ROR1 EXPRESSION AND FUNCTION IN B-CELL MALIGNANCIES

By

Vincent T. Bicocca

A DISSERTATION

Presented to the Department of Cell & Developmental Biology
and the Oregon Health & Science University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

November, 2011

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Vincent T. Bicocca

has been approved

_____

Dr. William Skach/ Chair

_____

Dr. David Parker

_____

Dr. Jan Christian

_____

Dr. Philip Streeter

_____

Dr. Rosalie Sears

_____

Dr. Brian Druker/ Advisor

TABLE OF CONTENTS

List of Figures

Abbreviations

ALL – Acute lymphoblastic leukemia

BCR – B-cell receptor

CAR – Chimeric antigen receptor

CD – Cluster of differentiation

CLL – Chronic lymphocytic leukemia

CML – Chronic myelogenous leukemia

EFS – Event-free survival

FACS – Fluorescent activated cell sorting

Fz – Frizzled domain

GIST – Gastrointestinal stromal tumor

Ig – Immunoglobulin

IgH – Immunoglobulin heavy-chain

IgL – Immunoglobulin light-chain

IP – Immunoprecipitate

ITAM – Immunoreceptor tyrosine-based activation motif

Kr – Kringle domain

NS – Non-specific

Nt – Nucleotide

NT – No treatment

PCR – Polymerase chain reaction

RAPID – RNAi-Assisted Protein Target Identification

RT-PCR – Reverse-transcriptase PCR

qRT-PCR – Quantitative real-time PCR

SLC – Surrogate light-chain

Tm – Melting temperature

WT – Wild type

Acknowledgements

I am deeply indebted to the many people who have made this work possible. First and foremost, I must thank my mentor and advisor, Dr. Brian Druker, for his support and patience throughout my time in his lab. I would also like to thank Dr. Jeffrey Tyner for his attention and guidance during my graduate work. In addition, I must acknowledge all the members of the Druker and Deininger lab, both past and present, for the years of collaboration and friendship.

I am also very grateful to the members of my dissertation advisory committee, Drs. William Skach, Jan Christian, Roselie Sears, David Parker, and Michael Deininger, for all their guidance and willingness to share ideas and reagents. A thank you must also be extended to Dr. Markus Muschen for his help with xenographs and microarray analysis.

Finally, I must thank my family and friends. To my parents, Vince and Barbara, and brother, Matthew, thank you for imbuing in me a strong work ethic, a love of learning, and an endless fascination with the natural world. To my wife, Caridad, I am forever indebted to you for your support over the past 5 years; I am truly fortunate to have such a collaborator with whom to explore this life. To my daughter, Sophia, thank you for always lifting my spirits and brightening every day.

This work has been supported by the Howard Hughes Medical Institute, William Lawrence & Blanche Hughes Foundation, National Cancer Institute, Leukemia & Lymphoma Society, and Tartar Trust Fellowship.

Abstract

Aberrant tyrosine kinase activity is commonly implicated in the pathogenesis of leukemia and other cancers. Identification of these leukemogenic tyrosine kinases has proven invaluable for diagnostic and prognostic stratification of patients as well as for the development of novel strategies for therapeutic intervention. The Druker lab has previously demonstrated that siRNA screening of mononuclear cells from leukemia patients can determine sensitivity to loss of individual tyrosine kinases. With the goal of uncovering novel viability-dependent tyrosine kinases in leukemia patients, we have employed an RNAi-assisted protein target identification (RAPID) assay to screen cytogenetic subtypes of acute lymphoblastic leukemia (ALL). ALL is the most common pediatric cancer, accounting for one-quarter of all childhood malignancies. Childhood ALL has a primarily B-cell precursor phenotype and is characterized by chromosomal abnormalities, primarily translocations and duplications. One of the most common recurring translocations associated with pediatric ALL, t(1;19)(q23;p13.3), generates the E2A-PBX1 fusion product. Here I show unique viability-dependent expression of a receptor tyrosine kinase, ROR1, in the t(1;19) ALL background. In addition, I identify a kinase inhibitor, dasatinib, with significant activity against t(1;19) ALL cells due to its capacity to inhibit tyrosine kinases necessary for transduction of pre-B-cell receptor (pre-BCR) signaling. Finally, I show that ROR1 and the pre-BCR activate mutually compensatory signaling pathways, suggesting that optimal therapeutic regimens would include agents targeting both pathways.

Chapter 1

Introduction

1.1 Pediatric Acute Lymphoblastic Leukemia

Cancer is one of the most common cause of non-accidental death in children 1-14 years of age in the United States[1]. Acute Lymphocytic Leukemia (ALL) is the most common cancer in children and accounts for more than one quarter of the malignancies diagnosed in children under the age of 15[2]. In the United States, childhood leukemia will be diagnosed in approximately 3,500 children each year; three out of four of these patients will have ALL[2].

Acute leukemias are highly aggressive disorders that occur when hematopoietic progenitor cells undergo malignant transformation into undifferentiated precursor cells with abnormal proliferation and longevity. These aberrant lymphoblasts (in ALL) have evolved a selective growth advantage that fosters uncontrolled cell proliferation and results in replacement of normal marrow tissues and hematopoietic cells[3].

Childhood ALL has a primarily B-cell precursor phenotype (80-85% express B-lineage markers[4]) and is characterized by chromosomal abnormalities such as translocations and aneuploidy[5]. Hyperdiploidy events, in which greater than 46 chromosomes are present, account for approximately 35% of chromosomal changes identified in childhood B-cell precursor ALL patients, and translocations are present in another 30% of patients[6]. Unlike epithelial cancers, which usually possess an intra-clonally diverse karyotype containing multiple chromosomal deletions and translocations, the chromosomal changes observed in pediatric leukemia usually reflect a single abnormality[7]. This distinction indicates that such chromosomal abnormalities are not the product of chromosomal or genomic instability; rather, they exist as single

events that occur with minimal concurrent genetic abnormalities, are stable, and are intra-clonally conserved[6].

Specific recurring chromosomal translocations found in leukemia are selectively associated with distinct subtypes of the disease. ALL, in particular, is characterized by reciprocal translocations that result in aberrant in-frame chimeric genes that produce modified transcription factors. The aberrant transcription factor activity of these fusion products is thought to disrupt cell differentiation and initiate leukemogenesis[8]. The diverse functional characteristics of these lesions may also impact the heterogeneity of each leukemic subtype. As a result of this heterogeneity, each subtype of ALL presents its own challenge for the clinic[9].

The challenge for translational studies in pediatric ALL is three-fold: 1) improve cure rates, 2) identify patients with a higher risk of relapse, and 3) decrease the burden of therapy for patients with a low risk of relapse. The survivability of pediatric ALL has improved dramatically since the 1970's, but there remains room for improvement[10]. Pediatric ALL patients are classified into risk groups based upon clinical features such as age of the patient, white blood cell count, the genetics of their leukemia, and initial response to therapy[9]. For example, patients who are classified as low or average risk typically respond well to induction therapy and have a >90% chance of achieving 5-year event-free survival (EFS). High and very high risk patients, however, can expect a 75-90% and <75% 5-year EFS, respectively. Favorable recurring genetic changes, such as the presence of double trisomy 4 and 10 or a 12:21-translocation, will usually classify a patient as low or average risk and assignment of standard induction therapy. However, patients with hypodiploidy, *MLL* rearrangements, or the 9;22-translocation are classified

as very high risk and will likely receive high risk induction therapy. Other genetic abnormalities, such as the 1;19-translocation, can classify a patients as average or high risk depending upon their initial response to standard induction therapy[9].

Improving the 5-year EFS of all pediatric ALL risk groups, but especially the high and very high risk patients, with better drugs and better drug combinations is a major goal of pediatric ALL research. This may be achieved by identifying patient specific biomarkers that predict individual risk of recurrence and can be used to tailor their therapy for improved treatment. In addition, this can allow for decreasing a patient's burden of therapy. Current cytotoxic therapies can have long-lasting, and often life-threatening, side-effects ranging from cardiac dysfunction to secondary malignancy[11, 12]. As a result, the long-term goal of pediatric ALL research is to both increase patient EFS, and to do so with less toxic therapies that reduce the patients therapeutic burden and minimizes side-effects.

1.2 Tyrosine Kinases as Therapeutic Targets

The aberrant regulation or overexpression of tyrosine kinases plays a critical role in the pathogenesis of leukemia as well as other common malignancies. The mammalian tyrosine kinase gene family consists of 91 members. These signaling proteins play a critical role in diverse cellular processes, including proliferation, differentiation and apoptosis. Oncogenesis can result from disruption of any of these processes, so it is not surprising that aberrant regulation of tyrosine kinase signaling is often observed in leukemia and many other common cancers[13]. For example, the development of chronic myelogenous leukemia (CML) can be linked to a single translocation event that results in a portion of the BCR gene from chromosome 22 being fused to the end of the ABL

gene on chromosome 9[14] and the subsequent production of the BCR-ABL fusion protein[15, 16]. This faulty signaling protein triggers white blood cells to rapidly and persistently divide through its constitutive ABL tyrosine kinase activity[17]. The therapeutic compound imatinib (Gleevec) targets this aberrant tyrosine kinase and selectively shuts it off, killing the cells that produce it and leaving healthy cells unharmed[18]. And while CML is characterized by the presence of BCR-ABL, this oncogene has been identified in additional forms of leukemia, including approximately 5% of patients with B-cell precursor ALL[19].

While BCR-ABL is the prototypical oncogenic tyrosine kinase, aberrantly regulated tyrosine kinases have been described in other forms of leukemia, as well as in various malignant tumors. TEL-PDGFRβ and FIP1L1-PDGFRα are activated tyrosine kinase fusion products (similar to BCR-ABL) that are responsible for leukemogenesis in subsets of patients with chronic myelomonocytic leukemia and idiopathic hypereosinophilic syndrome, respectively [20, 21]. Activating point mutations in the cytokine receptor tyrosine kinase c-KIT are found in as many as 90% of gastrointestinal stromal tumors (GIST) and the receptor tyrosine kinases EGFR and HER2 are overexpressed or possess point mutations in certain lung and breast tumors[22-24].

As with the use of Gleevec in BCR-ABL-positive CML, specific small molecule inhibitors of oncogenic tyrosine kinases have proven to be effective therapies. Gleevec's off-target activity against c-KIT is a critical weapon in the treatment of gastrointestinal stromal tumors (GISTs)[25]. Compounds that target multiple tyrosine kinases such as sunitinib (Suntel) and dasatinib (Spycel) are used to treat renal cell carcinoma, Gleevec-resistant GISTs, CML, and BCR-ABL positive ALL[26-28]. The EGFR and HER2

receptor tyrosine kinase inhibitors erlotinib (Tarceva) and lapatinib (Tykerb) are FDA approved for treatment of certain lung and breast tumors[29, 30].

In addition to small molecule inhibitors, monoclonal antibodies have been developed to interact with and disrupt the activity of receptor tyrosine kinases present on the surface of cancer cells. This strategy has been employed with limited clinical success in treating breast cancer. The monoclonal antibody trastuzumab (Herceptin) was designed to target HER2 (ErbB2), a receptor tyrosine kinase that is overexpressed in 20-30% of breast cancers[31, 32]. HER2 overexpression has traditionally been a poor prognosis indicator, and breast tumors with this marker have an increased risk of recurrence. Trastuzumab therapy has proven clinically efficacious when used as an adjuvant treatment and when used in combination with more traditional therapies, reducing the risk of relapse for HER2-positive patients[33].

Cancer therapies, such as antibodies and small molecule inhibitors, which target the specific oncogenic lesion critical to cancer survival, are extremely valuable, as they allow for a personalized therapeutic approach that minimizes and may even eliminate the side-effects of traditional chemotherapies[34, 35]. In addition, targeted drugs, like imatinib and dasatinib, have been used in combination with standard cytotoxic chemotherapy and have proven to improve treatment. For example, imatinib in combination with intensive chemotherapy improves 5-year EFS for patients with BCR-ABL-positive ALL, and suggests that these patients no longer need hematopoietic stem cell transplant as first line therapy[36]. The value of reducing therapeutic side effects is particularly prevalent in the treatment of both pediatric malignancies and cancers in the elderly. The infirmed condition of elderly cancer patients can often immediately

eliminate the use of aggressive chemotherapy regimens (even if they are the best course of treatment) because the side-effects are simply not tolerable[37, 38]. Pediatric malignancies, on the other hand, present a very different problem with respect to chemotherapy side-effects. Children, in general, are very robust and are capable of tolerating extremely aggressive chemotherapy regimens that would be impossible with elderly patients and most adults. While this characteristic is beneficial in treating a pediatric cancer, it can result in debilitating and life-threatening side effects that a pediatric cancer survivor may deal with for the rest of his or her life[12]. Adoption of therapeutic regimens that supplement or, preferably, replace high side-effect traditional chemotherapies with low side-effect kinase inhibitor therapies could have the potential to increase patient EFS while simultaneously reducing the destructive side-effects of traditional chemotherapy.

1.3 RNAi-Assisted Protein Target Identification (RAPID)

Many techniques have been employed to identify novel mutant genes in cancer. Large-scale sequencing of cancer genomes has uncovered a multitude of mutations; the functional role of these genetic anomalies, however, remains largely unclear[39, 40]. An alternative approach, involving phospho-proteomic profiling of cancer cells, guides subsequent sequencing analysis to likely sources of mutations[41]. Each of these approaches has met with varying degrees of success, but they have also revealed the need for an assay that could provide information of greater diagnostic relevance. An assay that yields functional information about relevant genes would allow rapid molecular genetic profiling of malignancies.

RNAi technology provides a valuable opportunity to selectively reduce the expression of specific genes, thus allowing impact assessment of an individual gene on cancer cell survival[42]. For the first time, our laboratory has utilized this technology to screen the entire tyrosine kinome for genes critical to cancer cell proliferation and viability[43]. This rapid screening approach provides functional data that can identify genes integral for malignant cell growth, and this is accomplished independent of a gene's mutational status. Using this approach, I have identified the largely unstudied, receptor tyrosine kinase-like orphan receptor 1 (ROR1) as a critical target for cell proliferation and viability in a patient diagnosed with pediatric B-cell precursor ALL carrying the recurrent t(1;19)(q23;p13) gene rearrangement (**Figure 1.1**).

The following chapters provide the first evidence of conserved ROR1 expression and functional significance in pediatric ALL. Chapter 2 details the expression profile of ROR1 in normal B-cell development as well as its consistent expression in 1;19-translocated pediatric ALL and other B-cell malignancies. Chapter 3 outlines the functional significance of ROR1 within the context of pre-B malignancies and provides the first evidence of pathway activation in B cells. Additionally, I identify the small-molecule tyrosine kinase inhibitor dasatinib as having potential clinical efficacy against ROR1-positive B-cell malignancies via inhibition of the pre- and mature B-cell receptor. Together, this work identifies ROR1 as a conserved viability-dependent target in t(1;19) ALL and provides the first evidence of ROR1 function in normal B-cell development and leukemia.

