BV173 and CD34⁺ CML cells. (A) A representative example of a FACS histogram of the surface CXCR4 expression in SHC and DNM2-knockdown BV173 cells with or without 1 μM IM treatment for 24 hours. (B) The expression of surface CXCR4 was quantified from three independent experiments, which were normalized to the CXCR4 expression in BV173 SHC cells. (C) A representative example of a FACS histogram of the surface CXCR4 expression in SHC and DNM2-knockdown CD34⁺ CML cells. (D) The levels of surface CXCR4 expression were quantified from two patient samples, which were normalized to the CXCR4 expression in CD34⁺ CML SHC cells. (E) The migratory tendency of BV173 SHC and DNM2-knockdown cells towards CXCL12 was tested in the transwell setting. The cells were first treated with or without 1 μM IM for 24 hours. Then, the cells were separated by a porous transwell insert from the serum-low RPMI 1640 media containing 500 nM CXCL12 and incubated at 37° C for 4 hours. The numbers of the cells that migrated to the bottom chambers were counted. Three independent transwell/migration assays were performed. Values shown are the mean ± SEM. * = p<0.05, ** = p<0.01 and *** = p<0.001 (ANOVA).

5.2.5 Suppression of DNM2 or BCR-ABL kinase activity affects ROS production in BCR-ABL⁺ and primitive CML cells

In CML cells, overproduction of reactive oxygen species (ROS) causes genomic instability, which has been recognized as one of the main causes of disease progression to the advanced phase [202, 208, 209]. Recently, one study has demonstrated the ability of DNM2 to potentiate ROS production in lung epithelial cells [342]. Given these two pieces of information, I was interested to investigate whether DNM2 could be one of the sources of ROS overproduction in CML cells.

To address this question, I stained different types of BCR-ABL⁺ cells using the CellROX deep red reagent that turns red upon ROS oxidization. Compared to SHC control cells, knockdown of DNM2 using shDNM2b and shDNM2d in K562 cells significantly reduced ROS production by 71% and 50%, respectively (Figure 5.7A & B). The reduction of ROS production
was also observed in BV173 and CD34+ CML cells with DNM2 suppression (26% and 41%, respectively) (Figure 5.7C - F). Furthermore, IM treatment significantly attenuated ROS production in BV173 cells and CD34+ CML cells compared to SHC controls by 65% and 70%, respectively, which agreed with previous findings that BCR-ABL functions as a main driver of ROS overproduction in CML cells. Notably, the lowest intracellular ROS accumulation was observed in DNM2-knockdown BV173 and CD34+ CML cells exposed to IM (9% and 20%, respectively) (Figure 5.7C - F). These results suggested that DNM2 could be another contributor of ROS overproduction in CML cells, and concurrent inhibition of DNM2 and BCR-ABL can further reduce ROS production.
5.2.6 DNM2 suppression affects the activation of autophagy in CML cells

Given the fact that DNM2 has recently been shown to play a direct role in regulating autophagic flux [366, 443, 444] and both endocytosis and ROS production can promote the activation of autophagy [247, 445-447], I was prompted to determine the biological influences of DNM2 on autophagy. Western blot analysis was carried out on four key autophagy proteins, ULK-1, Beclin-1, LC3-I/LC3-II and p62, in BCR-ABL+ cell lines and primary CD34+ CML cells under normoxic and nutrition-rich conditions. ULK-1 and Beclin-1 are required for initiation of autophagosomes, so the upregulation of ULK-1 and Beclin-1 are expected during activated autophagy [242]. Conversely, p62, functioning as a chaperone protein, recruits and targets ubiquitinated proteins to autophagosomes, and undergoes degradation within autolysosomes with these polyubiquitin-tagged proteins [237]. Therefore, high autophagic flux is usually accompanied by a decrease in p62 protein levels (Figure 5.8A). The levels of LC3-II
can be observed to be upregulated or downregulated depending on the stage of the autophagic flux, wherein LC3-II accumulates during the formation of autophagosomes but degrades at the late stage of the autophagy process [236, 448]. Therefore, the protein levels of LC3-II, combined with p62, serve as a better marker to interpret the stages of autophagic flux and whether autophagy is activated or inhibited. Compared to SHC control cells, K562 transduced with shDNM2b and shDNM2d exhibited decreased ULK-1, Beclin-1 and LC3-II, while strong and mild increased levels of p62 were observed in shDNM2b and shDNM2d cells, respectively (Figure 5.8B). Similarly, relative to SHC control cells, suppression of DNM2 in BV173 and CD34+ CML cells caused a reduction of ULK-1, Beclin-1 and LC3-II but accumulation of p62 (Figure 5.8B & C). These findings showed strong correlation with the changes observed in endocytosis and ROS production when DNM2 was depleted in BCR-ABL+ cells (Section 5.2.3 & 5.2.5). These data suggested that DNM2 may participate in maintaining the autophagic process under non-stimulated conditions in CML cells, possibly by directly functioning as an autophagy mediator and/or through upregulating endocytosis and ROS production.
Figure 5.8. The effects of suppression of DNM2 on key autophagy regulators in CML cell lines and primary CD34+ CML cells. (A) Schematic overview of changes of ULK-1, Beclin-1, LC3-II and p62 that correspond to the activation or suppression of autophagy. (B) Western blotting analysis of ULK-1, Beclin-1, LC3-II and p62 under the influence of DNM2 suppression in BCR-ABL+ cells. (C) Western blotting analysis of ULK-1, Beclin-1, LC3-II and p62 in SHC and DNM2-knockdown primary CD34+ CML cells.
5.3 Discussion and future directions

Recently, an increasing body of evidence has indicated that deregulated DNM2 is implicated in development and progression of many types of malignancies. In solid tumors, the deregulation of DNM2 activity exhibits strong associations with metastatic and invasive phenotypes, largely attributed to its mediation of cell mobility and migration through directly regulating the kinetics of focal adhesion turnover and actin cytoskeleton dynamics in the formation of membrane protrusions [371-375]. In hematopoietic malignancies, the exact pathological roles of DNM2 have not yet been well characterized. A few studies suggested that in T cell leukemias, the defective DNM2 due to mutations or the downregulation of DNM2 leads to inactivation of T cells so that the impaired immune surveillance fails to detect and eliminate neoplastic formation [378, 379].

In this present study, I demonstrated an oncogenic role of DNM2 in CML by showing that DNM2 knockdown caused inhibition of cell growth, induction of apoptosis, and increased sensitivity to TKI treatments in various BCR-ABL+ cell lines and pre-treatment CD34+ cells from IM-nonresponders. Moreover, similar phenotypes were recapitulated in these cells upon MitMAB treatments, suggesting that DNM2 inhibition is detrimental to the survival of CML cells. Interestingly, DNM2 suppression greatly impaired the colony-forming ability of CD34+ CML cells in short- and long-term culture assays, and the effects were further enhanced in the presence of TKIs. These results highlighted a new role of DNM2 in maintaining survival and contributing to TKI resistance CML stem/progenitor cells. Therefore, it may be worth
considering a concurrent inhibition of DNM2 and BCR-ABL as a more effective approach to target rare leukemic stem cell populations.