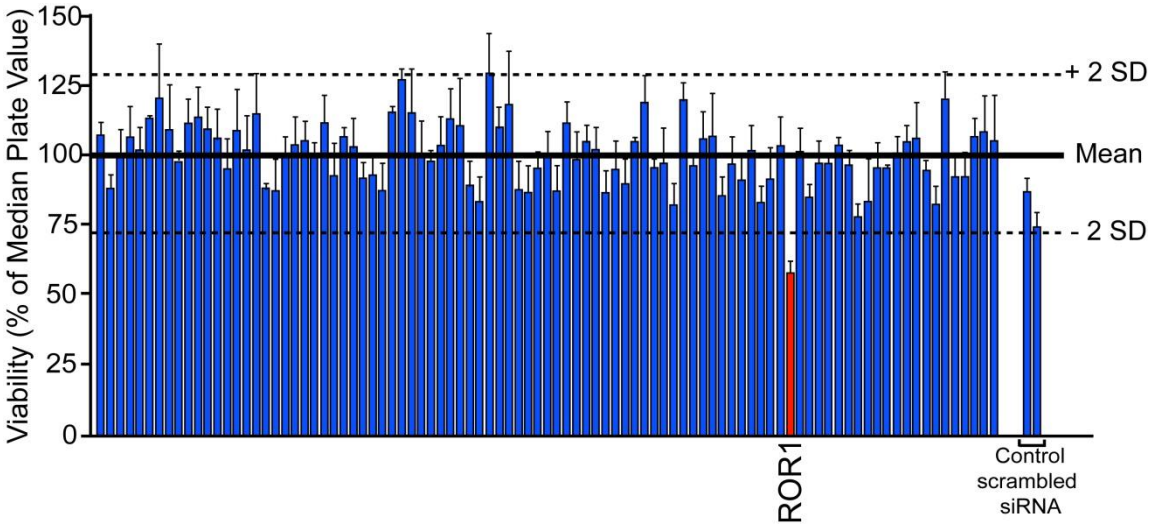


Figure 1.1. RAPID assay identifies ROR1 as a viability dependent target in t(1;19) ALL. White Blood cells (2.25 x 10^7) from a t(1;19)-positive ALL patient (07-112) were suspended in siPORT buffer and incubated with 1 μM siRNA from an siRNA library individually targeting each member of the tyrosine kinase family as well as N-RAS, K-RAS, and single and pooled non-specific siRNA controls. Cells were electroporated on a 96-well electroporation plate at 1110 V (equivalent of 150 V), 200 μsec, 2 pulses. Cells were replated into culture media and cell viability was determined by addition of a tetrazolium salt (MTS assay) at day 4 post-electroporation. Values represent percent mean (normalized to the median value on the plate) ± s.e.m (n = 3).

Chapter 2

ROR1 expression in B-cell malignancy

The work detailed in this chapter was presented in abstract form at the 52nd annual meeting of the American Society of Hematology in Orlando, FL, December 6th, 2010 and is currently under revision for publication. The contributions of co-authors are detailed in Appendix II.

2.1 Abstract

Aberrant tyrosine kinase activity is commonly implicated in the pathogenesis of leukemia and other cancers. Identification of these leukemogenic tyrosine kinases has proven invaluable for diagnostic and prognostic stratification of patients as well as for the development of novel strategies for therapeutic intervention. Our lab previously demonstrated that siRNA screening of mononuclear cells from leukemia patients can determine sensitivity to individual tyrosine kinases. With the goal of uncovering novel viability-dependent tyrosine kinases in leukemia patients, we have employed an RNAi-assisted protein target identification (RAPID) assay to screen cytogenetic subtypes of acute lymphoblastic leukemia (ALL). ALL is the most common pediatric cancer, accounting for one-quarter of all childhood malignancies. Childhood ALL has a primarily B-cell precursor phenotype and is characterized by chromosomal abnormalities, primarily translocations and duplications. One of the most common recurring translocations associated with pediatric ALL, t(1;19)(q23;p13.3), generates the E2A-PBX1 fusion product. Here I show conserved expression of a receptor tyrosine kinase, ROR1, in the t(1;19) ALL background.

2.2 Introduction

2.2.1 Receptor tyrosine kinase-like orphan receptor 1 (ROR1)

Human ROR1 is a 937-residue, single pass transmembrane protein that is highly expressed during early embryonic development[44, 45]. The extracellular ligand binding portion of ROR1 contains an immunoglobulin (Ig) domain, a frizzled (Fz) domain, and a kringle (Kr) domain proximal to the transmembrane region. The intracellular portion contains a tyrosine kinase domain and a tail containing proline-rich and serine/threonine-rich domains[44] (**Figure 2.1**).

ROR1 shares 58% amino-acid identity with its single homologue, ROR2. Overall, the ROR-family of RTKs is most closely related to Muscle Specific Kinase (MuSK), but the kinase domain of ROR-family proteins is most closely related to neurotrophic tyrosine kinase (Trk). The two ROR-family proteins are conserved in mice and *Drosophila*. Murine Ror1 and Ror2 share 97 and 92% amino-acid identity with their human counterparts, and *Drosophila* express the equivalent ROR-proteins Dnrk and Dror[45]. Interestingly, unlike their *Drosophila* relatives, the mammalian ROR-family possesses abnormal amino-acid substitutions in their canonical ATP binding motifs. Purified kinase assays show that although both ROR1 and ROR2 possess these aberrant substitutions, ROR2 maintains its kinase activity, while ROR1 does not[44, 46]. The proline-rich cytoplasmic tails of ROR1 and ROR2 are unique to the mammalian ROR-family and contain binding motifs that suggest potential binding partners that possess Src homology 3 (SH3) domains[47]. In addition, ROR1, but not ROR2, possesses a consensus XPPXY motif within its proline-rich tail that suggests potential

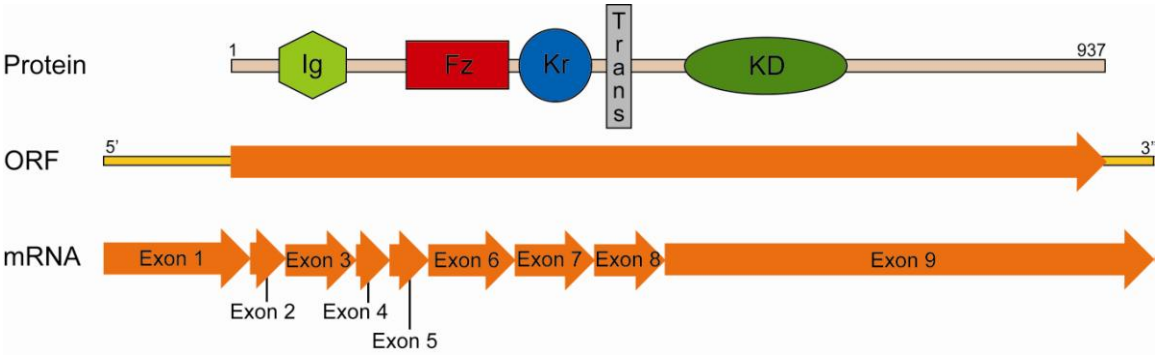


Figure 2.1. Diagram of the ROR1 gene product. ROR1 is comprised of an extracellular immunoglobulin (Ig), frizzled (Fz), and kringle (Kr) domain and an intracellular kinase domain (KD). The ROR1 transcript is composed of 9 exons with a 399 base pair 5' untranslated region (UTR) and 169 base pair 3' UTR.

interaction with WW domain proteins and SH2 domain proteins[48]. Similarly, the cytoplasmic regions of ROR2 and Dnrk, but not ROR1 or Dror, possess a YALM motif capable of interacting with SH2 domain proteins[49]. Together, these homology studies suggest ROR1 likely possesses a distinct mechanism of action from its closest relatives (due to lost kinase activity and unique intracellular protein-protein interaction motifs) and may function in a discrete manner.

Invertebrate studies of the ROR-family homologue CAM-1 in *C. elegans* has greatly expanded our understanding of ROR function in development; however, these studies again suggest the developmental function of mammalian ROR-family member ROR2 has been conserved from its ancestral homologues, while ROR1 may have evolved disparate function. Unlike *Drosophila* ROR-family members, the extracellular region of *C. elegans* ROR-family homologue CAM-1 is highly conserved, possessing the Ig, Fz, and Kr domains of the mammalian proteins. Study of ROR function using CAM-1 in *C. elegans* has shown ROR-capacity to sequester WNT ligands, recruit DLIXIN-1 to the cell membrane, and mediate WNT effects on cell polarity [50-53]. These studies have greatly expanded our understanding of ancestral ROR-family members' role in development, link the ROR/CAM-1 Fz domain to WNT activity, and support studies investigating ROR1 and ROR2 in vertebrate systems.

The extracellular Fz-domain present in ROR-family proteins has made these receptors particularly intriguing due to the role of these domains in WNT signaling. The Fz-domain is a conserved cysteine-rich domain found in FRIZZLED proteins that binds WNT ligands[54]. WNT signaling, in general, is of particular importance for its role in hematopoietic stem cell proliferation and leukemic development[55]. Wnt5a, in

particular, has been shown to promote expansion of undifferentiated progenitor cells in vitro and increased human hematopoietic stem cell (HSC) re-population in NOD-SCID xenograph models[56, 57]. Studies of vertebrate ROR2 have shown evidence of WNT binding and signaling, in particular, Wnt/β-catenin transcription activation is inhibited by Wnt5a mediated activation of Ror2[58-61]. In addition, the Ror1 and Ror2 mouse double knockout phenotype strongly resembles that of Wnt5a-/- null mice, displaying dwarfism, shortened limbs, facial abnormalities, ventricular septal defects in the heart, and lung development abnormalities[62, 63]. Importantly, Ror1-deficient mice, though possessing a fatal respiratory defect, do not exhibit the cardiac abnormalities observed in the double mutant or the skeletal abnormalities that are the primary phenotype of Wnt5a loss[64]. Ror2-deficient mice, on the other hand, do exhibit skeletal abnormalities and ventricular septal heart defects[65], and this phenotype is enhanced in the double mutant[64]. Together, these knockout studies suggest independent functions for Ror1 and Ror2 as well as genetic interaction between the two. In particular, these studies consistently link Ror2 to Wnt signaling, and present the first evidence for Ror1 functioning in a similar capacity, perhaps in cooperation with Ror2.

The work detailing ROR2 function in WNT signaling, combined with the mouse knockout studies suggesting Ror1 may cooperate with Ror2 in Wnt5a function, have driven investigation of ROR1 as a potential WNT ligand receptor. Studies of ROR1 in mammalian systems show it can influence WNT5A activity in multiple tissue types; however, the conclusions of these studies have often come with minimal experimentation, and even at the expense of contradictory experimental results, and suggest significant work remains to fully understand the functional role of ROR1 in

WNT5A signaling. The first report of ROR1 interacting with a WNT-ligand utilized an epitope-tagged WNT5A construct to coimmunoprecipitate an epitope modified ROR1 construct in HEK293 cells. This experiment was the first to show ROR1 could act as a receptor for WNT5A[66]. Interestingly, this study does not report an influence on LEF/TCF activity, as described in ROR2 studies[58-60], and instead suggests ROR1 influences NFκB activity. Surprisingly, a subsequent study, which also utilized exogenous expression of epitope-tagged proteins in HEK293 cells, failed to corroborate this result. Here, epitope-tagged Wnt5a failed to coimmunoprecipitate exogenous Ror1, but did successfully precipitate Ror2[67]. In whole brain lysates, however, Ror1 immunoprecipitates contained both Ror2 and Wnt5a, suggesting Ror1 may require heterodimerization with Ror2 to bind Wnt5a. This study also showed that both Ror1 and Ror2 were required for Wnt5a induced synapse formation in hippocampal neural cultures. Though these two studies generated opposing results while performing the same experiment, they managed to come to the same conclusion: ROR1 interacts with WNT5A to modulate non-canonical WNT signaling. Additional studies have also generated data supporting a ROR1/WNT5A interaction. In HeLaS3 cells, loss of ROR1 or ROR2 prevents RAC activation and FRIZZLED2 internalization in response to WNT5A stimulation[68]. In breast cancer cell lines, incubation with WNT5A conditioned media results in ROR1 Ser/Thr-phosphorylation and WNT5A stimulation of cells expressing a ROR1-LRP6 chimera (extracellular ROR1 and intracellular LRP6) activates a TCF-luciferase reporter[69]. Though questions remain, these studies show consistent evidence of ROR1 modulating WNT5A signaling in both developmental tissues and cell culture systems.

Beyond ROR1's expression profile during development, growing evidence of ROR1 expression in adult tissues suggests a ROR1 signaling importance that is not yet fully realized. Interest in ROR1 and hematologic malignancies has grown rapidly in the past four years, after the most common form of leukemia, chronic lymphocytic leukemia (CLL), was discovered to consistently express high levels of ROR1 at the cell surface[70, 71]. Subsequent studies (overlapping with my own work described below) that looked at ROR1 expression in additional malignancies have shown high levels of ROR1 expression in various hematopoietic malignancies[72-74]. Prior to these studies, ROR1 expression was thought to be seen only during embryonic development and lost postnatally. In murine systems, Ror1 is expressed during development in the craniofacial region and the developing limb, cardiovascular and respiratory systems, the digestive systems, and in the nervous system and the thymus. This expression profile corroborates the knockout phenotype (described above) in suggesting a role for Ror1 in respiratory, cardiac and skeletal development[44, 45, 75, 76]. ROR1 expression in normal adult tissue has been somewhat controversial. Expression of a truncated Ror1 variant in adult neural tissue has been reported and contested[75, 77]. In addition, *ROR1* mRNA is detected in adult adipose, pancreatic, lung and ovarian tissue; however, expression levels appear significantly lower than what is seen in CLL (10-100-fold less) and confirmation of protein expression has not been performed[78]. As a whole, my work described below and the work of Hudecek et al.[78] provides the first evidence of ROR1 expression in normal hematopoietic tissue.

2.2.2 B-cell development

Developmental progression of B lymphopoiesis and generation of mature B cells is a complex, highly regulated process characterized by distinct marker protein expression and successive recombination of the immunoglobulin (Ig) gene loci[79, 80](**Figure 2.2**). B-cell development originates in the bone marrow with a pool of multipotent stem cells possessing a germline configuration of the Ig loci. The first step in B-cell differentiation begins with recombination of the Ig heavy-chain locus (IgH). Rearrangement of D_H to J_H (the Diversity (D) and Joining (J) segments of the heavy-chain locus) occurs at early pro-B stage of development and is followed by V_H (Variable (V)) to DJ_H rearrangement at the late pro-B stage[81, 82]. The recombination reaction responsible for immunoglobulin gene rearrangement is mediated by expression of the RAG1-RAG2 protein complex[83, 84]. The RAG protein complex is responsible for generating double-stranded DNA breaks in gene segments possessing specific recombination signal sequences[85]. As a result, regulation of RAG protein expression is critical to temporally controlled recombination of immunoglobulin genes during differentiation. Productive VDJ recombination results in production of the immunoglobulin heavy-chain μ (Igμ). Igμ is expressed by pre-B cells as a component of the pre-B-cell receptor (pre-BCR) complex (discussed in detail in Chapter 3)[86]. Transient expression and signaling from the pre-BCR represents an important checkpoint in B-cell development by providing immediate feedback regarding the functionality of heavy-chain recombination[87-89].

Functional signaling from the pre-BCR initiates clonal expansion and ensures only cells with signaling-competent receptors can mature further[90]. Pre-BCR signaling

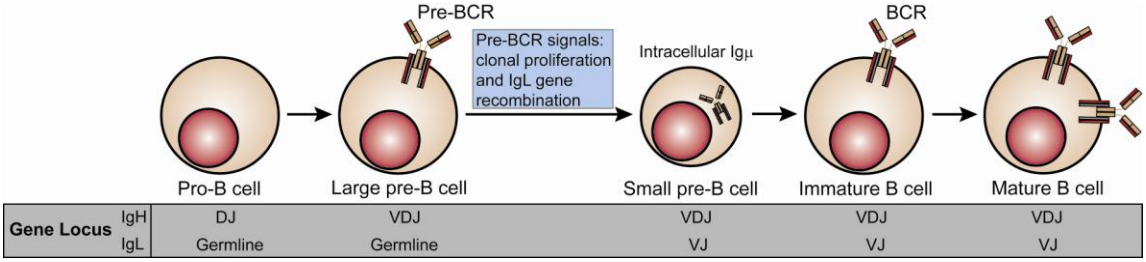


Figure 2.2. Development of human B-lineage cells. $CD34^{+}$ pro-B cells are the first stage of B-lineage differentiation and possess the first heavy-chain gene rearrangement (D-J). Large pre-B cells have undergone heavy-chain VDJ recombination and express Igμ as a critical component of the pre-BCR. Transition from large to small pre-B cells involves internalization of the pre-BCR, loss of the surrogate light-chain, and recombination of light-chain (V-J). Immature B cells have rearranged heavy- and light-chain and express mature-BCR. Alternative splicing, isotype switching, and somatic hypermutation yields mature B cells with membrane and secreted Ig. Adapted from Herzog S. et al[80].

is responsible for negative feedback regulation of pre-BCR components, and leads to downregulation of the complex and attenuated signaling[91]. Loss of pre-BCR signaling leads to upregulation of RAG proteins and facilitates recombination of immunoglobulin light-chain (IgL). Functional rearrangement of IgL allows assembly of the mature-BCR consisting of Igμ and IgL[92-94].

The intrinsic checkpoints utilized during B-cell differentiation ensure selection of only viable B-cell progenitors for maturation and elimination of defective progenitors[95]. These checkpoints, however, can be disrupted or disregulated after an oncogenic event. The recurrent translocations observed in ALL generate oncogenes that interfere with normal B-cell maturation and result in pools of malignant or pre-malignant B-cell progenitors arrested at specific stages of development[8, 96]. Because different translocation result in different oncogenes with unique mechanisms of transformation, the stage at which a malignant progenitor is arrested is dependent upon the oncogene that cell harbors. As such, ALL patients are categorized into distinct subsets of disease based upon the translocation in their leukemic blasts, and are treated accordingly[9]. Of particular importance are the four most common recurring translocations observed in pediatric ALL: 9;22 (BCR-ABL), 12;21 (TEL-AML), 1;19 (E2A-PBX1), and assorted chromosome 11 translocations (MLL fusions)[8]. These translocations account for approximately 55% of all observed translocations and nearly 90% of recurring translocations. The ALL subtype possessing the 1;19 translocation that generates E2A-PBX1 is particularly interesting, as it is arrested at intermediate stage of B-cell development (where the B-progenitor is expressing the pre-BCR)[97], whereas the other three ALL subtypes are all arrested at earlier stages of development

(that still express the surface marker CD34 and do yet express the pre-BCR)[98-101]. This characteristic of t(1;19) ALL proves to be important to our understanding of ROR1 expression.

As described above, discovery of ROR1 expression in CLL and assorted other B-cell malignancies has brought new attention to the function of ROR1 and interest in it as a target for therapeutic intervention. ROR1 expression in CLL and Mantle cell lymphoma is interesting, as these are both mature B-cell malignancies. Analysis of equivalent normal B-cell progenitor populations shows that ROR1 is not normally expressed at these stages of development[71, 78]. However, surface expression analysis of B-cell progenitor populations (early, early intermediate, late intermediate, and mature) shows ROR1 expression during the early and late intermediate stages of development[78]. While it is unclear from these studies why ROR1 expression in mature B-cell malignancies does not coordinate with ROR1 expression in intermediate B-cell development, they do provide the first evidence of a connection between ROR1's function in normal B-cell development and its function in B-cell malignancies.

2.2.3 1;19-translocated ALL

One of the most common, recurring translocations found in ALL patients is t(1;19)(q23;p13), which is observed in approximately 5% of all pediatric ALL cases as well as 1-2% of adult ALL cases. It is also rarely found in T-ALL and acute myeloid neoplasms. Greater than 90% of patients with t(1;19) exhibit blasts with expression of cytoplasmic Igμ and an absence of the pro-B lineage marker CD34 on the cell surface, indicating that these blasts are typically arrested at a later stage of B-cell differentiation

(small pre-BII) than most other subsets of ALL patients[102]. The 1;19 translocation results in the fusion transcription factor complex *E2A-PBX1*[103].

E2A belongs to the class I family of basic helix-loop-helix proteins and is widely expressed. E2A is capable of homodimerization to activate transcription or heterodimerizing with tissue-specific helix-loop-helix proteins to activate transcription[104, 105]. Importantly, E2A is well known to play a critical role in the regulation of lymphocyte differentiation. Early studies showed E2A proteins were capable of binding the E box motifs present in the Ig promoter, and subsequent knockout studies showed that B cell development was arrest at the pro-B stage in E2A-deficient mice[106, 107]. In addition to playing a critical role in regulating pre-BCR component expression during the pro-B to pre-BII transition stage of development, E2A continues to regulate pre-BCR expression during the pre-BCR to mature-BCR transition as well as necessary regulators of this process. Because t(1;19) ALL is arrested at the pre-BII small stage of development, the role E2A plays in the progenitor transition from pre-BCR-positive to mature BCR-positive is particularly important. E2A plays a major role in the coordinated internalization of the pre-BCR, downregulation of the surrogate light-chain, and recombination of light-chain[108-111]. Together, these processes are all required for proper assembly of the mature BCR, and disruption of these steps with the E2A-PBX1 fusion product contributes to B-cell developmental arrest and the t(1;19) ALL phenotype.

While E2A plays an extensive role in normal lymphocyte development, PBX1 is not normally expressed in the lymphoid compartment[112]. PBX proteins are a functionally and biochemically unique subclass of homeodomain proteins and their

ability to interact with and modulate HOX protein action has been the major focus of their investigation. HOX proteins are a class of transcription factors that bind DNA via their helix-turn-helix homeodomain, and PBX-family proteins have been shown to modulate their activity through cooperative DNA binding activity. As PBX1's HOX cooperativity motif is retained following the E2A-PBX1 translocation, effects of the translocation on PBX1's interaction with HOX proteins and their subsequent role in leukemogenesis has been a major focus of study[113, 114].

Based upon what we know of wild-type E2A and PBX1, we can begin to construct a model of how fusion of these proteins disregulates B-cell development, facilitates leukemogenesis, and culminates in acute leukemia. In normal cells there are two normal copies of the PBX1 locus on chromosome 1 and two normal copies of the E2A locus on chromosome 19. In normal lymphocyte progenitors, only E2A (possessing a helix-loop-helix domain and two activation domains) is expressed. The 1;19-translocation results in expression of only one normal copy of E2A (in the case the predominant non-reciprocal translocation[115]) and one copy of N-terminal E2A fused to C-terminal PBX1. This fusion protein contain the two activation domains of E2A (but not the DNA-binding helix-loop-helix domain) and the dimerization, homeodomain, and HOX binding motif of PBX1 (but not the transcriptional repressor and MEIS/PREP-interaction domains)[116].

There are several significant consequences to this arrangement. First, as we mention above, E2A is critical to normal B-cell development and following the 1;19-translocation progenitor B-cells are left with only one functional copy of E2A. In addition, the E2A-PBX1 fusion gene retains the activation domains of wild-type E2A,

but no longer interacts with E2A specific DNA binding sites. As a result, E2A-PBX1 binds cofactors critical for wild-type E2A function but does not localize these cofactors to appropriate E2A bindings sites. This action effectively sequesters important E2A cofactors (such as p300/CBP and the SAGA complex), further abrogating the endogenous activity of E2A[117, 118]. Along similar lines, these cofactors that would normally be destined for E2A-specific binding sites are now being localized to PBX1-associated sites[119]. The second major consequence of E2A-PBX1 expression is the presence of PBX1 in B-cell progenitors that normally lack its expression. As we discussed above, PBX1 is particularly interesting due to its interactions with HOX-family proteins. In fact, in demonstration of the critical role of HOX protein interaction, deletion studies have shown that the homodimerization domain of PBX1 is completely expendable, whereas the HOX cooperativity motif of PBX1 is necessary but not sufficient for recapitulation of the E2A-PBX1 oncogenic phenotype[113, 120]. Similarly, the PBX1 homeodomain, which cooperatively act with HOX protein to bind DNA, is also required for transcriptional activation, but is, again, not sufficient for recapitulation of the E2A-PBX1 phenotype[119, 121]. Together, these studies show that the inherent DNA-binding activity of PBX1 is not required for transformation, and it is PBX1 ability to associate with HOX-family transcription factors to mediate DNA-binding and cofactor recruitment that is responsible for E2A-PBX1 transformative capabilities.

Importantly, discussed above are just the two primary consequences of E2A loss and PBX1 gain [(1. Sequestration and association of E2A-specific cofactors with E2A-PBX1 (2. Activated transcriptional activity of PBX1/HOX in B-cell progenitors] resulting from the 1;19-translocation. Because each component of the E2A-PBX1

chimera gains and loses wild-type function, the E2A-PBX1 fusion product has unique acquired functions that also contribute to oncogenesis. Normally, PBX1/HOX transcriptional activity is regulated, in part, by the N-terminal transcriptional repressor domains of PBX1 and PBX1 can act as a repressor of HOX transcriptional activity[122]. These domains, however, are lost following the 1;19-translocation, allowing E2A-PBX1 to activate PBX1/HOX-target genes normally repressed by PBX1[123]. In addition, PBX1 is typically shuttled between the nucleus and cytoplasm in a highly regulated manner dependent upon the N-terminal region of PBX1 lost following translocation[124, 125]. Ectopic expression studies show that while overexpressed PBX1 is diffuse throughout the cell, E2A-PBX1 is localized exclusively in the nucleus[126]. Nuclear accumulation of E2A-PBX1 represents an additional mechanism of disrupting regulation of HOX/PBX1 interaction and subsequent gene expression. Finally, the two activation domains found on the N-terminal region of E2A, which are known interact with the p300/CBP and SAGA complex co-activators, are important for the transformative properties of E2A-PBX1[117, 118]. Deletion of these domains inhibits transactivation in cell lines models, and blocks transcriptional activation of a reporter gene in fibroblasts[119].

In addition to the unique functional properties of E2A-PBX1, demonstrated largely in vitro, its respective contributions to oncogenesis have been demonstrated in a variety of in vivo assays, inducing myeloid, T-lymphoid, and B-lymphoid malignancies in mouse models[127-130]. On a molecular level, E2a-Pbx1 has been shown to contribute to lymphoid neoplasia via enhanced expression of Bmi-1 leading to subsequent repression of the *INK4A-ARF* tumor suppressor locus[131]. In addition, siRNA mediated

loss of E2A-PBX1 in t(1;19) ALL cell lines impairs cell viability, in part, by disrupting the aberrant transcriptional activation of *EB1* and *WNT16B*, both of which are thought to contribute to cellular transformation[132, 133].

Although t(1;19) ALL patients generally exhibit 80-85% relapse free survival rates[134], many of these patients must tolerate intensive chemotherapy regimens to achieve remission. Further, there remains a subset of patients with t(1;19) who experience a relapse. Salvage therapies including stem cell transplant remain suboptimal with less than 50% retrieval[9]. Therefore, these patients may benefit with the addition of tyrosine kinase inhibitors to salvage therapies to improve outcomes. Additionally, standard cytotoxic drugs exhibit toxicities that can give rise to a multitude of long term side effects and secondary malignancies[11]. As such, a gene targeted drug could offer great benefit to these patients by reducing rates of relapse and allowing patients to be treated with less intensive chemotherapy regimens while still exhibiting similar or more favorable outcomes.

2.3 Materials and Methods

2.3.1 Patient Samples. This study was approved by the Institutional Review Board of Oregon Health & Science University and the Children's Oncology Group. Peripheral blood or bone marrow aspirate samples were obtained with informed consent from all patients. Mononuclear cells were isolated by density gradient centrifugations. Cells were cultured in RPMI-1640 medium supplemented with 20% FBS (Atlanta Biologicals, Lawrenceville, GA), L-glutamine, insulin/transferrin/sodium selenite, penicillin/streptomycin, fungizone (Invitrogen, Carlsbad, CA), and 10^{-4} M 2-mercaptoethanol (Sigma). Aliquots of cells were used for DNA, RNA, and protein extractions.

2.3.2 Cell Culture. RCH-ACV, Kasumi-2, MHH-cALL2 and REH cells were obtained from DSMZ and cultured in RPMI supplemented with 10% FBS (or 20% for MHH-cALL2), penicillin/streptomycin, L-glutamine, and fungizone.

2.3.3 RT-PCR. Total RNA (Qiagen, RNeasy) from each sample was used to synthesize cDNA (Invitrogen SuperScript III) with random hexamer primers. Target genes were amplified using primers given in Appendix II.

2.3.4 Gene Expression Microarray. A meta-analysis was performed for gene expression microarray analyses of pediatric ALL patient samples and normal B-cell progenitor populations as previously described[135]. Datasets were processed and normalized using the RMA algorithm and normalization was validated based on even expression levels for a set of 7 reference genes (HPRT, COX6B, GUSB, GAPDH, PGK, ACTB and B2M) among all tissue samples studied. Gene expression values for the ROR1 probesets (211057_at and 205805_s_at) and the IGHM probeset (212827_at) were studied.

2.3.5 Immunoblotting and Flow Cytometry

All cells were lysed in sample buffer (75 mM Tris pH 6.8, 3% SDS, 15% glycerol, 8% β-mercaptoethanol, 0.1 % bromophenol blue) and separated by SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore, Billerica, MA) and subjected to immunoblot analysis with antibodies specific for ROR1 (R&D Systems, Minneapolis, MN), E2A-PBX1 (BD Biosciences, San Jose, CA), STAT3 and pSTAT3 (Cell Signaling), or β-actin (Millipore). Proteome Profiler Human Phospho-Kinase Array assays (R&D Systems) were performed and analyzed according to the manufacturer's protocol. Data was analyzed using ImageJ. For flow cytometry, cells were immunostained with antibody specific for ROR1 (R&D Systems) or Goat IgG (Jackson ImmunoResearch), washed 3 times with PBS wash buffer containing 2% FBS, then stained with Donkey Anti-goat IgG-Phycoerythrin (R&D Systems). Samples were analyzed on a BD FACSAria.

2.3.6 Mutational Analysis and Cloning

In patient 07-112, PCR products generated from ROR1 primers beginning in the exon 1 and ending in exon 9 were subcloned using the TOPO-TA cloning kit (Invitrogen) were sequenced using vector and gene specific primers.

2.3.7 Statistical Analyses

For cell growth and viability assays, a Student's t test was carried out for each siRNA treatment compared with non-specific siRNA.

2.4 Results

2.4.1 ROR1 expression in patient 07-112

To identify new tyrosine kinase gene targets in ALL patients, we tested clinical specimens from pediatric ALL patients by gene-silencing with an siRNA library that collectively targets the tyrosine kinome. Cells were electroporated with pre-validated siRNAs that individually target each tyrosine kinase as well as non-specific control siRNA[136]. After four days in culture, cells were subjected to an MTS assay for assessment of cell viability. Evaluation of the t(1;19)-positive sample 07-112 revealed hypersensitivity to siRNA targeting the receptor tyrosine kinase ROR1 (**Figure 1.1**). Other ALL cases with normal karyotype did not exhibit sensitivity to ROR1 silencing. Further evaluation by RT-PCR revealed overexpression of *ROR1* in sample 07-112 at levels comparable to artificial *ROR1* overexpression in Ba/F3 cells, while samples 07-230 and 07-266 exhibited little or no detectable *ROR1* expression (**Figure 2.3**).

Genomic DNA from patient 07-112 was used to determine the mutational status of ROR1 and confirmed the presence of only wild-type (WT) ROR1. Surprisingly, qualitative *ROR1* PCR of cDNA from patient 07-112 revealed the expression of four previously undescribed *ROR1* spice variants (described in further detail in **Figure 2.3**). Though interesting, the significance of these variants remains unclear as I have found no evidence of their expression at the protein level. I hypothesize that these splice variants are bystander products resulting from a combination of elevated *ROR1* expression levels and the aberrant mRNA processing often observed in malignant backgrounds[137].