One intriguing question is whether the oncogenic activities of DNM2 are BCR-ABL-dependent or independent. On one hand, given the findings that BCR-ABL phosphorylates DNM2 in the BAD complex in BCR-ABL+ cells (Section 4.2.8), it is tempting to speculate that BCR-ABL is involved in regulating DNM2 activity since tyrosine phosphorylation is critical for DNM2 activation [343, 344]. On the other hand, previous studies have shown that JAK2 and the Src family kinases Src, Lyn and Hck are all able to phosphorylate/activate DNM2, and these tyrosine kinases all show abnormally high activities in CML cells [449, 450]; therefore, it is likely that these kinases might also participate in mediating or increasing DNM2 activity. Hence, I propose that BCR-ABL (as shown in my thesis) and other tyrosine kinases including the abovementioned could all or partially contribute to phosphorylation-mediated DNM2 activation, presumably leading to oncogenic DNM2 with aberrantly high activity. In order to examine this hypothesis in more details, the following experiments could be considered. Firstly, DNM2-knockdown BCR-ABL+ cells could be transduced with a non-phosphorylatable DNM2 mutant (Y231F/Y597F) [343, 344, 357], followed by testing whether these cells can exhibit similar phenotypes as SHC control cells without DNM2 suppression. The experimental results would confirm if tyrosine phosphorylation of DNM2 (by BCR-ABL and/or other kinases) is necessary for the DNM2’s oncogenic ability in CML cells. Secondly, one could treat BCR-ABL+ cells with specific
inhibitors such as Src family kinase inhibitors or JAK2 inhibitors [451, 452]. In these cells, co-IP assays like what was described in Section 4.2.8 could be performed to evaluate if any of these inhibitors cause a reduction in DNM2 phosphorylation, which could possibly lead to identification or rejection of other tyrosine kinases besides BCR-ABL, which are involved in DNM2 phosphorylation.

The next question I investigated was how DNM2 may exert its oncogenic effects on CML cells at the molecular level. It was shown that DNM2 deletion resulted in decreased endocytosis, ROS production and autophagy in BCR-ABL+ K562, BV173 and CD34+ CML cells, which suggested that DNM2 is involved in mediating these three essential cell activities in CML stem/progenitor cells. Given the fact that DNM2 is one of the key components in the endocytic machinery, it is not surprising that DNM2 also regulates the endocytic process in CML cells. Based on the recently identified roles of DNM2 in ROS production and autophagy [342, 366, 443, 444], I also linked DNM2 with these two cellular functions in CML cells. Possible mechanisms of how DNM2 may participate in ROS production and autophagy activation will be discussed later in this section.

In CML, uncontrolled BCR-ABL kinase activity has been shown to affect many cellular activities including cell division, proliferation, apoptosis, differentiation and adhesion/migration [79, 453]. However, to my knowledge, the endocytic process is one of few cellular functions that have not been directly linked to CML pathogenesis. In this study, the experimental finding of IM reducing transferrin uptake in K562, BV173 and CD34+ CML cells provided the first
evidence that the BCR-ABL kinase activity is implicated in the endocytic process of CML cells, potentially causing deregulation of this cellular process (Figure 5.5). In the past two decades, a growing body of evidence has implicated the deregulation of endocytosis with cancer development and progression. The deregulated endocytosis-mediated defective trafficking of surface receptors, increased kinetics of focal adhesion turnover and excessive degradation of cadherin-based intercellular junctions have been recognized as hallmarks of tumor cells [346, 454, 455]. For CML, since this is the first study that links the BCR-ABL kinase activity to endocytosis, mechanistically, little is known about how BCR-ABL affects endocytosis. Based on some findings in this study, I propose that one possible way for BCR-ABL to control endocytosis is through phosphorylation-mediated activation of DNM2, the essential protein in membrane fission. An example that might provide some evidence to support this hypothesis, albeit weak, is the illustrated internalization of CXCR4 in CML cells (Section 5.2.4)

Defective bone marrow adhesion is a major cause of bone marrow egress of immature CML blast cells, and BCR-ABL-mediated impairment of CXCL12-CXCR4 is believed to be one of the underlying mechanisms that contribute to this phenomenon [178]. It has been shown that the BCR-ABL kinase activity is directly linked to the downregulation of CXCR4 transcripts, whereas TKI treatments result in restoration of CXCR4 surface expression in CML cells, which causes migration of leukemic cells to the protective CXCL12-enriched bone marrow [167, 178]. In this study, I have shown that compared to SHC controls, DNM2 knockdown BV173 cells exhibited significantly higher surface levels of CXCR4 (Figure 5.6A & B), suggesting that
DNM2 is directly involved in endocytosis of CXCR4 in BCR-ABL+ cells. In addition, it was observed that IM was also able to increase the surface expression of CXCR4 (Figure 5.6A & B), which agrees with previous findings that IM inhibits the BCR-ABL kinase activity, leading to restoration of CXCR4 expression [167]. However, given that BCR-ABL might mediate DNM2 activity by phosphorylation, there could be another possible mechanism that contributes to IM-mediated elevation of surface CXCR4 expression: IM inhibits the BCR-ABL kinase activity, which causes reduction of phosphorylation-mediated DNM2 activation, thereby resulting in decreased internalization of CXCR4. I understand that the data I present in this study does not provide enough evidence to support the second data interpretation. Here, I simply present another possibility that BCR-ABL might be able to suppress CXCR4 expression at the protein level through endocytosis. Future work will be needed to verify this hypothesis. In general, since this study suggested the endocytic process might be deregulated in CML, future studies could focus on determining whether other endocytic mediators are also deregulated in CML cells. As the functional roles of more endocytic proteins are revealed in CML, we will have a better idea of how deregulated endocytosis contributes to CML leukemogenesis.

In the past two decades, many studies have demonstrated that elevated ROS production causes increased oxidative DNA damage and genomic instability, leading to TKI resistance and malignant progression to the fatal blast crisis phase in CML [197, 199-202]. In this thesis, it was shown that DNM2 might contribute to ROS overproduction in CML cells as DNM2 suppression significantly attenuated ROS accumulation inside BCR-ABL+ cells (Figure 5.7). One possible
mechanism of DNM2 potentiating ROS production in CML cells might be through facilitating the assembly of NADPH oxidase. NADPH oxidase (Nox), especially Nox2 and Nox4, is recognized as another source of ROS in BCR-ABL+ cells [207, 456]. Unlike mitochondria that produce ROS as by-products, the primary function of Nox family proteins is to generate ROS. In CML cells, the upregulated Nox positive regulators p22^{phox} and p47^{phox} promote the activation of Nox family members, resulting in ROS overproduction [457, 458]. Furthermore, Nox2 was earlier found to regulate the redox milieu of TKI-resistant CML cells [456]. Moreover, genetic or pharmacological inhibition of Nox strongly decreased the viability of CML cells in vitro and tumor load in vivo, and the effects were further enhanced when combined with TKIs [213, 458]. Interestingly, one study from Singleton et al. presents biological connections between DNM2 and the NADPH oxidase complex [342]. In lung endothelial cells, activated DNM2 by c-ABL phosphorylation under hypoxic conditions promotes the activation of NADPH oxidase through recruiting key components required for the formation of the NAPDH oxidase complex, leading to ROS production. During CML leukemogenesis, a similar mechanism might be exploited by leukemic cells, but with a lot less conditional restraints. In CML cells, under physiological conditions, the highly expressed DNM2 has greater chances to interact with and get phosphorylated/activated by BCR-ABL and/or other abovementioned tyrosine kinases that possess abnormally elevated activities. The activated DNM2 could in turn strongly promote the NADPH oxidase activity and ROS generation. To test this hypothesis, the activity of the NADPH oxidases by using lucigenin-derived chemiluminescence assay [459] could be
compared between DNM2-knockdown CML and control cells. Moreover, it would be interesting to determine if DNM2 suppression impairs the formation of NADPH oxidases by testing whether DNM2 suppression affects translocation of Nox regulatory subunits such as p47phox from cytosol to the plasma membrane and co-localization of these regulatory subunits with the integral transmembrane protein gp91phox [460, 461].