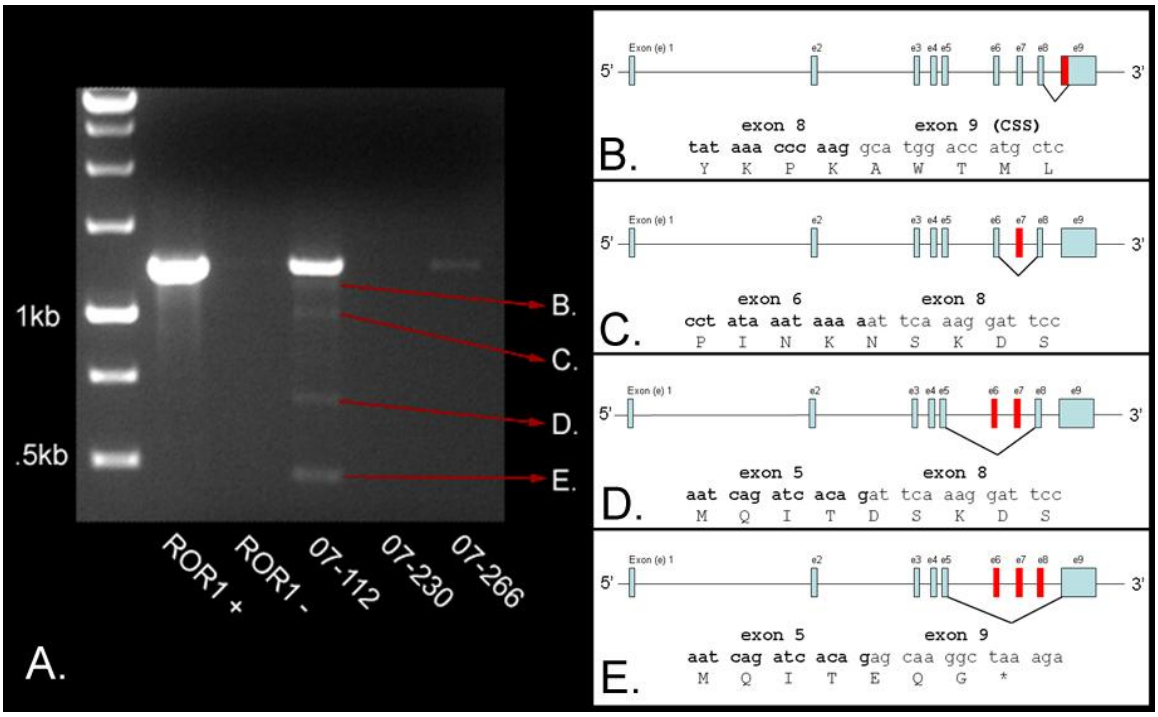


Figure 2.3. *ROR1* expression in E2A-PBX1-positive ALL patient 07-112. (A) cDNA from the murine pro-B cell line Ba/F3 transformed with wild-type human ROR1 (ROR1+) and normal parental Ba/F3 cells (ROR1-) were employed as positive and negative controls. Wild-type *ROR1* as well as four additional *ROR1* splice variants (B.–E.) are highly expressed in patient 07-112. Two representative E2A-PBX1-negative childhood B-cell precursor ALL patients are included; patient 07-230 shows no expression of *ROR1*, while patient 07-266 shows limited expression of wild-type *ROR1*. **(B)** The *ROR1* splice variant Δ9 CSS is the result of an aberrant splicing event that links the end of exon 8 to a cryptic splice site (CSS) located 100 base-pairs within exon 9. This splice variant produces an out-of-frame product that results in a receptor tyrosine kinase isoform terminating 12 amino acids after the abnormal splice site and eliminating the protein's tyrosine kinase domain. The CSS observed in this variant was predicted independently with a high degree of certainty by the splice site prediction programs NNSPLICE 0.9, NetGene2, and GENSCAN[138-141]. **(C)** The splice variant Δ7 *ROR1* is the result of an in-frame excision of exon 7 that produces a *ROR1* product missing its extracellular Kr domain. **(D)** The splice variant Δ6-7 *ROR1* is the result of an in-frame excision of exons 6 and 7, resulting in a *ROR1* product missing both its extracellular ligand-binding Fz and Kr domains. **(E)** The splice variant Δ6-8 *ROR1* is the result of an out-of-frame excision of exons 6, 7, and 8 that produces a truncated soluble isoform without a Kr, Fz or kinase domain.

2.4.2 ROR1 overexpression is conserved in t(1;19) ALL

To test whether the ectopic expression of *ROR1* observed in t(1;19) patient 07-112 was uniformly detectable in all t(1;19) ALL samples, I obtained ten pediatric ALL samples (generously provided by the Children's Oncology Group ALL Biology Lab) and two cell lines that are positive for t(1;19) and compared them with five pediatric ALL samples and two cell lines that are t(1;19)-negative. I observed that all t(1;19)-positive samples exhibited overexpression of *ROR1* while none of the t(1;19)-negative samples or normal white blood cells displayed the same phenotype (**Figure 2.4a**). Overexpression of ROR1 protein was also observed by immunoblot and FACS analysis on t(1;19)-positive cells (**Figure 2.4b,c**).

2.4.3 ROR1 expression in t(1;19) ALL is a product of B-cell differentiation arrest

To assess the extent and exclusivity of ROR1 expression in a larger cohort of patient samples, I examined microarray meta-analysis data generated from pediatric ALL patients and normal B-cell progenitors[135]. I compared t(1;19) ALL patients with those carrying t(9;22) (*BCR-ABL*), t(12;21) (*ETV6-RUNX1*), or patients with MLL (11q23) gene rearrangements. In addition, I evaluated *ROR1* levels in distinct, normal B-lineage progenitor populations ($CD34^+$ Lin^-, pro-B, pre-BI, pre-BII large, pre-BII small, and immature B cells). I observed significantly higher levels of *ROR1* expression on every t(1;19) patient compared with all patients from each of the other leukemic subsets. Similarly, t(1;19) patients showed significantly higher levels of *ROR1* expression compared with normal B-cell progenitor populations at the earliest stages of B-lineage development ($CD34^+$ Lin^-, pro-B, and pre-BI). However, when compared with

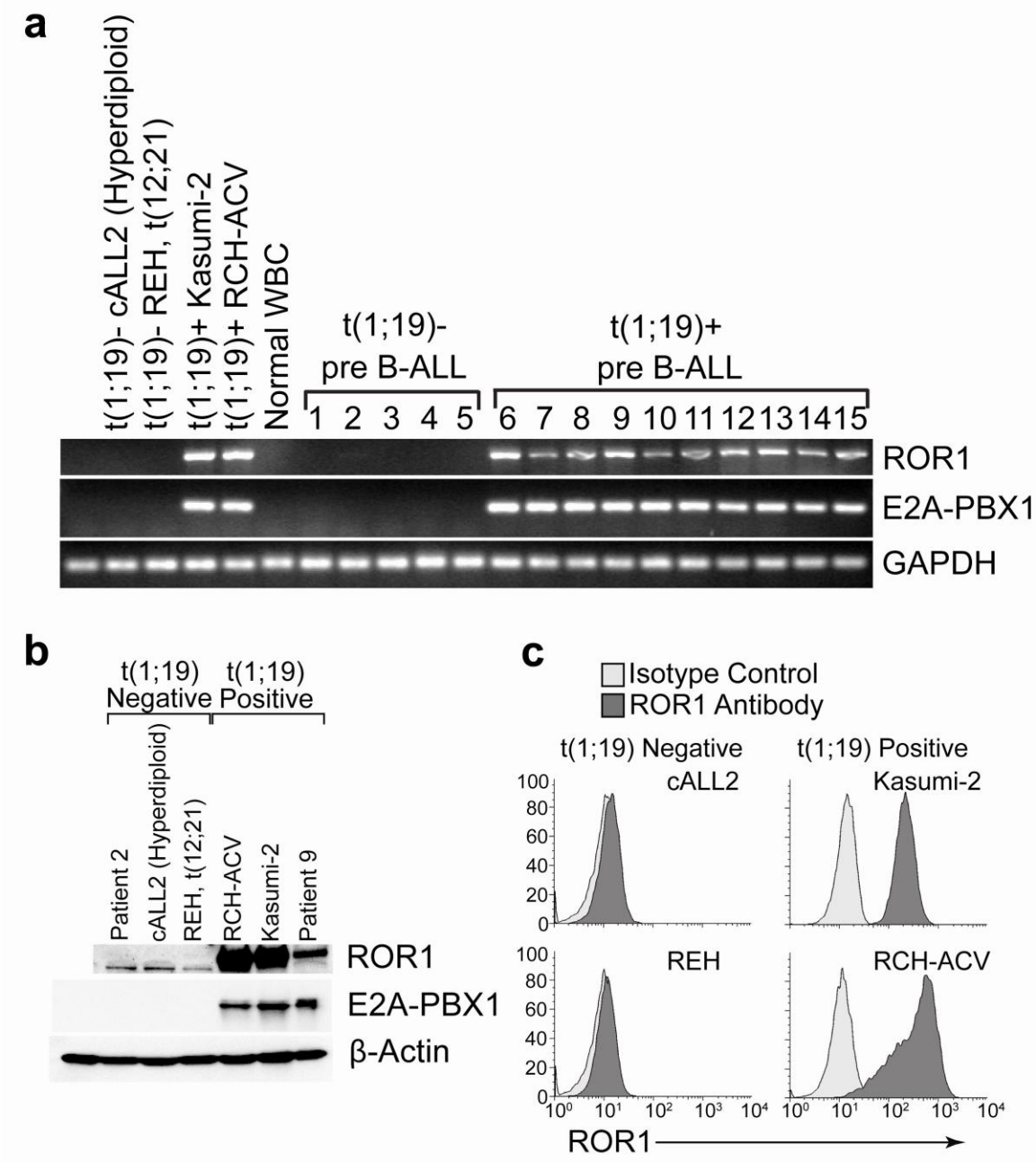


Figure 2.4. ROR1 overexpression is conserved in the t(1;19) ALL background. (A) cDNA derived from t(1;19)-positive and negative cell lines and primary patient samples was amplified using primers specific for ROR1, E2A-PBX1, or GAPDH and PCR products were analyzed by gel electrophoresis. **(B)** Whole cell extracts derived from t(1;19)-positive and negative cell lines and primary patient samples were subjected to immunoblot analysis using antibodies specific for ROR1, E2A-PBX1, or β-ACTIN. **(C)** Flow cytometric analysis of t(1;19)-positive and negative cell lines was performed using specific polyclonal anti-human ROR1 antibodies (dark grey histogram) versus isotype control (light grey histogram).

normal B-lineage cells at an intermediate stage of B-cell development (large/small pre-BII and immature B), I observed high levels of *ROR1*, similar to those seen on t(1;19) cells (**Figure 2.5**).

These results support recent findings showing ROR1 cell surface expression on intermediate stages of normal B-cell development[78]. Importantly, this and other studies did not observe ROR1 expression on normal, mature B cells and plasma cells[66, 70, 78]. Hence, in non-malignant B-lineage cells, ROR1 expression appears to be absent at the earliest stages of development, becomes highly expressed at intermediate/late stages of B-lineage development, and is then downregulated in normal, mature B cells. Interestingly, the vast majority of blasts from t(1;19) patients are arrested at this intermediate/late stage of B-lineage development (small pre-BII). As such, these data suggest that high ROR1 expression in t(1;19) may be a product of the comparatively mature differentiation state of these malignant blasts and may not be due to aberrant transcription profiles of the chimeric transcription factor E2A-PBX1. Subsequent examination of the E2A-PBX1 transcription factor in t(1;19) cell lines supports this hypothesis, since siRNA mediated knockdown of E2A-PBX1 in the t(1;19) cell line RCH-ACV showed a corresponding knockdown of the E2A-PBX1 transcriptional target *WNT16B* [133], but had no effect on expression of *ROR1* (**Figure 2.6**).

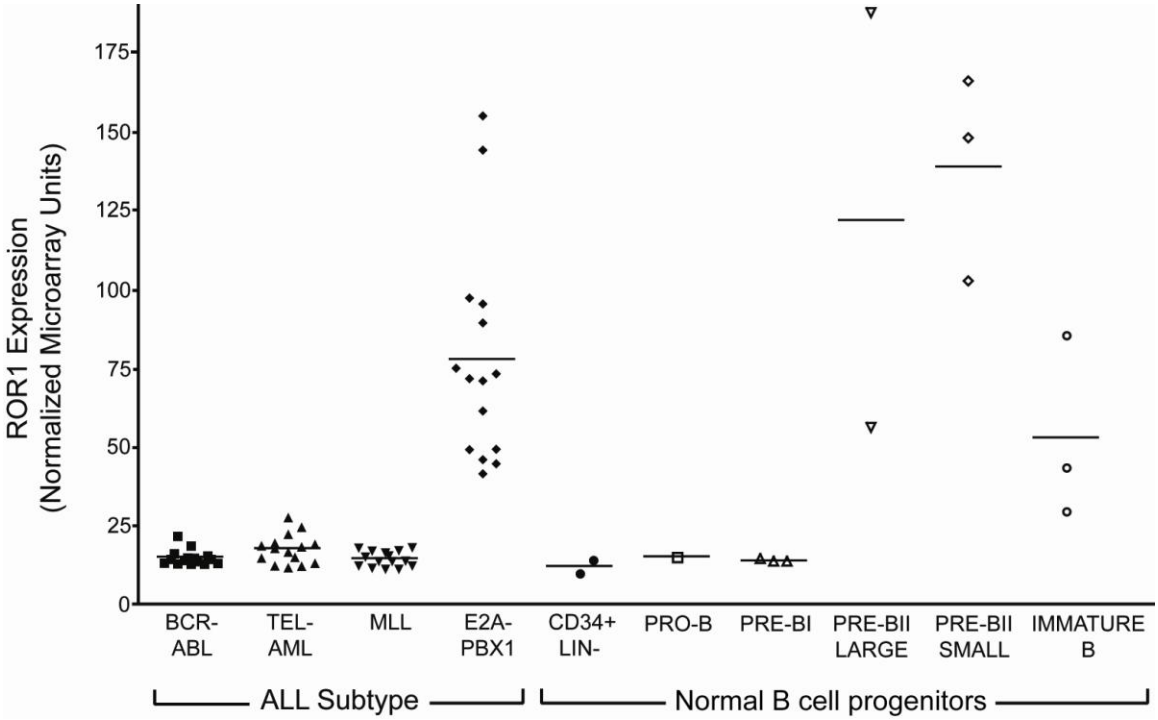


Figure 2.5. ROR1 is normally expressed during intermediate B-cell development. Gene expression microarray data for pediatric ALL patients and normal B-cell progenitor populations were compiled into a meta-analysis. Patients with MLL gene rearrangements, t(9;22) (BCR-ABL), t(12;21) (TEL-AML), or t(1;19) (E2A-PBX1) (n=15 for each subset) and non-malignant B-cell progenitor populations (CD34+ Lin-, pro-B, pre-BI, pre-BII large, pre-BII small, and immature B) (n=15 total) were examined and Affymetrix intensity values for ROR1 are plotted for each individual patient sample.

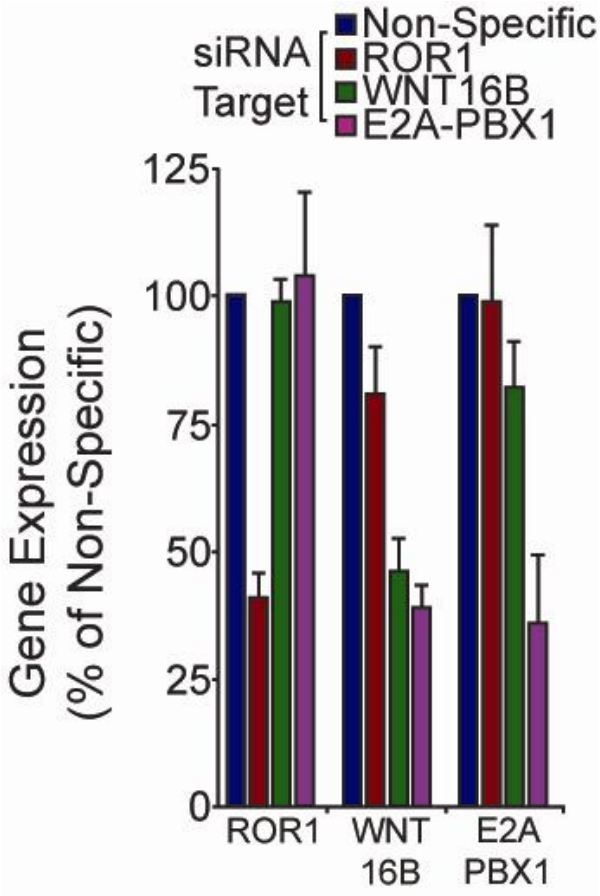


Figure 2.6. Loss of E2A-PBX1 has no effect on ROR1 expression. siRNA mediated depletion of the E2A-PBX1 chimeric transcription factor in RCH-ACV cells result in a concomitant loss of the E2A-PBX1 transcriptional target *WNT16B*. *ROR1* expression, however, is unaffected by loss of E2A-PBX1.

2.4.4 ROR1 expression is conserved in other intermediate/mature B-cell malignancies

ROR1 expression in B-cell malignancies was originally described in CLL. This is interesting when compared to t(1;19) ALL, as the coordinate normal B-cell progenitor population of t(1;19) ALL (small pre-BII) also expresses ROR1, whereas the normal coordinate population of CLL (Mature naïve and memory B cell) do not express ROR1 due their mature differentiation state[78]. This contrast suggests mature B-cell malignancies may retain ROR1 expression beyond its normal expression state, and that retention of ROR1 may be critical for disease progression. To determine if ROR1 expression is conserved in intermediate and mature B cell malignancies beyond t(1;19) ALL and CLL, I examined primary samples from a patient diagnosed with t(17;19) ALL and Burkitt's leukemia. The blasts from both these patients present characteristics shared by t(1;19) ALL, displaying expression of Igμ and lacking CD34. As expected, both of the primary samples showed high *ROR1* expression levels, similar to those observed in t(1;19) ALL and CLL primaries (**Figure 2.7**). Together, these data support a model of conserved ROR1 expression in intermediate to mature B-cell malignancies.

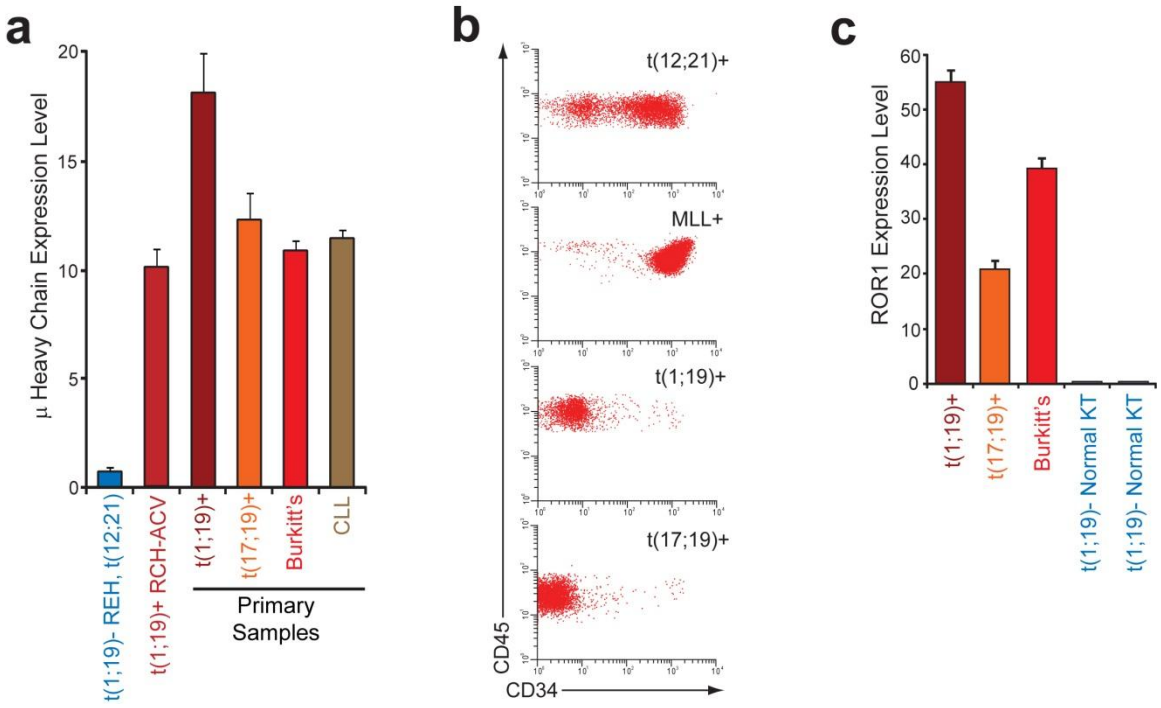


Figure 2.7. ROR1 is expressed in other intermediate/mature B-cell malignancies. **(A)** cDNA derived from REH, RCH-ACV, t(1;19) ALL, t(17;19) ALL, Burkitt's lymphoma, and CLL primary samples was analyzed for relative *ROR1* mRNA expression using quantitative PCR. cDNA was amplified using primers specific for *Igμ* and *GUSB*, and *Igμ* levels were normalized to *GUSB*. **(B)** t(12;21) ALL, MLL translocated ALL, t(1;19) ALL, and t(17;19) ALL primary samples were analyzed for CD34 surface expression by flow cytometry. Samples were gated upon CD45-positive blasts. **(C)** cDNA derived from t(1;19) ALL, t(17;19) ALL, Burkitt's lymphoma, and normal karyotype ALL primary samples was analyzed for relative *ROR1* mRNA expression using quantitative PCR. cDNA was amplified using primers specific for *ROR1* and *GAPDH*, and *ROR1* levels were normalized to *GAPDH*.

2.5 Discussion

These findings offer new insight into the functional importance of ROR1 in t(1;19) ALL as well as in the normal B-cell maturation process, but still little is known about the biological role of ROR1 in these settings. I show that ROR1 upregulation in t(1;19) ALL is a product of B-lineage development arrest at the pre-BII stage of B-cell development and not a result of aberrant regulation by the E2A-PBX1 transcription factor generated by the 1;19 translocation. My studies also show ROR1 is upregulated in t(17;19) ALL and Burkitt's leukemia/lymphoma, suggesting ROR1 may be expressed in most B-cell malignancies arrested at an intermediate/mature stage of development. This hypothesis is supported by recent findings showing ROR1 expression in cell lines and primary samples derived from patients with Mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL)[66, 70, 78, 142]. Importantly, the expression of ROR1 on cells from the mature B-lineage malignancies, CLL, MCL and Burkitt's, demonstrates a critical deviation from the normal distribution of ROR1-expression observed in B-cell development. While ROR1-expression is observed only in the intermediate stage of normal B-cell development, it is observed in both intermediate and mature B-cell malignancies. This important distinction suggests that retention of ROR1-expression into the mature stages of B-cell maturation plays a role in maintenance of cell viability of these mature, malignant B-cell clones. Unfortunately, a complete understanding of the molecular interactions that regulate expression from the ROR1 locus and its biochemical function within these cells are unresolved questions that must still be addressed.

The work described in Chapter 2 of my thesis identifies ROR1 as a consistent therapeutic target in t(1;19) ALL and other B-cell malignancies. The best mechanism

for therapeutic targeting of ROR1, however, remains unclear. The functional significance of ROR1 and its therapeutic potential as a target of tyrosine kinase inhibitors will be discussed in the subsequent chapter. But, regardless of ROR1 function in intermediate/mature B-cell malignancies, its conserved expression on the surface of these cells makes it a promising target for immunological based therapies. Immunotherapies that utilize chimeric antigen receptor (CAR)-modified T-cells targeting B-cell lineage-specific surface markers, such as CD19 and CD20, are actively being investigated in clinical trials for B-cell malignancies[143-145]. My data suggest that a similar strategy targeting ROR1 could prove valuable in treating most intermediate/mature B-cell malignancies. In fact, this strategy is already in development by a group at the Fred Hutchinson Cancer Research Center and showing promising results, whereby T-cells that have been engineered to express a ROR1-CAR efficiently lyse primary ROR1-expressing CLL and MCL samples[78]. However, due the nature of the therapy (introducing a T-cell that targets a self-antigen) it could present significant complications. As discussed in the introduction, expression of ROR1 in the adult (or post-embryonic) brain is controversial but likely will not present an issue, as the lone study reporting ROR1 in the brain describes a truncated version that lacks transmembrane and extracellular components that result in its localization in the cytoplasm. More concerning is the work of Hudecek et al.[78], who describe *ROR1* expression in adipose, pancreatic, lung, and ovarian tissue at the RNA level. While, these results have not been confirmed to show ROR1 expression at the cell surface, it could present a significant obstacle in the use of ROR1 as a target in CAR-T-cell

therapy. Further study addressing the potential toxicity of targeting ROR1 surface expression will be critical for advancing ROR1-targeting as a viable therapeutic option.

Finally, the controlled, transient expression of ROR1 during select stages of B-cell development, suggests an important role for ROR1 in supporting the differentiation process. Studies of ROR1's role in B-cell development have not been performed, so the effects of ROR1 loss or overexpression on B-cell development are not known. The concomitant upregulation of ROR1 expression with assembly and signaling from the pre-BCR suggests a potential physical interaction or signaling connection between the two. Downregulation of ROR1 following light-chain recombination and assembly of the mature-BCR supports the idea that transient ROR1 expression may support pre-BCR function during the pre-BCR to mature-BCR transition. The following chapter presents the first investigation of the connection between ROR1 and the pre-BCR.

Chapter 3

Compensatory Signaling from ROR1 and the Pre-B-Cell Receptor Promote Survival of t(1;19) Acute Lymphoblastic Leukemia

The work detailed in this chapter was presented in abstract form at the 53nd annual meeting of the American Society of Hematology in San Diego, CA, December 11th, 2011 and is currently under revision for publication. The contributions of co-authors are detailed in Appendix II.

3.1 Abstract

Aberrant tyrosine kinase activity is commonly implicated in the pathogenesis of leukemia and other cancers. Identification of these leukemogenic tyrosine kinases has proven invaluable for diagnostic and prognostic stratification of patients as well as for the development of novel strategies for therapeutic intervention. Our lab previously demonstrated that siRNA screening of mononuclear cells from leukemia patients can determine sensitivity to individual tyrosine kinases. With the goal of uncovering novel viability-dependent tyrosine kinases in leukemia patients, we have employed an RNAi-assisted protein target identification (RAPID) assay to screen cytogenetic subtypes of acute lymphoblastic leukemia (ALL). ALL is the most common pediatric cancer, accounting for one-quarter of all childhood malignancies. Childhood ALL has a primarily B cell precursor phenotype and is characterized by chromosomal abnormalities, primarily translocations and duplications. One of the most common recurring translocations associated with pediatric ALL, t(1;19)(q23;p13.3), generates the E2A-PBX1 fusion product. In Chapter 2, I show conserved expression of the receptor tyrosine kinase ROR1 in t(1;19) ALL and other intermediate/mature B-cell malignancies. Here, I show that t(1;19) ALL is dependent upon ROR1 expression for viability. In addition, I identify a kinase inhibitor, dasatinib, with significant activity against t(1;19) ALL cells due to its capacity to inhibit tyrosine kinases necessary for transduction of pre-B-cell receptor (pre-BCR) signaling. Finally, I show that ROR1 and the pre-BCR activate mutually compensatory signaling pathways, suggesting that optimal therapeutic regimens would include agents targeting both pathways.

3.2 Introduction

3.2.1 The pre-B-cell receptor complex

The pre-BCR complex signals and is regulated in a manner similar to the mature-BCR[146, 147]. Both complexes are composed of a central transmembrane immunoglobulin (Ig) complex, which consists of a heterodimer of Igα and Igβ as well as the transmembrane form of the Ig heavy-chain (IgH)[80]. Likewise, signaling from the two complexes is initiated by the SRC-family of tyrosine kinases, which phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) present on the intracellular portion of Igα and β[148]. Phosphorylated Igα and β recruits the tyrosine kinase SYK which, in turn, activates downstream effector pathways (**Figure 3.1**)[149].

Despite the conserved components of the pre-BCR and BCR, it is a small difference between the two complexes that confers significant functional differences. Unlike the mature receptor, the pre-BCR lacks the Ig light-chain (IgL); instead, two proteins, λ5 and VpreB, come together to form the surrogate light-chain (SLC)[150]. And unlike the polymorphic IgL, all SLCs are identical. The effect of which is a pre-BCR that lacks the ability to bind conventional ligand. Because of this characteristic, the pre-BCR does not possess a stimulated signaling response similar to the BCR. Instead, the pre-BCR has basal signaling activity that is critical for maturation through the pro-B-cell/pre-B cell check point and initiation of IgL gene recombination[87, 151].

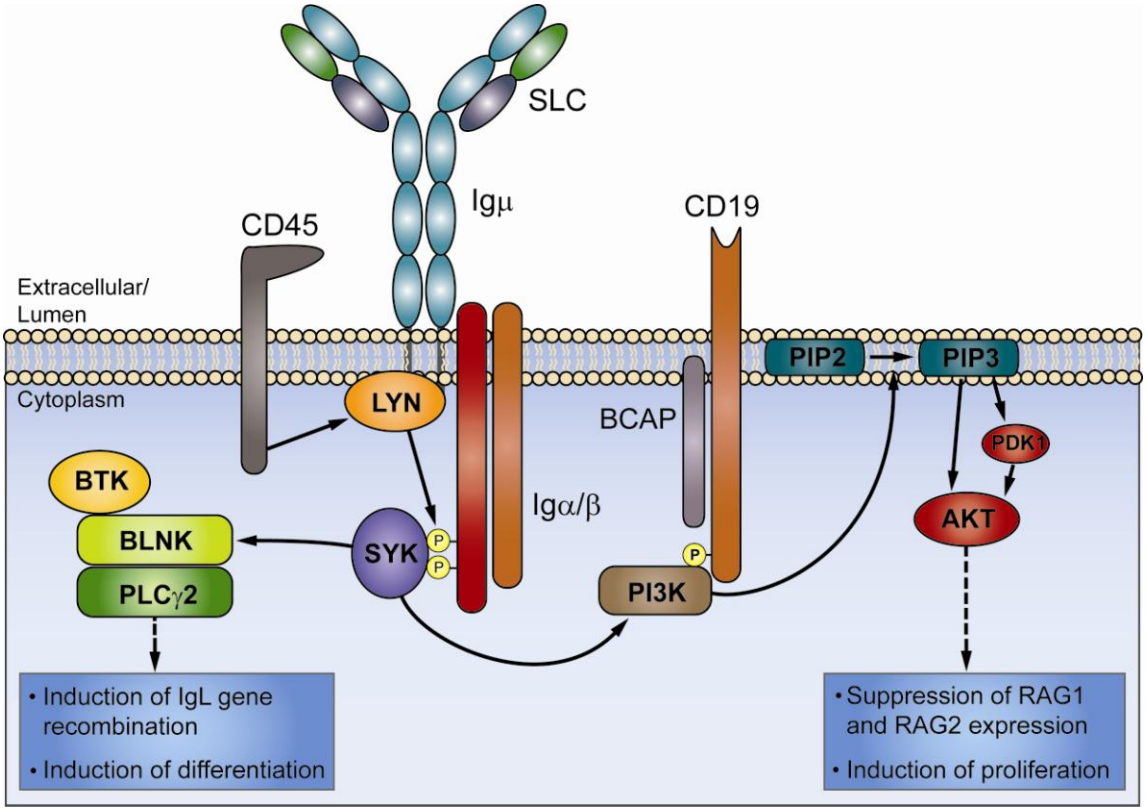


Figure 3.1. Structure and function of the pre-BCR. The central transmembrane component of the pre-BCR consists of the Igα and Igβ heterodimer and the Ig-heavy chain, μ. Extracellular Igμ associated with the surrogate light-chain (SLC), which is composed of the proteins λ5 and VpreB. The co-receptors CD45 and CD19 act as positive regulators of pre-BCR signling. CD45 dephosphorylates SRC-family kinases associated with the pre-BCR (LYN is used as an example here), activating them, and allowing phosphorylation of Igα and Igβ. Phosphorylation of Igα and Igβ initiates recruitment and activation of SYK, which activates downstream signaling pathways. CD19 and BCAP facilitate SYK mediated activation of the PI3K/AKT pathway by forming a docking site for PI3K. AKT activation following association with PIP3 and phosphorylation by PDK1 leads to suppression of the recombination protein RAG1 and RAG2 as well as induction of proliferation. The opposing BLNK/PLCγ2 pathway, is also activated by SYK, but is mediated by a multi-protein complex formed around the scaffold protein BLNK. Activation of BTK and PLCγ2 by association with BLNK leads to induction of IgL recombination, downregulation of SLC expression, and initiation of differentiation. Figure adapted from Herzog et al. and Monroe[79, 80].