It has been reported that TKI treatments trigger the activation of autophagy, which functions as a pro-survival mechanism for CML cell to withstand TKI-induced cytotoxicity [254, 255]. Therefore, it has been proposed that the concomitant treatment of CML with autophagy inhibitors and TKIs could be a more effective means to minimize TKI resistance [257-260]. In the past five years, the regulatory roles of DNM2 in several key steps of the autophagy process have been uncovered, which include autophagosome maturation and lysosome regeneration [366, 443, 444]. Therefore, it is likely that highly expressed DNM2 may directly enhance autophagy in CML cells. It is also worth noting that autophagy is affected by both endocytosis and ROS signaling. The endocytic pathway has been shown to have a profound impact on efficient autophagic flux through (1) being one of the sources of the autophagocytic membrane, (2) providing the critical components (e.g. ULK-1 and Beclin-1) of the essential regulatory protein complexes and (3) delivering lysosomal enzymes such as hydrolases, permeases and ATPase for lysosomal enzymes [446, 447]. The interplay between ROS and autophagy has been intensively studied in recent years. ROS function as signaling molecules to stimulate autophagy through transcriptional activation of key regulators participating in the autophagosome
formation. Activated autophagy, in contrast, suppresses ROS production in order to eliminate the deleterious oxidative effects by removing damaged mitochondria [247, 445]. Hence, DNM2 may also have an indirect impact on the autophagic process through regulating endocytosis and ROS production in CML. It was observed that DNM2 suppression inhibited autophagy in K562, BV173 and primary CML cells, evidenced by reduced ULK-1, Beclin-1 and LC3-II but accumulated p62 (Figure 5.8B & C). These findings highly correlated with the changes observed in endocytosis and ROS production when DNM2 was suppressed (Section 5.2.3 & 5.2.5), which supports the idea that DNM2 could directly and/or indirectly affect autophagy in CML cells. Our lab has recently shown that the expression levels of key autophagy mediators such as ATG4, ATG5 and Beclin-1 are significantly increased in primitive CML cells compared to normal bone marrow cells [253]. Furthermore, it was also shown that basal autophagy was essential for BCR-ABL-mediated leukemogenesis [462]. These studies suggest that the level of baseline autophagy in primitive CML cells is higher than that of their normal counterparts and is required for CML maintenance. My study thus provides new evidence that DNM2 may be one of the proteins that maintain relatively higher basal levels of autophagy in primitive CML cells.

In conclusion, this study established an oncogenic role of DNM2 in CML stem/progenitor cells and demonstrated that DNM2 suppression severely impaired survival of primitive CML cells. Furthermore, this study suggested that DNM2 may exert its oncogenic effects by deregulating three essential biological functions - endocytosis, ROS production and autophagy.
Chapter 6: General Summary and Future Directions

6.1 Summary and significance

Chronic myeloid leukemia used to be a lethal hematopoietic malignancy, and patients with this disease inevitably faced death within 3-5 years [463]. The advent of TKIs has greatly changed people’s perspectives about CML, and it is now considered a manageable disorder instead of a deadly disease. However, drug resistance and early disease relapse have emerged as significant clinical setbacks. Hence, we are still facing a challenging journey before achieving the ultimate goal of finding the cure for CML. In the past 15 years, a great deal of research has been conducted to further elucidate BCR-ABL-dependent mechanisms that contribute to CML leukemogenesis and BCR-ABL-independent factors that protect primitive CML cells, particularly CML stem cells for the survival of TKI treatments. My work here presents the structural and functional characterization of the AHI-1 SH3 domain, identification of a new AHI-1-mediated protein complex containing BCR-ABL and DNM2 and the oncogenic roles of DNM2 in CML, which may address some unsolved problems and provide new clues to future studies.

In Chapter 3, I investigated the biological functions of the AHI-1 SH3 domain and the unique structural features of this domain. Firstly, it was shown that the AHI-1 SH3 domain played an important role in sustaining CML cellular survival and was involved in mediating TKI response/resistance. Secondly, the crystal structure of the AHI-1 SH3 domain revealed that this
domain: (1) may contain a fourth binding pocket due to the unusual C-terminal helix, (2) has three large negatively charged patches scattered on the surface, and (3) a His residue instead of Tyr or Phe located in the center of the binding interface, which may all affect ligand binding affinity and specificity. Using the PD1R peptide, known to interact with the PI3K SH3 domain, I modeled the binding pattern between the AHI-1 SH3 domain and its ligands, which showed that an “Arg-Arg-Trp” stack may form within the binding interface. Thus, the crystal structure of the AHI-1 SH3 domain provides a valuable tool for identification of key interaction sites in the regulation of drug resistance and for the development of possible small molecule inhibitors for CML and other AHI-1-related diseases in the future.

Chapter 4 focuses on investigation of molecular determinants of the interaction between AHI-1 and DNM2 and characterization of a BAD protein complex formed by BCR-ABL, AHI-1 and DNM2. This study started with the identification of DNM2 as a new AHI-1 interacting partner in the affinity purification/mass spectrometry setting, and showed that DNM2 expression was significantly increased in primary CML cells, especially in the stem/progenitor population. Furthermore, the interaction between AHI-1 and DNM2 was found to be mainly dependent on the SH3-PRD recognition and seemed to occur in endosomal compartments. Next, in silico screening and modeling were exploited to determine the possible PXXP motif within the DNM2 PRD as the putative binding site of the AHI-1 SH3 domain. Lastly, I demonstrated that AHI-1 recruited DNM2 to BCR-ABL to form the BAD protein complex, within which BCR-ABL may phosphorylate DNM2, presumably regulating DNM2 activity. Through this study, a new
AHI-1-mediated protein complex was discovered and another BCR-ABL target - DNM2 was identified, which could help to further decipher BCR-ABL signaling networks in CML.

Chapter 5 details the characterization of the oncogenic functions of DNM2 in primitive CML cells and exploration of possible mechanisms of DNM2 exerting its oncogenic effects. It was shown that knockdown of DNM2 in BCR-ABL\(^+\) and TKI-insensitive CD34\(^+\) stem/progenitor cells led to reduced survival, increased apoptosis, and the impaired ability of these cells to produce colonies, and all of these effects were enhanced with TKI treatments. These results established that DNM2 played an oncogenic role in CML stem/progenitor populations and exhibited that concurrent inhibition of BCR-ABL and DNM2 may be a potential treatment strategy to target more primitive CML cells. Moreover, stable suppression of DNM2 strongly affected endocytosis, ROS production and autophagy in BCR-ABL\(^+\) cell lines and CD34\(^+\) CML stem/progenitor cells. These findings highlighted the significance of DNM2 in the regulation of endocytosis, ROS production and autophagy in CML, suggesting that DNM2 could be considered as an interesting therapeutic target in CML.