The Igα and β heterodimer component of the transmembrane pre-BCR complex is both necessary and sufficient for pre-BCR signal transduction, and associated intracellular SRC-family kinases are required for signal activation[152, 153]. Three Src-family kinases in particular, Blk, Fyn, and Lyn, have been shown to have somewhat redundant activity and loss of all three in B-cell progenitors blocks transition through the pro-B-cell/pre-B-cell checkpoint[148]. SRC-family kinases are activated at the pre-BCR by the associated phosphatase CD45. CD45 dephosphorylates an inhibitory tyrosine residue on the SRC, allowing it to initiate signaling by phosphorylating the ITAM domain of Igα and β[154, 155]. Phosphorylated ITAM-associated tyrosine residues acts as docking sites for recruitment and activation of the SH2-domain-containing kinase, SYK, a critical activator of the pre-BCR's downstream effector pathways[149, 156, 157].

Downstream of activated SYK are two critical regulatory pathways. The first pathway is mediated by PI3K and AKT and is required for proliferation. The second pathway is mediated by a signaling complex organized by BLNK (aka SLP65) and is responsible for differentiation. The proliferation pathway is initiated by SYK and SRC-family kinase mediated phosphorylation of the pre-BCR co-receptors CD19 and BCAP (B-cell PI3K adaptor)[158]. Phosphorylation of CD19 and BCAP results in recruitment and activation of PI3K, which phosphorylates its substrate, PIP_2, resulting in generation of PIP_3. The pleckstrin-homology domain of AKT and PDK1 binds PIP_3 at the cell membrane, where PDK1 phosphorylates and activates AKT[159, 160]. AKT plays a critical role in regulating diverse pre-B-cell functions, including suppression of RAG recombination proteins, inhibition of IgL recombination, and induction of cell proliferation[161]. The FOXO family of transcription factors serve as important

downstream effectors of AKT. These proteins are inhibited by AKT mediated phosphorylation, which excludes them from the nucleus and activates their degradation. FOXO transcription factors have been shown to regulate diverse processes required for B-cell differentiation, including transcriptional regulation of RAG1, the CDK inhibitor p27, and several receptor tyrosine kinases[162-164]. The differentiation pathway is initiated by activation of BLNK by SYK phosphorylation, which allows it to serve as a scaffolding protein for recruitment and association with BTK, PLCy2 and GRB2[165, 166]. Signaling through this multiprotein complex results in termination of SLC expression and upregulation of the RAG recombination proteins and IRF4. The result of which, is an opening of the *IgL* (*Igκ*) locus and initiation of light-chain recombination. This, in turn, drives differentiation of pre-BCR-positive cells to mature-BCR-positive cells[167-169].

Because SYK activates both of these pathways, careful regulation of SYK activity by pre-BCR co-receptors, associated kinases and adaptor proteins is critical to prevent uncontrolled cellular expansion and malignancy. For example, loss of BLNK expression (which participates in the pro-differentiation pathway and can impair AKT signaling) is associated with increased pre-BCR expression and high incidence of pre-B-cell leukemias in mice[96]. Similarly, reduction of BLNK expression has been observed in the malignant blasts of patients with pre-B ALL[170]. These studies illustrate how loss of a pro-differentiation component of the pre-BCR, not only disrupts differentiation but can influence the pro-proliferation pathway. Theoretically, this same principal can be used to treat pre-B ALL. Inhibitor mediated disruption of the pro-proliferation pathway, (with an AKT inhibitor, for example) should, in principle, support activation of the pro-

differentiation pathway and result in activation FOXO proteins, induction of cell cycle arrest, and initiation of differentiation.

The work of Hudecek et al.[78], combined with my work detailed in Chapter 2, has now thoroughly detailed the expression profile of ROR1 during B-cell development. Hudecek et al. shows consistent surface expression of ROR1 during the early and late intermediate stages of development, while my work looks at *ROR1* expression at the RNA level and demonstrates a more detailed expression profile, exhibiting upregulation at the large pre-BII stage of development, retained elevated expression at the small pre-BII stage, and decreased (but still present) expression at the immature B-cell stage. Importantly, the expression profile of ROR1 distinctly correlates with assembly and signaling from the pre-BCR. The work detailed in Chapter 3 investigates ROR1's relationship with the pre-BCR and their role in B-cell development and ALL.

3.2.2 The small-molecule tyrosine kinase inhibitor dasatinib

The success of imatinib as a treatment for CML ushered in a new generation of cancer therapeutic development based upon strategic targeting of viability dependent proteins[171]. Much of this work began with next generation inhibitors of BCR-ABL being developed in response to relapse of patients receiving imatinib therapy. The primary mechanism of CML relapse stems from kinase domain mutations in BCR-ABL[172]. At least 25 amino acid mutations have been identified throughout the ABL-kinase domain that reduce sensitivity to imatinib. Amplification of the BCR-ABL fusion gene in CML cells represents an alternative mechanism of relapse. Developing more potent BCR-ABL inhibitors that would inhibit the most common relapse mutations and overcome fusion gene amplification became an immediate priority for CML

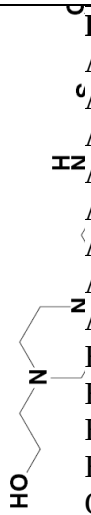
treatment[173]. Two approaches were used to find second generation inhibitors of BCR-ABL that would target imatinib resistant BCR-ABL mutant clones. One method made small modifications to the existing imatinib molecule to alter its binding affinity and screen it against ABL mutants[174]. The second method took preexisting drugs, designed to target other kinases, and screened them for activity against Abl. This second method led to the development of dasatinib[175]. Dasatinib was originally developed as an inhibitor of SRC-family kinases, but showed potent activity against wild-type BCR-ABL as well as many imatinib resistant mutants[176]. Phase 1 trials of dasatinib showed patients with imatinib-resistant BCR-ABL mutations (except T315I) had a favorable response to therapy. Likewise, patients with mutation-independent resistance responded well to dasatinib. These favorable results led to FDA approval of dasatinib for treatment of imatinib-resistant CML[177].

Dasatinib's original use, as a SRC-family kinase inhibitor, has also proven valuable and it is this activity that interests us in dasatinib as a potential therapy for B-cell malignancies. As described above, SRC-family kinases are critical activators of the pre-BCR, and loss of these kinase (LYN, BLK, and FYN) results in abrogation of pre-BCR signal. Inhibition of pre-BCR signaling and its downstream activation of AKT represents an interesting and novel means of treating pre-BCR-positive pre-B malignancies. In addition, because the following chapter will interrogate the connection between the pre-BCR and ROR1, dasatinib serves as a valuable tool to selectively disrupt the pre-BCR signal.

While dasatinib is primarily an inhibitor of ABL and SRC-family kinases, it does possess activity against other kinases. It is important that these activities be taken into

consideration when performing inhibitor experiments with dasatinib. **Figure 3.2** shows dasatinib affinities for all kinases with binding constants in a relevant dosage range (<500nM)[178].

Dasatinib



Kinase Target	K_d (nM)	Kinase Target	K_d (nM)
ABL1	0.53	FYN	0.79
ABL1 (E255K)	2.1	GAK	2.6
ABL1 (T315I)	590	HCK	0.35
ABL2	0.17	KIT	0.62
ACVR1B	330	LCK	0.2
ACVR2A	210	LIMK2	86
ACVRL1	460	LYN	0.57
ADCK3	190	MAP3K4	310
BLK	0.21	MAP4K5	45
BMX	1.4	NLK	260
BRAF	500	p38-alpha	27
BTK	1.4	p38-beta	410
CSF1R	0.58	PDGFRA	0.47
CSK	1	PDGFRB	0.63
DDR1	0.69	PKMYT1	130
DDR2	3.2	PTK6	7.8
EGFR	120	RIPK2	31
EPHA1	4.1	SNF1LK	3.9
EPHA2	0.85	SNF1LK2	6.4
EPHA3	0.093	SRC	0.21
EPHA4	1.2	SRMS	13
EPHA5	0.24	STK36	210
EPHA8	0.24	TESK1	13
EPHB1	0.45	TGFBR1	33
EPHB2	0.39	TGFBR2	230
EPHB3	6.9	TNK2	5.6
EPHB4	0.34	TNNI3K	11
ERBB4	55	TXK	2.1
FGR	0.5	YES	0.3
FRK	.31	ZAK	45

Figure 3.2. Kinase targets of dasatinib. Dasatinib's chemical structure and binding constants for relevant kinases are presented. Binding constants for dasatinib against 317 kinases were determined as described by Karaman MW et al[178]. Kinases with K_d's below 500nM are included. The ABL1 mutant T315I is included for comparison.

3.3 Materials and Methods

3.3.1 Patient Samples. This study was approved by the Institutional Review Board of Oregon Health & Science University and the Children's Oncology Group. Peripheral blood or bone marrow aspirate samples were obtained with informed consent from all patients. Mononuclear cells were isolated by density gradient centrifugations. Cells were cultured in RPMI-1640 medium supplemented with 20% FBS (Atlanta Biologicals, Lawrenceville, GA), L-glutamine, insulin/transferrin/sodium selenite, penicillin/streptomycin, fungizone (Invitrogen, Carlsbad, CA), and 10^{-4} M 2-mercaptoethanol (Sigma). Aliquots of cells were used for DNA, RNA, and protein extractions.

3.3.2 Primary leukemia cell xenograft. 2×10^6 cells from a bone marrow biopsy of a patient with t(1;19)-positive ALL were inoculated via tail vein injection into sublethally irradiated (250 cGy) NOD/SCID mice. When the mice became terminally ill because of overt leukemia, they were sacrificed and leukemia cells were harvested from bone marrow and spleen. Leukemic infiltration was confirmed by flow cytometry and cells were suspended in culture media (RPMI-1640 medium supplemented with 20% FBS, L-glutamine, insulin/transferrin/sodium selenite, penicillin/streptomycin, fungizone, and β-mercaptoethanol).

3.3.3 Cell Culture. RCH-ACV, Kasumi-2, MHH-cALL2 and REH cells were obtained from DSMZ and cultured in RPMI supplemented with 10% FBS (or 20% for MHH-cALL2), penicillin/streptomycin, L-glutamine, and fungizone.

3.3.4 RT-PCR. Total RNA (Qiagen, RNeasy) from each sample was used to synthesize cDNA (Invitrogen SuperScript III) with random hexamer primers. Target genes were amplified using primers given in Supplementary Table 1.

3.3.5 Gene Expression Microarray. A meta-analysis was performed for gene expression microarray analyses of pediatric ALL patient samples and normal B-cell progenitor populations as previously described[135]. Datasets were processed and normalized using the RMA algorithm and normalization was validated based on even expression levels for a set of 7 reference genes (HPRT, COX6B, GUSB, GAPDH, PGK, ACTB and B2M) among all tissue samples studied. Gene expression values for the ROR1 probesets (211057_at and 205805_s_at) and the IGHM probeset (212827_at) were studied.

3.3.6 Immunoblotting and Flow Cytometry

All cells were lysed in sample buffer (75 mM Tris pH 6.8, 3% SDS, 15% glycerol, 8% β-mercaptoethanol, 0.1 % bromophenol blue) and separated by SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore, Billerica, MA) and subjected to immunoblot analysis with antibodies specific for ROR1, BTK, LYN, phospho-SYK, (R&D Systems, Minneapolis, MN), E2A-PBX1 (BD Biosciences, San Jose, CA), AKT, ERK1/2, SYK, CD79a, phospho-LYN, phosphor-ERK1/2, phosphor-MEK, phospho-CD79a, phospho-AKT (Cell Signaling), 4G10 Phosphotyrosine or β-actin (Millipore). Proteome Profiler Human Phospho-Kinase Array assays (R&D Systems) were performed and analyzed according to the manufacturer's protocol. Data was analyzed using ImageJ. For flow cytometry, cells were immunostained with antibody specific for ROR1 (R&D Systems) or Goat IgG (Jackson ImmunoResearch), washed 3 times with

PBS wash buffer containing 2% FBS, then stained with Donkey Anti-goat IgG-Phycoerythrin (R&D Systems). Samples were analyzed on a BD FACSAria.

3.3.7 Cell Viability Experiments

For proliferation and viability assays, cells were electroporated with 1-2 μM siRNA at 250 V, 0.2 ms, 2 pulses and incubated for 96 hours at which point a CellTiter 96 AQ_{ueous} One solution cell proliferation assay was performed (Promega, Madison, WI). Absorbance values were normalized to control wells containing cells electroporated with non-specific siRNA. For apoptosis studies, cells were stained with Guava Nexin Reagent (Millipore) and analyzed on a Guava EasyCyte Plus Flow Cytometry System (Millipore).

3.3.8 Statistical Analyses

For cell growth and viability assays, a Student's t test was carried out for each siRNA treatment compared with non-specific siRNA.

3.4 Results

3.4.1 ROR1 is a viability dependent target in t(1;19) ALL

Since the two t(1;19) cell lines, RCH-ACV and Kasumi-2, recapitulated the ROR1 expression profile observed in t(1;19)-positive primary specimens (**Figure 2.4**), I next tested whether these cell lines were also sensitive to ROR1 silencing, as we observed with sample 07-112 in the RAPID assay (**Figure 1.1**). I treated both cell lines as well as the control t(1;19)-negative cell line REH with siRNA specific for ROR1. Consistent with the results from sample 07-112, both RCH-ACV and Kasumi-2 cells were sensitive to ROR1 silencing. Loss of ROR1 resulted in reduced viability as well as reduced cell outgrowth (**Figure 3.3**). In addition, to confirm that this loss of viability is not the result of off-target effects of the pooled ROR1 siRNA, RCH-ACV cells were treated with 3 individual siRNA duplexes that target different portions of ROR1 mRNA. This resulted in reductions of RCH-ACV cell viability that were proportional to the silencing capacity of each of the siRNA duplexes (**Figure 3.4a**). Similarly, I designed an siRNA duplex that targets only the 3'-UTR of ROR1 and stably overexpressed an ORF-only ROR1 variant possessing a V5 epitope-tag in RCH-ACV cells. As expected, overexpression of ROR1-V5 in these cell rescued the viability effect of ROR1 loss following knockdown of only endogenous ROR1 (**Figure 3.4b,c**).

I next confirmed that this finding of ROR1-dependence in t(1;19) ALL samples was also reproducible in early passage t(1;19) cells propagated by xenograft into immunocompromised NOG mice. I obtained xenograft cells derived from a t(1;19) patient and tested for ROR1 overexpression as well as for sensitivity to ROR1

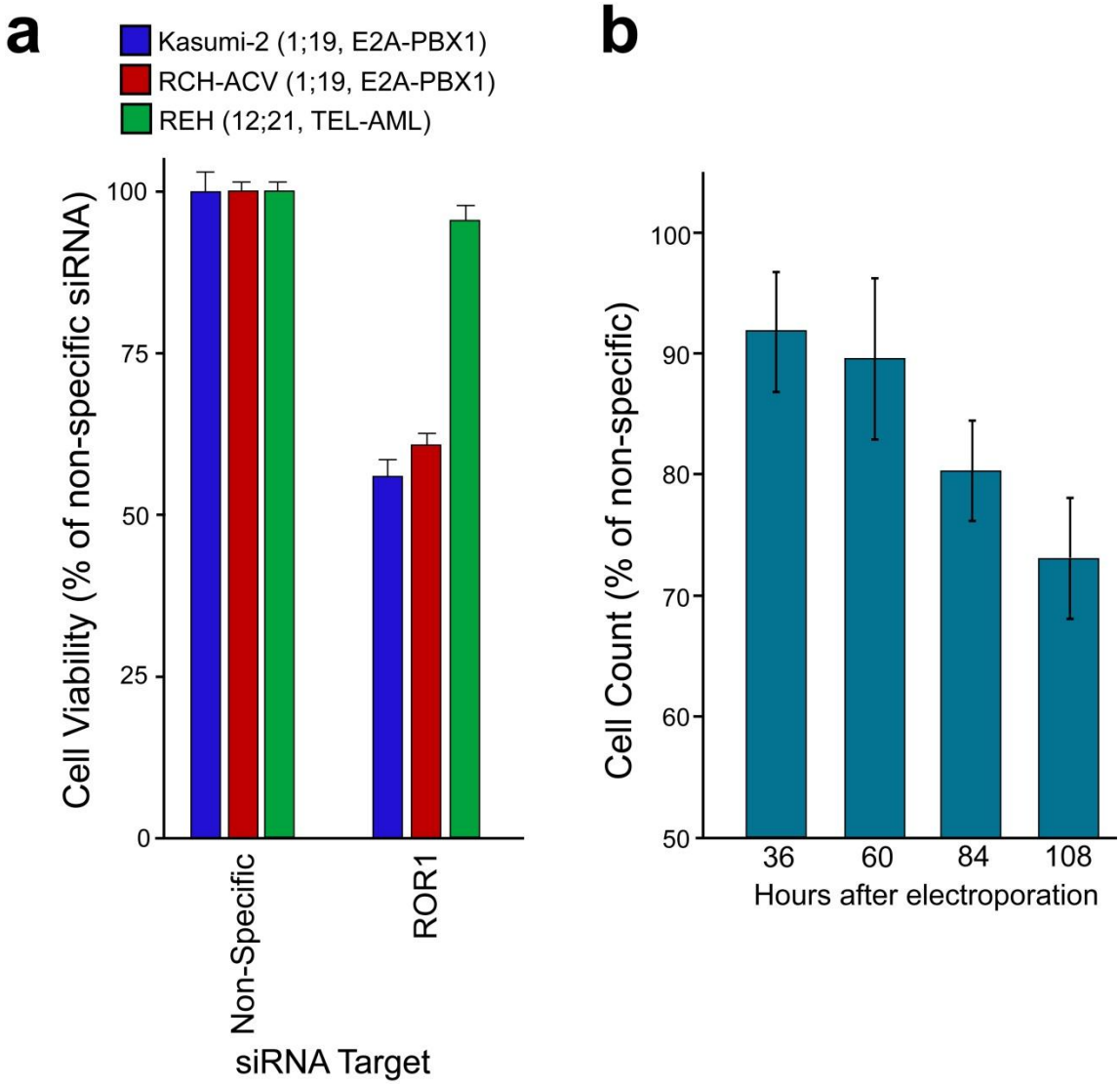


Figure 3.3. ROR1 dependent viability in t(1;19) ALL cell lines. (**A**) **(**RCH-ACV or Kasumi-2 cells (both t(1;19)-positive) as well as REH cells (t(12;21)-positive) were electroporated in the presence of non-specific siRNA or siRNA targeting ROR1 and plated into culture media. After 4 days, cells were subjected to an MTS assay to measure cell viability. Values represent percent mean (normalized to non-specific control wells) ± s.e.m. (n = 10). (**B**) ROR1 loss impairs t(1;19) ALL cell growth. RCH-ACV cells electroporated with non-specific control siRNA or ROR1-specific siRNA were grown in culture for 36 hours before performing cell counts every 24 hours. Due to electroporation conditions, RCH-ACV doubling rates are slower than when compared to unelectroporated RCH-ACV control cultures (data not shown). Still, cells treated with ROR1-specific siRNA show significantly impaired cellular outgrowth compared with non-specific siRNA (**B**).

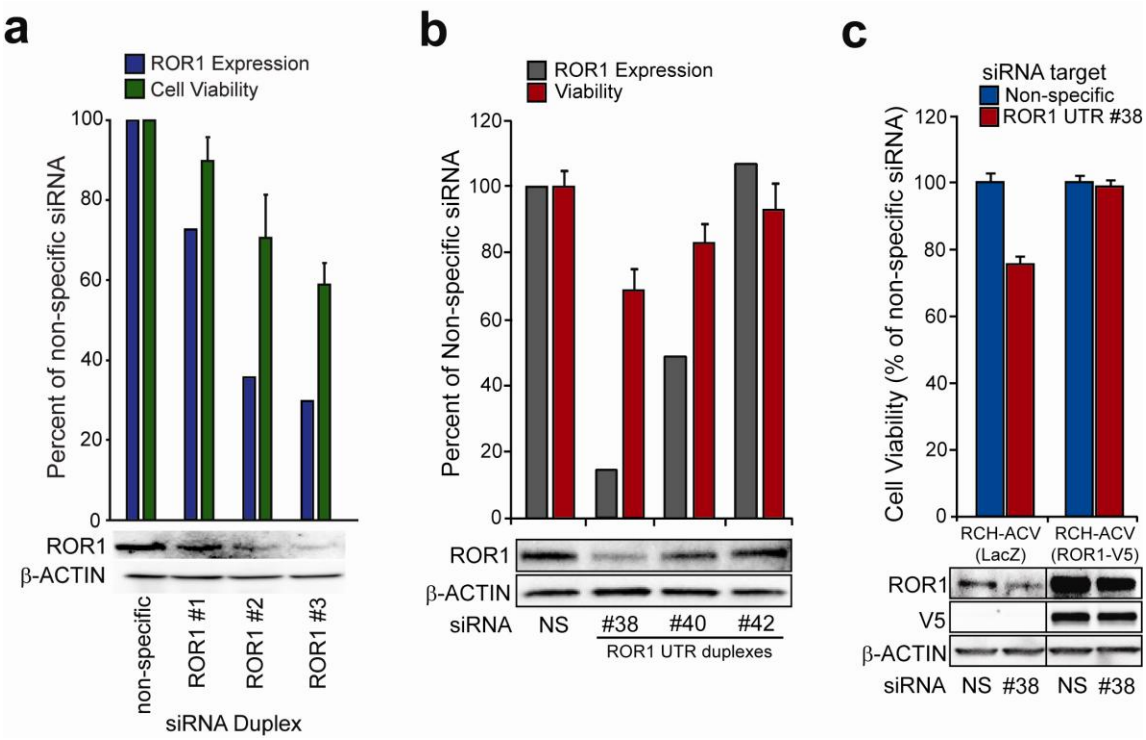


Figure 3.4. Sensitivity to pooled ROR1 siRNA is a ROR1-specific effect. (**A**) RCH-ACV cells were electroporated in the presence of an individual duplex of non-specific siRNA or 3 individual duplexes of siRNA targeting different portions of ROR1 and cells were plated into culture media. After 3 days, cells were lysed and subjected to immunoblot with antibodies specific for ROR1 or β-ACTIN. After 4 days, parallel cultures of cells were subjected to an MTS assay to measure cell viability. Values represent percent mean (normalized to non-specific control wells) ± s.e.m. (n = 6). (**B**) RCH-ACV were treated as described above, only 3 individual siRNA duplexes design to target the UTR regions of the ROR1 transcript were used for ROR1 knockdown. Cells were assessed for efficiency of ROR1 knockdown and viability. (**C**) RCH-ACV cells stably expressing ROR1-V5 or a LacZ control were treated as described above using a single non-specific siRNA or ROR1 UTR duplex #38. Cells were assessed for efficiency of ROR1 knockdown and viability.

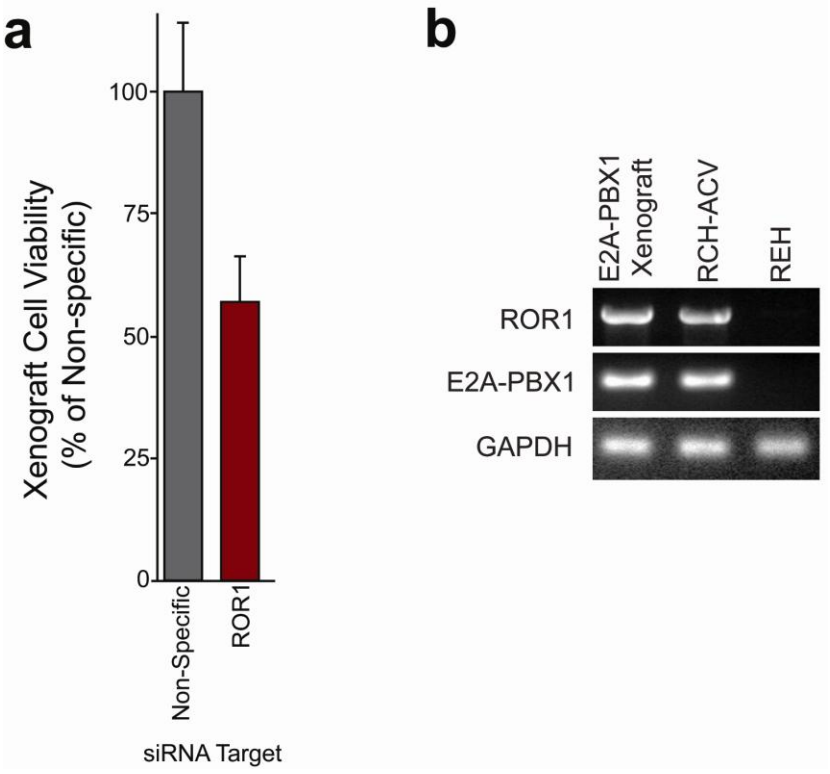


Figure 3.5. ROR1 dependent viability in early passage t(1;19) ALL xenographs. (A) Primary cells from a t(1;19) ALL patient were propagated in NOD-SCID mice lacking the IL-2 receptor γ chain. Xenograft cells were harvested from bone marrow and spleen of overtly leukemic mice and electroporated with non-specific or ROR1-targeting siRNA. After 4 days, cells were subjected to an MTS assay to measure cell viability. Values represent percent mean (normalized to non-specific control wells) ± s.e.m. (n = 4). **(B)** Primary cells from a t(1;19) ALL patient were propagated in a xenograft mouse model as above and RNA was harvested from cell extracts. PCR was performed on cDNA with primers specific for ROR1, E2A-PBX1, or GAPDH. The t(1;19) positive (RCH-ACV) and negative (REH) cell lines were included for comparison.

siRNA. I found that both features were recapitulated in these early passage t(1;19) xenograft cells (**Figure 3.5**).

3.4.2 Dasatinib impairs t(1;19) ALL viability

The unique and consistent overexpression of ROR1 in the t(1;19) pediatric ALL setting as well as the consistent sensitivity of t(1;19) cells to ROR1 siRNA makes ROR1 an appealing candidate target for therapeutic intervention in this subset of patients. The classification of ROR1 as a cell-surface, receptor tyrosine kinase suggests several potential strategies for targeting of the ROR1 protein, including small-molecule kinase inhibitors and immunological reagents such as ROR1-specific antibodies or T-cell chimeric antigen receptors (CARs). Since small-molecule kinase inhibitors have yielded promising clinical results for a wide range of other dysregulated kinases, I chose to first assess whether a candidate kinase inhibitor could be identified for the t(1;19) subset of ALL. To accomplish this, I employed a library of 66 small-molecule kinase inhibitors with a broad range of target specificities. Although there is no information about the potential of these compounds for targeting of ROR1, I conducted an initial cytotoxicity screen with two t(1;19) cell lines (RCH-ACV and Kasumi-2) as well as one ROR1-negative pediatric ALL cell line (REH). I observed that the FDA-approved drug dasatinib exhibited effective killing of the ROR1-positive cell lines while the ROR1-negative cell line was insensitive to dasatinib to the highest dose tested (**Figure 3.6a**). To determine whether this observation in patient-derived cell lines was also true in primary cells taken directly from ALL patients, I tested the effects on cell viability of leukemia cells from ten ALL patients of varying disease subsets over graded

concentrations of dasatinib. Two of these ten patient samples were obtained from t(1;19)-positive patients and both samples tested were highly sensitive to dasatinib, with IC_{50} values of approximately 2 and 12 nM, while the eight other ALL samples did not achieve IC_{50} values even at the highest tested concentration (1000 nM) (**Figure 3.6b**). These results purposely exclude 9;22-translocated pediatric ALL samples, as they express the BCR-ABL oncogene and are known to be sensitive to dasatinib. Importantly, examination of ROR1 levels in these two t(1;19) patients confirmed ROR1 overexpression similar to levels observed on all other t(1;19) samples previously tested (**Figure 3.6c**).

3.4.3 Dasatinib disrupts pre-BCR signaling in t(1;19) ALL

The dual sensitivity of t(1;19) ALL cells to dasatinib and ROR1 siRNA suggests that ROR1 may represent a target of dasatinib and this may mechanistically explain dasatinib sensitivity in the setting of t(1;19). However, evaluation of the phosphorylation state of ROR1 following immunoprecipitation surprisingly showed no evidence of endogenous ROR1 tyrosine phosphorylation (**Figure 3.7**). Likewise, *in vitro* kinase activity assays revealed no significant ROR1 kinase activity, a finding consistent with previous studies showing no ROR1 kinase activity[44, 46].

Since dasatinib is a small-molecule kinase inhibitor, I hypothesized that dasatinib sensitivity of t(1;19) cells must be mediated by alternative kinases with intact catalytic activity that are expressed in these cells. As noted above, greater than 90% of t(1;19) specimens derive from cells that are arrested at the small pre-BII stage of B-lineage development, at which stage one can also observe expression of the cytoplasmic pre-BCR complex, as shown by expression of Ig heavy-chain, μ (**Figure 3.8**). As such,

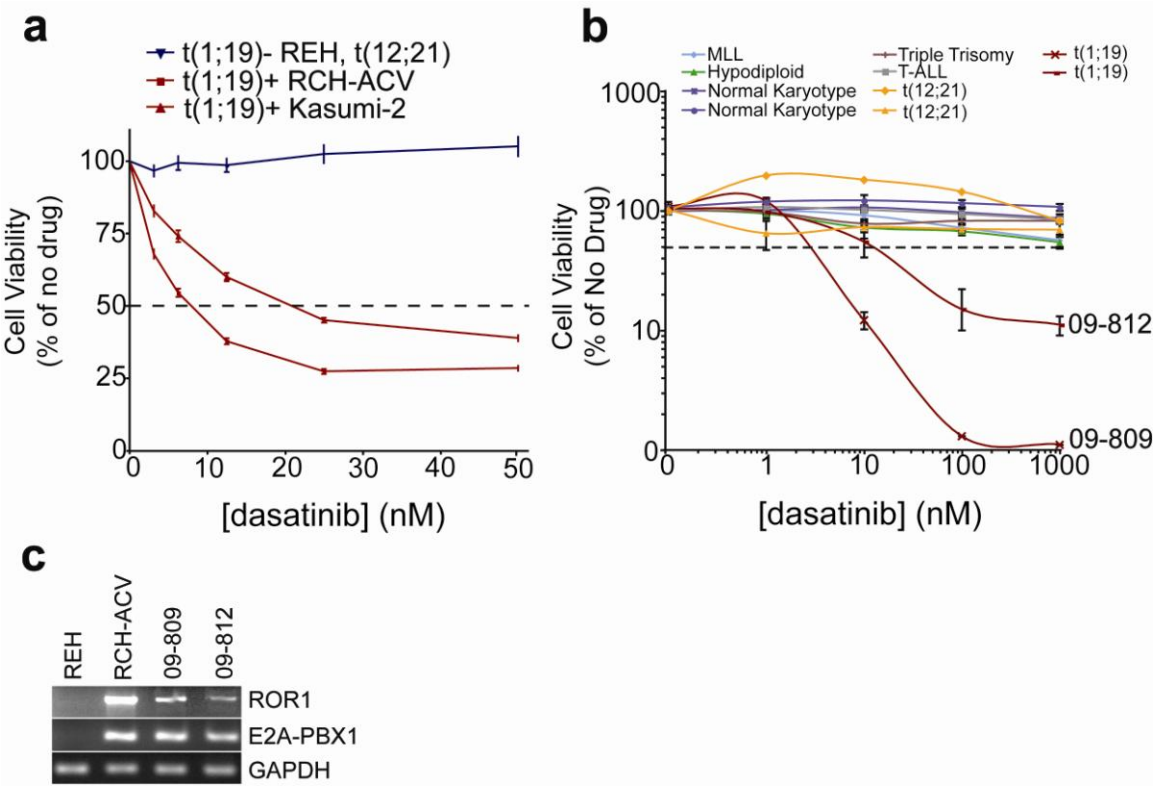


Figure 3.6. Dasatinib sensitivity in t(1;19) ALL. (A) RCH-ACV, Kasumi-2, and REH cells were cultured in graded concentrations of the kinase inhibitor, dasatinib, for 3 days at which time cells were subjected to an MTS assay for measurement of cell viability. Values represent percent mean (normalized to no-drug control wells) ± s.e.m. (n = 6). **(B)** Malignant cells from 10 pediatric ALL patients exhibiting a variety of chromosomal translocations (2 positive for t(1;19)) were cultured in graded concentrations of the kinase inhibitor, dasatinib, for 3 days at which time cells were subjected to an MTS assay for measurement of cell viability. Values represent percent mean (normalized to no-drug control wells) ± s.e.m. (n = 3). **(C)** cDNA derived from primary samples of t(1;19) ALL patients 09-809 and 09-812 was amplified using primers specific for ROR1, E2A-PBX1, or GAPDH. The t(1;19) positive (RCH-ACV) and negative (REH) cell lines were included for comparison.