### 6.2 Future directions

Given the biological significance of the AHI-1 SH3 domain in contributing to TKI resistance in CML cells, targeting of the AHI-1 SH3 domain by dissociating the SH3 domain-mediated interaction between AHI-1 and its binding partners could be a potential approach to minimize TKI resistance and eliminate primitive CML cells. With the crystal
structure of this domain now available, it would be feasible to design AHI-1 SH3 domain inhibitors with high affinity and specificity. Solving the crystal structure of the AHI-1 SH3 domain complexed with its ligand in future experiments would help to provide more accurate structural details of the binding interface of AHI-1 SH3 domain-mediated protein interactions. The peptide ligand, derived from the DNM2 PRD with verified high affinity and specificity to the AHI-1 SH3 domain (see Discussion in Chapter 4), could be used as a binding partner in such co-crystallization experiment.

Although targeting the SH3 domain is in general a challenging task, unique structural features of the AHI-1 SH3 domain identified in this study could make it possible to develop promising inhibitors. It is possible that “PXXP” contained inhibitory peptides that possess certain structural elements could specifically bind to and target the AHI-1 SH3 domain. Firstly, a “compass” Arg residue ought to be included in the inhibitory peptide since the potential Arg-Arg-Trp hydrophobic stack can greatly enhance the binding affinity. Secondly, more attention should be focused on designing elements that interact with the unusual P4 pocket that is absent in most other SH3 domains. Therefore, targeting this site could confer an enhanced binding specificity of the inhibitory ligand to the AHI-1 SH3 domain. Recently, it was reported that Rhodium (II) conjugates on peptide ligands drastically increased the binding affinity between the metallopeptides and Src family as well as Abl kinases (Kd: 10 – 80 nM) due to complex formation of Rhodium and histidine residues near the SH3 binding interface [464]. Since there is a His residue in the center of the binding interface and sets the boundary between
the P1 and P2 pockets in the AHI-1 SH3 domain, Rhodium could be conjugated within the “PXXP” core motif of potential inhibitory peptides to further increase both binding affinity and specificity. If certain peptides contain all these structural characteristics and fit well into the binding interface, which can be verified based on the information obtained from the co-crystal structure, they would hold promise to act as strong inhibitors to disrupt the interactions between the AHI-1 SH3 domain and its natural ligands. Subsequently, the synthesized peptide inhibitors of the AHI-1 SH3 domain could be selected and tested for direct binding to the AHI-1 SH3 domain in biochemical assays such as isothermal titration calorimetry or microscale thermophoresis. The candidate inhibitors would then be tested to see if they have therapeutic potentials in primitive CML cells in the presence or absence of TKIs or other relevant inhibitors both in vitro and in vivo.

In addition, it has been shown that DNM2 was directly implicated in the deregulation of endocytosis, ROS production and autophagy in CML stem/progenitor cells, and DNM2 suppression significantly impaired the survival of primitive CML cells. These findings suggested DNM2 is an interesting, potential target in CML treatment. To validate that, it will be necessary to test the toxicity of DNM2 inhibition in normal BM cells, e.g. by MitMAB treatment or other DNM2 inhibitors. Using this information, a therapeutic window between normal and CML cells could be established. Furthermore, it would be intriguing to evaluate the significance of DNM2 in inducing CML or a CML-like disease in mice in vivo. Investigation of the disease latency of conditional DNM2-knockout mice in the hematopoietic system in a
BCR-ABL transgenic background (i.e. DNM2\(^{-/-}\)/BCR-ABL\(^{+}\) mice) would illustrate whether DNM2 is needed to induce and maintain CML. These mouse models have been shown to be valuable in establishing the oncogenic functions of Wnt and sonic hedgehog signaling pathways in CML leukemogenesis [156-159]. Alternatively, comparing the ability of control vs. DNM2-knockdown CML stem/progenitor cells to engraft immunodeficient mice could shed light on whether DNM2 plays a role in sustaining the long-term repopulating property of the more rare leukemic stem cells, since this model does not induce a lethal disease in mice [465, 466].

To investigate additional details of the oncogenic roles of DNM2, transcriptome/proteome profiling analysis of DNM2-knockdown CD34\(^{+}\) CML cells in comparison to control cells could be performed. By RNA-seq (RNA sequencing) analysis and CyTOF (Cytometry by Time of Flight), the altered expression of specific genes/proteins and abnormal signaling pathways caused by DNM2 deregulation could be identified, which would greatly help to further elucidate the underlying mechanisms through which DNM2 contributes to CML leukemogenesis.

Based on the results from the transferrin uptake assay, a speculation was made that the upregulated DNM2 and/or BCR-ABL might potentiate the endocytosis process in CML cells. However, whether the kinetics of endocytosis is increased in CML cells compared to normal cells is still not fully defined. Therefore, it would be useful to compare transferrin uptake between normal BM and primary CD34\(^{+}\) CML cells.

Lastly, one interesting observation in this study was that the overexpression of full-length
Ahi-1, but not the Ahi-1 SH3Δ mutant in BCR-ABL-transduced BaF3 cells, significantly increased transferrin uptake, ROS production and autophagy as compared to parental BCR-ABL-transduced BaF3 cells (Figure A.17 – 19 in Appendix). These results suggested that AHI-1 is also implicated in regulating endocytosis, ROS production and autophagy activation, probably through its SH3 domain. Now, given the fact that all three components of the BAD complex – BCR-ABL, AHI-1 and DNM2 are involved in endocytosis, ROS production and autophagy activation in CML cells, it is tempting to speculate that the AHI-1-mediated BAD protein complex can contribute to deregulation of all these essential cellular functions in CML cells. In order to test this hypothesis, various functional assays could be performed on multiple BCR-ABL inducible BaF3 cell lines co-transduced with different AHI-1 and DNM2 constructs, such as cells co-expressing full-length AHI-1 and DNM2 and cells co-transduced with full-length AHI-1 and modified DNM2 containing mutations in the PRD, which disrupt the interaction between these two proteins. Other important cell lines needed to be generated are the cells co-transduced with AHI-1 mutants containing mutations within the SH3 domain combined with DNM2 mutants that have mutations in the PRD; however, these mutants are still able to interact with relatively high affinity. To achieve this, for example, the residues that make up the binding pockets of the AHI-1 SH3 domain could be mutagenized to the corresponding residues comprising the binding interface of the Grb2 SH3 domain (Figure A.20), while within the DNM2 PRD, the APPIPSR (P5) sequence, assuming that this is the putative binding site of the AHI-1 SH3 domain, could be converted to PPPVPPR (Figure 4.7B, E & F), which is the
sequence of the binding ligand of the Grb2 SH3 domain [467]. Once these cell lines are generated, various phenotypes including DNM2 phosphorylation, transferrin uptake, ROS production and changes of autophagy mediators could be compared between them. The results from these experiments would provide information on whether the interaction between AHI-1 and DNM2 is directly involved in regulating these three cellular activities in BCR-ABL+ cells. Furthermore, once additional molecular determinates of the interaction between BCR-ABL and AHI-1 are identified (e.g. co-IP assays between different domain-deleted mutants of BCR-ABL and AHI-1), similar experimental settings could be used to test the importance of this interaction in DNM2 phosphorylation and ROS, endocytosis and autophagy regulation. Combining all these data, one would have more definitive answers whether the BAD protein complex contributes to CML leukemogenesis through regulating endocytosis, ROS production and autophagy activation.

Overall, this thesis study (1) identifies DNM2 as a new interacting protein with the SH3 domain of AHI-1 and reveals the crystal structure of the AHI-1 SH3 domain; (2) identifies a novel BAD protein complex in CML cells, (3) introduces DNM2, a new BCR-ABL target, to the CML field and suggests that DNM2-mediated deregulation of endocytosis, ROS production and autophagy may contribute to CML leukemogenesis; (4) generates new information on the importance of DNM2 in regulating leukemic stem/progenitor cell survival and TKI response/resistance in CML. Hence, this study lays new groundwork for future investigations and makes a contribution to understanding the unique properties of CML stem cells and the
molecular pathogenesis of CML.
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Appendix

A1 Expression of the AHI-1 WD40-repeat domain with an N-terminal 6xHis tag or 6xHis-MBP tag in E.coli

Possible protein sequences representing the AHI-1 WD40-repeat domain were predicted by Protein Homology/analogy Recognition Engine (PHYRE). 30 constructs with 5 different start residues (583E, and 600K, 607A, 512V and 560P) and 6 different end residues (E906, D919, E926, S972, A995 and S1011) were generated (Figure A.1) and cloned into a pET28HT vector, from which the protein constructs were tagged with an N-terminal 6xHis-TEV tag, or a pET28HMT vector, from which the protein constructs were fused with an N-terminal 6xHis-MBP-TEV tag.

All 60 protein constructs were expressed in the E. coli Rosetta (DE3) pLacI strain that contains codon rarely used in E. coli. Additional 6 protein constructs starting with 583E or 600K and ending with E906, D919 or E926 were also expressed in the E. coli BL21 (DE3) pLysS strain (kindly provided by Dr. Gregg Morin lab), which has tighter control of the expression of toxic proteins. Pilot studies were set to test the protein expression of 9 constructs fused with an N-terminal 6xHis-MBP-TEV tag, starting with 583E, 600K or 607A and ending with E906, D919 or E926. The recombinant proteins were induced with 0.5 mM IPTG at 37°C for 3-4 hours in the E. coli Rosetta (DE3) pLacI strain. However, protein expression was not observed after IPTG induction for all nine constructs, indicating that these constructs were not expressed in this condition (Figure A.2A, Lane 2 as compared to Lane 1, e.g. 607A-E926 with a 6xHis-MBP-TEV tag). A new expression condition was tried for these constructs, which was to induce protein
production with IPTG at 18°C for 20 hours. A low temperature slows down protein synthesis, which can therefore help with proper protein folding for better expression and solubility [468]. Indeed, all 9 protein constructs were strongly expressed in this condition (Figure A.2A, Lane 3 as compared to Lane 2, e.g. 607A-E926 with a 6xHis-MBP-TEV tag, the expression of the recombinant protein indicated by a red arrow). Hence, 18°C for 20 hours was used as a standard expression method to express all 60 protein constructs in E. coli.
Figure A.1. Protein constructs designed for expressing the AHI-1 WD40-repeat domain in E. coli. The protein sequence of human AHI-1 is shown. The start residues of the protein constructs are colored in red, while the end residues are colored in blue.
Unfortunately, all 60 protein constructs induced at 18°C for 20 hours in two *E. coli* strains were either insoluble (Figure A.2B, recombinant proteins present in the pellet but not in the supernatant, e.g. 607-A-E^{926} with a 6xHis-MBP-TEV tag) or not expressed (Figure A.2C, no protein produced after IPTG induction, e.g. 512-V-E^{906} with a 6xHis-TEV tag). In addition, reducing the amount of IPTG from 0.5 mM to 0.05 mM, which could also help to slow down the rate of protein production [469], did not solve the issues of expression and solubility (Figure A.2D & E, e.g. 607-A-E^{926} with a 6xHis-TEV tag). Therefore, it was difficult to obtain soluble recombinant proteins of the AHI-1 WD40-repeat domain with an N-terminal 6xHis tag or 6xHis-MBP tag in *E. coli*.

In the future, one could try to extract the AHI-1 WD40-repeat domain fused with an N-terminal 6xHis tag or 6xHis-MBP tag from inclusion bodies of *E. coli* cells and test if the soluble recombinant proteins could be obtained after refolding. In addition, the AHI-1 WD40-repeat domain could be expressed as a fusion protein with other protein tags such as a GST or SUMO tag, and the expression and solubility of the resultant recombinant protein could be tested.
Figure A.2. Expression and solubility of protein constructs of the AHI-1 WD40-repeat domain in E.coli. (A) *E.coli* cells transformed with the protein construct of $^{607}$A-$^{E^{926}}$ were induced with 0.5 mM IPTG at 37°C for 3 hours or 18°C for overnight. Control cells were obtained before IPTG induction. Expression of the recombinant protein was tested by coomassie-stained SDS-PAGE. (B) *E.coli* cells were sonicated and centrifuged. The supernatant and pellet fractions were obtained and ran on SDS-PAGE. (C) *E.coli* cells transformed with the protein construct of $^{512}$V-$^{E^{906}}$ were induced with 0.5 mM overnight at 18°C. Control cells were obtained before IPTG induction. (D) + (E) *E.coli* cells transformed with the protein construct of $^{607}$A-$^{E^{926}}$ were induced with 0.5 mM (D) or 0.05 mM IPTG (E) overnight at 18°C. *E.coli* cells were then sonicated and centrifuged. The supernatant and pellet fractions were obtained and ran on a SDS-PAGE. The recombinant protein was indicated by a red arrow.
A2  Expression of the AHI-1 WD40-repeat domain in insect Sf9 cells using transient transfection

Since the AHI-1 WD40-repeat domain was not successfully produced in E. coli, insect Sf9 cells were considered as an alternative. One of the major advantages of using Sf9 cells as expression hosts is that these eukaryotic cells contain chaperone machineries that can help with proper folding of overexpressed recombinant proteins [470]. Collaborating with Dr. Jinrong Min (an expert in research of the WD40-repeat domain from University of Toronto), 13 constructs with different starts and ends were designed: 12 constructs started with 1M, 160K, 330D or 435R and ended with P940, P1116, or E1196, while another one is the protein construct 600K–E926 (Figure A.3). These constructs were cloned into a pIEX2 vector, from which the protein constructs were fused with an N-terminal GST-6xHis-thrombin tag. In order to test whether the pIEX2 vector was a valid protein expression vector, a GFP reporter gene was cloned into this vector (kindly provided by Dr. Gregg Morin lab) and transfected into Sf9 cells using the transfection agent PEI (Polehtlenimine) [471]. It was shown that after 48-hr transfection, less than 10% of the cells became GFP+ (Figure A.4), indicating that the pIEX2 vector could be used to express recombinant proteins in Sf9 cells although the transfection efficiency was relatively low.
Protein constructs designed for expressing the AHI-1 WD40-repeat domain in insect Sf9 cells. The start residues of the protein constructs are colored in red, while the end residues are colored in blue.
Figure A.4. The test of the transfection efficiency in Sf9 cells using a GFP reporter protein. Sf9 cells were transfected with a pIEX2 vector, which contained a GFP reporter gene, for 48 hours using PEI. The images of the same field view were acquired using a LEICA DMIRE2 microscope in bright field (A) or with a FITC channel (B).

All 13 constructs were transfected into Sf9 cells, and after 48 hours the expression of resultant proteins was tested in the cell lysates. The western analysis exhibited that all of the 13 protein constructs were expressed as soluble proteins (Figure A.5). Based on the expression level, I selected a total 6 out of the 13 protein constructs for protein purification, including $^{160}\text{K} - P^{940}$, $^{160}\text{K} - E^{1196}$, $^{330}\text{D} - E^{1196}$, $^{435}\text{R} - P^{940}$, $^{435}\text{R} - E^{1196}$ and $^{600}\text{K} - E^{926}$.

The first column used for protein purification was a nickel affinity column that binds to the 6xHis tag (Figure A.6A, e.g. the protein construct $^{160}\text{K} - P^{940}$). The western blotting analysis using an anti-GST antibody showed that the majority of the POI (protein of interest) was bound to the column although some were lost in the flow-through (FT) fraction (Figure A.6A, Lane 2). After the POI was eluted from the column, the pooled eluate was dialyzed to remove imidazole...
and/or salts for the subsequent purification step, either a glutathione column or an ion-exchange column. However, for 5 out of the 6 protein constructs except for 600K – E926, white particles were observed during dialysis. After centrifugation, the white pellet was separated from supernatant, and the western blotting showed that the pellet contained a large amount of the POI, which indicated that the recombinant proteins formed aggregates during dialysis and precipitated out of the solution (data not shown). Different dialysis conditions including various pHs, salt concentrations or temperatures were tested to minimize protein aggregation (Figure A.6B, e.g. the protein construct 160K – P940). However, none of them prevented the POI from precipitating. The results suggested that these protein constructs were intrinsically unstable and more prone to proteolysis; therefore, they might not be good candidates for further purification.

![Western Blot](image)

**Figure A.5. The expression of 13 protein constructs in Sf9 cells using PEI transfection.** Sf9 cells were transfected with a pIEX2 vector containing different protein constructs for 48 hours using PEI. The cells were collected, lysed and centrifuged. The supernatant was collected and tested by western blotting using an anti-GST antibody.
Figure A.6. Purification of the recombinant protein $^{160}{\text{K}} - {P}^{940}$ with a nickel affinity column and the stability test using dialysis with different pHs. (A) The protein lysate containing $^{160}{\text{K}} - {P}^{940}$ with a GST-6xHis-thrombin tag was loaded on to a nickel affinity column and three elution steps (E1, E2 and E3) using 250 mM imidazole were performed. Finally, all three elution fractions were pooled (E pool). (B) The elution pool was dialyzed against 20 mM buffers with different pHs at 23°C for 2 hours. The samples then collected from the dialysis tubing and centrifuged to separate the supernatant (sup) from the pellet. Western blotting was performed using an anti-GST antibody.
Since the protein construct $^{600K-926}_{E}$ was stable during dialysis, more efforts were devoted to this construct. The protein lysate from 500 mL of Sf9 cell culture was loaded to a nickel affinity column. After intensive washes, the POI was eluted in three elution steps (Figure A.7A). The pooled eluate was then subjected to dialysis in a buffer of 300 mM NaCl, 20 mM Tris 8.0 and 5% glycerol for 2 hours at 23°C. Importantly, no precipitation was detected during this procedure, and the POI was intact after dialysis (Figure A.7A, Lane 8). Subsequently, the POI was collected from the dialysis tubing and loaded to a glutathione column, which binds to the GST tag. Although some proteins were lost in FT, a considerable amount of the POI was still retained in the column, which was eluted in two elution steps. Since the recombinant protein could go through two affinity columns and the dialysis procedure, it might suggest that this protein construct $^{600K-926}_{E}$ was relatively stable and had proper folding. Therefore, it was presented as a good candidate for further purification.

However, the corresponding coomassie-stained gel showed that in the elution fractions of the nickel affinity column, there were still a large amount of protein contaminants (Figure A.7B), which made it difficult to identify the band that represented the POI; in the elution fractions of the glutathione column, the band of the purified protein was not detected, indicating that the amount of the eluted POI was very little (Figure A.7B). One possible reason of this might be due to low yield production of the recombinant proteins caused by low transfection efficiency as mentioned above. Since less than 10% of Sf9 cells were transfected, the majority of cells did not express the POI. Therefore, a high level of endogenous Sf9 proteins could bind to the nickel
column via non-specific binding, which resulted in a large amount of protein contaminates in the elution. Furthermore, due to the low yield of the protein production and loss of proteins during the affinity purification and dialysis, it was expected that only a small amount of the POI was obtained after the glutathione column. Hence, in order to further purify this protein construct $^{600}K - ^{926}E$, other methods of protein expression in Sf9 cells should be used.
Figure A.7. Purification of the recombinant protein $^{600}$K – $^{E926}$ with two affinity columns. (A) The protein lysate containing $^{600}$K – $^{E926}$ with a GST-6xHis-thrombin tag was loaded on to a nickel affinity column and three elution steps (E1, E2 and E3) using 250mM imidazole were performed. Finally, all three elution fractions were pooled (E pool). The elution pool was dialyzed against 300 mM NaCl, 20 mM Tris 8.0 and 5% glycerol at 23°C for 2 hours. The samples then collected from the dialysis tubing and loaded to a second glutathione affinity column. Two elution steps (E1 and E2) using 10 mM reduced glutathione were performed. Finally, the two elution fractions were pooled. Western blotting was performed using an anti-GST antibody. (B) The corresponding coomassie-stained gel to the western blotting shown in A.
A3 Expression and purification of the AHI-1 WD40-repeat domain in insect Sf9 cells using the baculovirus system

One way of obtaining a high yield of recombinant proteins in Sf9 cells is using the baculovirus expression vector system (BEVS) [472]. Baculoviruses are double-strand, supercoiled DNA molecules in a rod-shaped capsid and are the most prominent viruses to infect insect cells [473]. BEVS has been used to express many recombinant proteins with high yield and accuracy [474]. A construct containing \(600^\text{K} - E^{926}\) with the 6xHis and thrombin cleavage sequence at the N-terminal was subcloned into a pDEST20 vector, from which the \(600^\text{K} - E^{926}\) construct was fused with a N-terminal GST-6xHis-thrombin tag. Subsequently, the pDEST20 vector was used to produce recombinant baculoviruses in Sf9 cells. As compared to the uninfected Sf9 cells, the cells infected by baculoviruses carrying the recombinant constructs exhibited a larger size and decreased density (Figure A.8A & B). Most importantly, it seemed that almost all the Sf9 cells got infected by baculoviruses, which could result in high yield of the tagged \(600^\text{K} - E^{926}\) protein. Furthermore, it was shown that the POI was expressed using BEVS (Figure A.8C).
Figure A.8. The test of the infection efficiency of baculoviruses containing the recombinant protein $6^{00}K - E^{926}$ in Sf9 cells and its expression. Comparison of the morphology of Sf9 cells uninfected (A) and infected with baculoviruses containing $6^{00}K - E^{926}$ with a GST-6xHis-thrombin tag (B) after 72-hour infection. The bright field images were acquired using a LEICA DMIRE2 microscope. (C) The expression of the GST-6xHis-thrombin tagged $6^{00}K - E^{926}$ using BEVS in Sf9 cells was tested by western blotting using an anti-GST antibody.

The protein lysate from a 500 mL culture of Sf9 cells infected with baculoviruses was loaded onto a nickel affinity column. The eluate from this column then underwent dialysis before loaded onto a glutathione column. The coomassie-stained gel showed that the POI was purified and enriched after the second glutathione column (Figure A.9). Subsequently, I tried to
test whether the POI was in the monomeric state by using a size exclusion column. Two major peaks were observed on the chromatogram (Figure A.10). The coomassie-stained gel showed that the POI (indicated by a red arrow) was eluted in the fractions corresponding to the first peak (Figure A.10A & B). Using the size standard provided by the column manufacture (GE Healthcare) (Figure A.10C), the molecular mass of the POI was above 665 kda, which is more than 10 times larger than the expected size (60 kda). This result suggested that the POI formed protein aggregates, perhaps due to improper folding.

It was noted that there was another strong band (indicated by yellow arrow) below the POI band in all the elution fractions of the size exclusion column (Figure A.10A); furthermore, this un-identified protein was co-purified with the POI from the first nickel affinity column (Figure A.9B). This contaminate protein was unlikely to be the degradation product of the POI, as this protein was not detected in the western blot probed by an anti-GST antibody (Figure A.9A). Due to the presence of the contaminant protein, there might be another explanation how the POI ended up in the protein aggregate. If the contaminant protein instead of the POI caused the protein aggregation, since the POI was tightly bounded to the contaminant protein, the POI would have been “dragged” into the protein aggregate. If this was the case, separation of these two proteins might be able to rescue the POI from aggregation.
Figure A.9. Purification of the recombinant protein $^{600\text{K}}-E^{926}$ with two affinity columns. (A) The protein lysate containing $^{600\text{K}}-E^{926}$ with a GST-6xHis-thrombin tag was loaded on to a nickel affinity column and three elution steps (E1, E2 and E3) using 250mM imidazole were performed. Finally, all three elution fractions were pooled (E pool). The elution pool was dialyzed against 300 mM NaCl, 20 mM Tris 8.0 and 5% glycerol at 23°C for 2 hours. The samples then collected from the dialysis tubing and loaded to a second glutathione affinity column. Two elution steps (E1 and E2) using 10 mM reduced glutathione were performed. Western blotting was performed using an anti-GST antibody. (B) The corresponding coomassie-stained gel to the western blotting shown in A. The input, E2 and E3 were not included in the coomassie gel.
Figure A.10. The test of the monomeric state of the recombinant protein $^{600}K - E^{926}$ by a size exclusion column. (A) 2 mL of protein samples were loaded on to HiLoad 16/60 superdex 75, and elution fractions corresponding to the peaks in the chromatograph (B) were collected and tested using a coomassie-stained gel. (B) The predicted elution position of the recombinant protein $^{600}K - E^{926}$ was marked using a red line. (C) The chromatograph provided by GE healthcare for HiLoad 16/60 superdex 75 indicates the relationship between the molecular weight and the elution position.
Therefore, two ways were tried to remove the contaminant protein from the POI. Firstly, after the glutathione column, the POI was cleaved by thrombin proteases (Figure A.11A, the POI, contaminate protein and cleaved product were indicated by red, brown and green arrows, respectively) and then loaded to an ion exchange (IEX) column. However, there was only one large peak observed in the chromatograph (Figure A.11B), and the coomassie-stained gel indicated that the cleaved protein product was co-eluted with the contaminate protein (Figure A.11A). These results suggested that the tag-free 600K – E926 was bound to the contaminate protein too strongly so that the ion exchange column was not able to separate them. Secondly, I partially denatured the POI using 6M urea and in this way, the binding between the POI and contaminate protein would be disrupted. Then, the POI was firstly purified by a nickel affinity column, and the result showed that the contaminant protein was no longer co-purified with the POI (Figure A.12A). However, it was observed that a large amount of the POI was lost in FT and wash fractions. Subsequently, the eluate of the nickel affinity column subjected to a step-wise dialysis, which was used to gradually remove urea for refolding the POI. However, during the dialysis process, the POI was precipitated out (Figure A.12B). There might be some reasons for this. Firstly, dialysis might not be a suitable protein refolding technique for the POI; therefore, in the future, other techniques such as dilution or using chemical additives could be considered [475]. Secondly, it was possible that the un-identified contaminant protein was a protein chaperone, and the POI, which was in a partially unfolded state, needed to be bound to the chaperone to remain soluble in the solution. Once the POI was denatured by urea, the
protein-chaperone association was disrupted. Assuming that the step-wise dialysis could result in refolding of the POI back to its native state, since its original form was defective in folding, the POI would still form aggregates and precipitate out of the solution. If this was the case, this protein construct $^{600}K - E^{926}$ would not be worth further pursuing.
Figure A.11. Protease cleavage of the recombinant protein $^{600}K - E^{926}$ and the ion exchange column used to separate the cleaved recombinant protein $^{600}K - E^{926}$ from the protein contaminants. (A) Protein samples that contained the recombinant protein $^{600}K - E^{926}$ were cleaved using the thrombin protease overnight at 23°C and loaded on to a monoQ column. The fractions according to the peak in the chromatograph (B) were collected and tested by a coomassie-stained gel. The tagged $^{600}K - E^{926}$, tag-free $^{600}K - E^{926}$ and un-identified contaminant protein were indicated by red, green and brown arrows, respectively.
Figure A.12. Purification of the urea-denatured recombinant protein $^{600}$K – $^{E926}$ using a nickel affinity column and refolding of the protein using a step dialysis. (A) Sf9 cells containing the recombinant protein $^{600}$K – $^{E926}$ were lysed and purified in the buffers containing 6M urea. Denatured protein samples were loaded on to a nickel affinity column, and three washes were used in the purification procedure. (B) The collected eluate was dialyzed against 4M, 2M, 1M urea-contained buffers in sequence. The samples obtained from the dialysis tubing in the buffer containing 1M urea were centrifuged to separate the supernatant from the pellet. The samples of the supernatant and the pellet were tested in a coomassie-stained gel.

Since purification of the AHI-1 WD40-repeat domain alone did not seem to be feasible, purification of a protein construct that contains both WD40-repeat and SH3 domains was tried. For some recombinant proteins, they can only fold properly when bound to their binding ligands or partners [476]. Therefore, I considered the protein construct $^{435}$R-$^{P^{116}}$, which contains the additional SH3 domain and was shown to be expressed and soluble in Sf9 cells (Figure A.5). Similar strategy was used to generate baculoviruses that contained the construct $^{435}$R-$^{P^{116}}$ and infect Sf9 cells. The resultant recombinant protein had an N-terminal GST-6xHis-thrombin tag.
The POI was purified by nickel and glutathione affinity columns in sequence, and the resultant purified products showed relatively high purity (Figure A.13). Subsequently, the POI was cleaved with the thrombin protease for 2 hours at 23°C, and the tag-free POI was obtained, shown by the coomassie-stained gel (Figure A.14A). Western blotting analysis using two different antibodies was performed to test whether the protease cleavage was complete and verify if the cleaved product was the actual tag-free POI. Western blotting using a GST antibody exhibited that up to 90% of the POI was cleaved, resulting in the accumulation of the GST-6xHis protein tags (Figure A.14B, indicated by red arrows). Western blotting using a C-terminal Ahi-1 antibody, which recognizes the SH3 domain of AHI-1, revealed that indeed the cleaved product was the tag-free POI that contained both WD40-repeat and SH3 domains (Figure A.14C). Subsequently, the sample that contained the tag-free POI and cleaved GST-6xHis tags was loaded onto another nickel affinity column in order to remove the cleaved protein tags. However, strangely, the POI could not be collected in the FT fraction (data not shown). The same results were observed in three independent experiments, suggesting somehow this tag-free POI was stuck on the column. Therefore, I intended to use other column chromatography to purify the tag-free POI from the cleaved protein tags and other protein contaminants. Firstly, the tag-free POI was dialyzed in a low salt buffer (10 mM NaCl) in order to load onto an ion exchange column. Unfortunately, the tag-free POI precipitated out of solution during dialysis. Therefore, further purification of the tag-free POI using an ion exchange column could not be preceded. Secondly, the tag-free POI was concentrated by a
protein concentrator to a small volume (2 mL) in order to get further purified by a size exclusion column. Before loading on to a size exclusion column, the protein sample recovered from the protein concentrator was subjected to centrifugation in order to remove any debris. However, a protein pellet was observed after centrifugation, and the coomassie-stained gel indicated that the tagged POI was in the pellet (data not shown). Based on all these results, I speculated that the protein construct $^{435}R$-$P^{1116}$ might still have folding issues (stuck to the affinity column) and also tended to form protein aggregates at a high concentration (precipitated after centrifugation). Hence, I stopped working on this construct at this point. In the future, the protein construct $^{435}R$-$P^{1116}$ with other protein tags such as a MBP or SUMO tag could be tested for protein purification.

In all, in this study, expression/purification of the AHI-1 WD40-repeat domain was performed using *E. coli* and insect Sf9 cells as expression hosts. It was found that all the protein constructs expressed in *E. coli* cells were either not expressed or insoluble, while all the protein constructs expressed in Sf9 cells were either unstable or had folding issues. Therefore, the goal of solving the crystal structure of this domain was not achieved.
Figure A.13. Purification of the recombinant protein $^{435}R - P^{1116}$ with two affinity columns. (A) The protein lysate containing $^{435}R - P^{1116}$ with a GST-6xHis-thrombin tag was loaded on to a nickel affinity column. An extra ATP wash was used to minimize contamination of chaperone proteins. Two elution steps (E1 and E2) using 250mM imidazole were performed. Finally, all two elution fractions were pooled. The recombinant protein $^{435}R - P^{1116}$ is indicated by a red arrow. (B) The elution pool was dialyzed against 300 mM NaCl, 20 mM Tris 8.0 and 5% glycerol at 23°C for 2 hours. The samples were collected from the dialysis tubing, loaded to a second glutathione affinity column and eluted using 10 mM reduced glutathione. (C) The corresponding western blotting (anti-GST antibody) to the coomassie-stained gel shown in A.
Figure A.14. Cleavage of the recombinant protein $^{435}R - P^{1116}$ using the thrombin protease. A sample that contained the purified recombinant protein $^{435}R - P^{1116}$ was cleaved with the thrombin protease for 2 hours or 16 hours at $23^\circ C$. The samples before and after cleavage were tested by a coomassie-stained gel (A) and western blotting using an anti-GST (B) or anti-Ahi-1 C-ter (C) antibody. The POI, tag-free POI and cleaved tags were indicated by red arrows.
% Identity to AHI-1 SH3 domain | Class II ligands
---|---
P67phox | XPXXPXK
Mona | XPXXPXK
P40phox | FSAAPPSPSR (DNM2 PRD P5)
Hck | XPXXPXK
Pex13 | XPXXPXK
BBC1 | XPXXPXK
Amphiphysin | P67phox 37%
| Mona 35%
| P40phox 32%
| Hck 27%
| Pex13 25%
| BBC1 24%
| Amphiphysin 23%
| Ponsin 21%

Figure A.15. The sequence alignment of the Class II ligands with the sequences to the N-terminus of the consensus sequences. The percentage of the sequence identity relative to the sequence of the AHI-1 SH3 domain is shown in the second column. Sequence alignment of the Class II ligands and P5 from DNM2 PRD with the sequences to the N-terminus of the consensus sequences is shown in the third column. The conserved proline residues within the PXXP motif are indicated in bold, the compass residues R/K are colored in red and the sequences to the N-terminus of the consensus sequences are indicated in italics/bold.
Figure A.16. Changes of phosphorylation of BCR-ABL in BCR-ABL-transduced human UT7 cells upon IM treatment. (A) Western blotting analysis of p-BCR-ABL in BCR-ABL-transduced UT7 cells with 24-hr treatments of 1, 2 or 5 μM IM. (B) Western blotting analysis of p-BCR-ABL in the BCR-ABL mutant T315I-transduced UT7 cells with 24-hr treatments of 1, 2 or 5 μM IM.
Figure A.17. Overexpression of Ahi-1 increases transferrin uptake in BCR-ABL+ cells. (A) BCR-ABL-transduced, BCR-ABL/Ahi-1 co-transduced or BCR-ABL/Ahi-1 SH3Δ co-transduced BaF3 cells were stained with 12 μg/ml Alexa Fluor 647-conjugated transferrin at 37°C for 30 minutes. (B) Intracellular transferrin signals were quantified from two independent experiments, which were normalized to the signals detected in the BCR-ABL-transduced BaF3 cells. The white scale bar represents 50 μm. Values shown are the mean ± SEM. * = p<0.05.
Figure A.18. The effects of overexpression of Ahi-1 on ROS production in BCR-ABL+ cells. (A) ROS were stained using CellROX deep red reagents at 4 μM in BCR-ABL-transduced, BCR-ABL/Ahi-1 co-transduced or BCR-ABL/Ahi-1 SH3Δ co-transduced BaF3 cells at 37°C for 30 minutes. (B) Intracellular ROS accumulation was quantified from three independent experiments, which were normalized to the signals detected in the BCR-ABL-transduced BaF3 cells. The white scale bar represents 50 μm. Values shown are the mean ± SEM. *** = p<0.001.
Figure A.19. The effects of overexpression of Ahi-1 on key autophagy regulators in BCR-ABL+ cells. (A) Overview of changes of ULK-1, Beclin-1, LC3-II and p62 that correspond to the activation or suppression of autophagy. (B) Western blotting analysis of ULK-1, Beclin-1, LC3-II and p62 under the influence of the Ahi-1 overexpression in BCR-ABL+ cells.
Figure A.20. Mutagenesis of the residues making up binding interface of the AHI-1 SH3 domain to the corresponding residues of the Grb2 SH3 domain. (A) Sequence alignment between the AHI-1 and Grb2 SH3 domains. The residues that make up the binding interfaces of the AHI-1 and Grb2 SH3 domains are highlighted in yellow. (B) The protein sequence of the AHI-1 SH3 domain after residue substitution to the corresponding residues in the Grb2 SH3 domain. The substituted residues are marked in red.