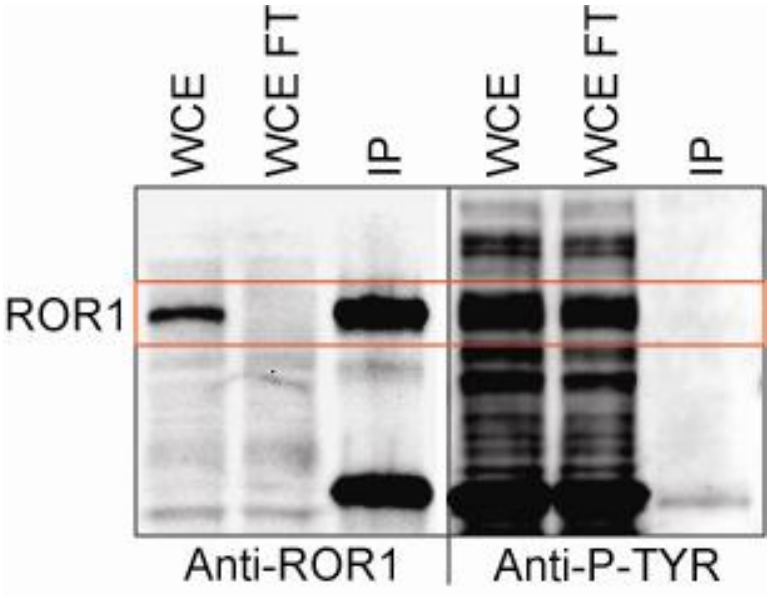


Figure 3.7. ROR1 shows no evidence of tyrosine phosphorylation in RCH-ACV cells. Whole cell extracts from RCH-ACV cells were incubated with ROR1 antibody, which was precipitated with Protein G agarose beads. The immunoprecipitate was eluted from beads in sample buffer and divided into two equal parts. The immunoprecipitates, as well as RCH-ACV whole cell extract (WCE) and ROR1 antibody-depleted whole-cell extract flow through (WCE FT), were subjected to SDS-PAGE followed by immunoblot analysis using antibodies specific for ROR1 or phospho-tyrosine (4G10).

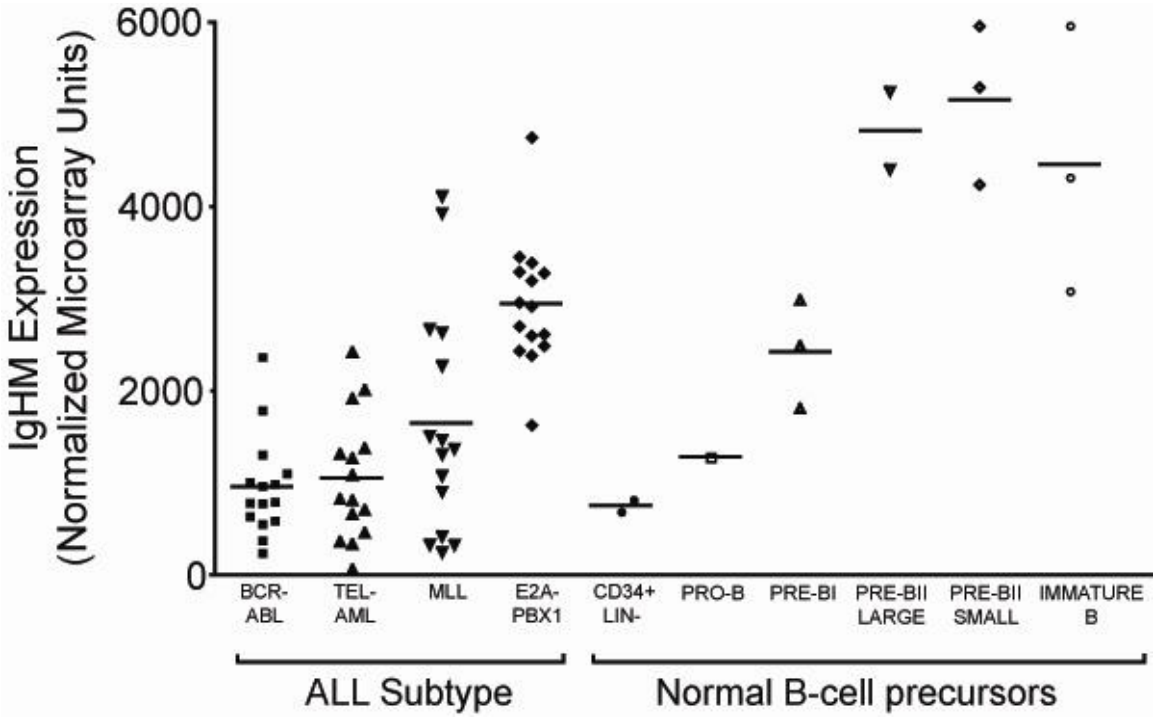


Figure 3.8. Ig heavy-chain, μ (Igμ), expression in B-cell ALL and normal B-cell development. A metaanalysis of gene expression microarray data generated from pediatric ALL patient samples and normal B-cell progenitor populations revealed significantly higher average Igμ expression levels in E2A-PBX1-positive ALL compared to other common translocations observed in pediatric ALL (BCR-ABL, TEL-AML, MLL rearrangements). The elevated Igμ expression in E2A-PBX1-positive samples correlates with the elevated expression levels observed in intermediate B-cell progenitor populations (pre-BI, pre-BII large/small).

I examined putative dasatinib targets that are known to mediate pre-BCR signaling. SRC-family kinases (particularly LYN, FYN and BLK) and the TEC-kinase BTK are established targets of dasatinib[178-180] and critical components of the pre-BCR and BCR signaling complexes[148, 165, 166, 181].

To determine whether dasatinib can, indeed, disrupt pre-BCR signaling I assayed the phosphorylation state of key components of the pre-BCR complex after dasatinib treatment. As expected, phosphorylation of BTK and the SRC-family kinase, LYN, is inhibited after dasatinib exposure. The additional loss of phosphorylation of SYK and Igα indicate complete disruption of the pre-BCR, as neither SYK nor Igα are direct targets of dasatinib but require SRC-family kinases for activation and function (**Figure 3.9a**).

To confirm that dasatinib sensitivity was, in fact, being mediated by the pre-BCR, I first showed that siRNA-mediated silencing of critical components of the pre-BCR (Igα and Igβ) could recapitulate the observed decrease in cell viability induced by dasatinib exposure in t(1;19) cells. Indeed, knockdown of Igα and Igβ results in significantly impaired cell viability, confirming the critical nature of the pre-BCR in t(1;19) ALL cell lines (**Figure 3.9b**).

Finally, disruption of pre-BCR signaling with dasatinib impairs cell proliferation and induces apoptosis in t(1;19) ALL cells, as demonstrated by cell counts and 7-AAD and Annexin-V staining (**Figure 3.10**).

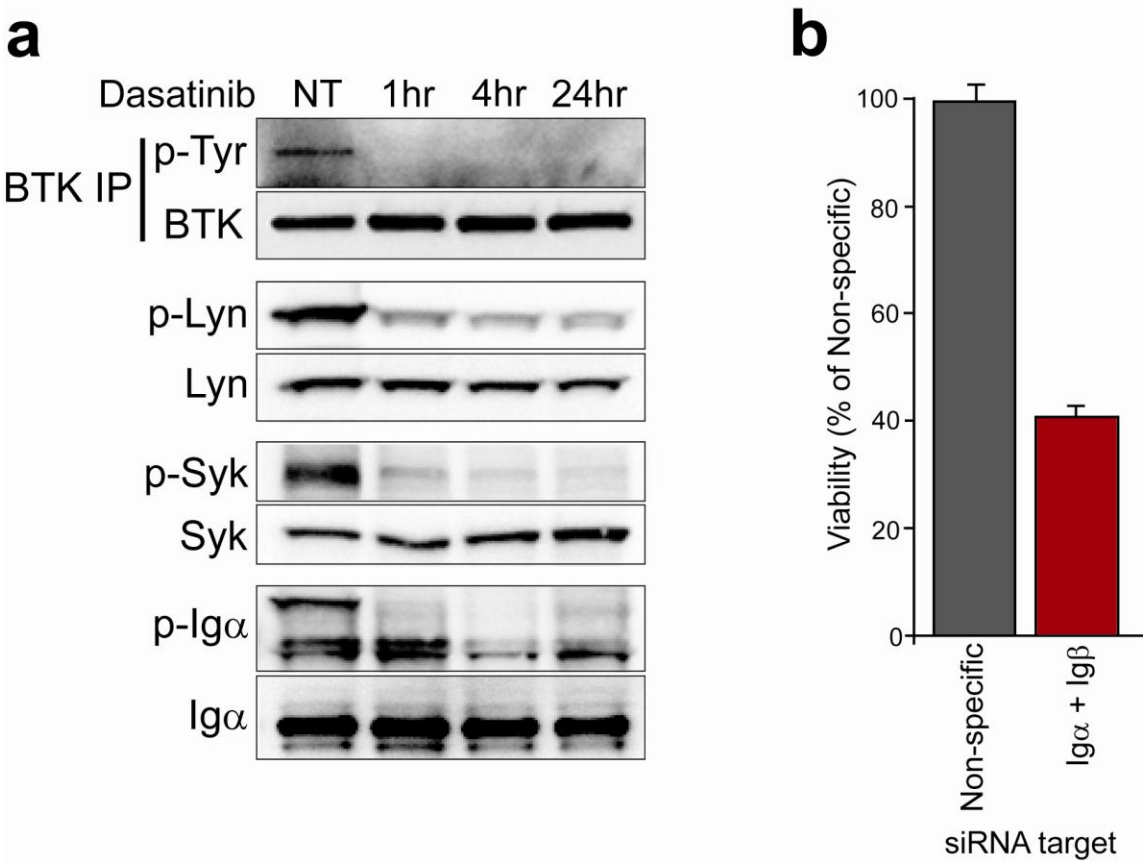


Figure 3.9. Dasatinib inhibits viability dependent preBCR activity. (A) RCH-ACV cells were treated with dasatinib (50 nM) over a time course and whole cell extracts were subjected to immunoblot analysis for total or phospho-LYN, SYK, and Igα. In addition, BTK was immunoprecipitated from the same cellular lysates and immunoprecipitates were immunoblotted for phosphotyrosine or total BTK. **(B)** RCH-ACV cells were electroporated with non-specific or Igα- and Igβ-targeting siRNA. After 4 days, cells were subjected to an MTS assay to measure cell viability. Values represent percent mean (normalized to non-specific control wells) ± s.e.m. (n = 5).

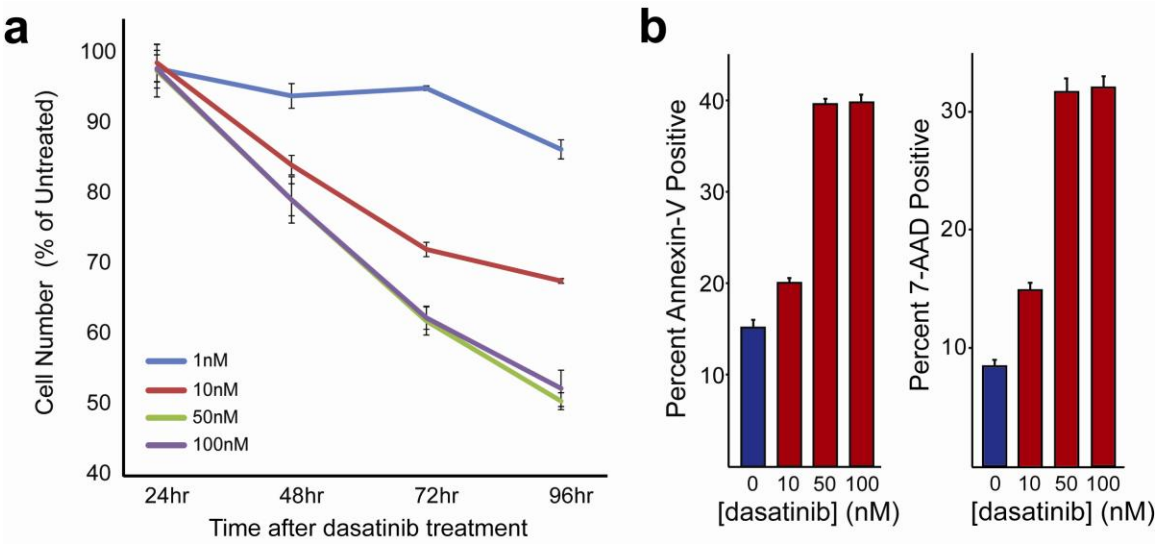


Figure 3.10. Dasatinib impairs t(1;19) ALL cell proliferation and induces apoptosis. **(A)** RCH-ACV cells were cultured in graded concentration of dasatinib for 96 hours. Cells were counted every 24 hours. **(B)** After 96 hours cells were harvested and stained with Annexin V-PE and 7-AAD and analyzed by flow cytometry to determine induction of apoptosis. Values represent the percentage of total positive cells ± s.e.m. (n = 3).

3.4.4 AKT is the critical downstream effector of the pre-BCR

Evaluation of the primary effector pathways activated by pre-BCR signaling revealed dasatinib treatment inhibits PI3K/AKT activation but has no effect on BLNK and PLCγ2 phosphorylation state (**Figure 3.11a,b**). This observation is in line with studies detailing downregulation or loss of BTK/BLNK/PLCγ2 signaling-complex components in pediatric ALL[170]. AKT inhibition occurs rapidly, within 15 minutes, and consistently shows the signs of recovery by 24 hours (**Figure 3.11b,c**).

As described above, AKT is a critical regulator of differentiation, proliferation and survival signaling pathways required during B-cell development. AKT's ability to phosphorylate and inhibit the FOXO family of transcription factor is particularly critical, as the FOXO family of transcription factors are known to regulate proapoptotic genes families and the RAG proteins employed for Ig rearrangements during B-lineage maturation. As I show in **Figure 3.11**, treatment of the t(1;19) ALL cell line RCH-ACV with dasatinib results in inhibition of AKT phosphorylation. Because I believe the viability effect of dasatinib on t(1;19) cells is mediated by AKT activity, I hypothesized that more continuous inhibition of AKT would increase the killing of t(1;19) ALL. To test this hyphothesis, I performed a constant ratio synergy test of RCH-ACV cells treated with dasatinib and the AKT inhibitor MERCK AKT1/2[182-184]. Treatment of RCH-ACV cells with MERCK AKT1/2 did not allow for recovery of AKT phosphorylation and I hyphothesized that the combination of the two drugs should result in an additive viability effect (**Figure 3.12a**). Analysis of the constant ratio drug treatment experiment, using the multidrug dose-effect analysis software Calcusyn, confirmed this hypothesis, and showed a moderate synergistic effect (**Figure 3.12b**).

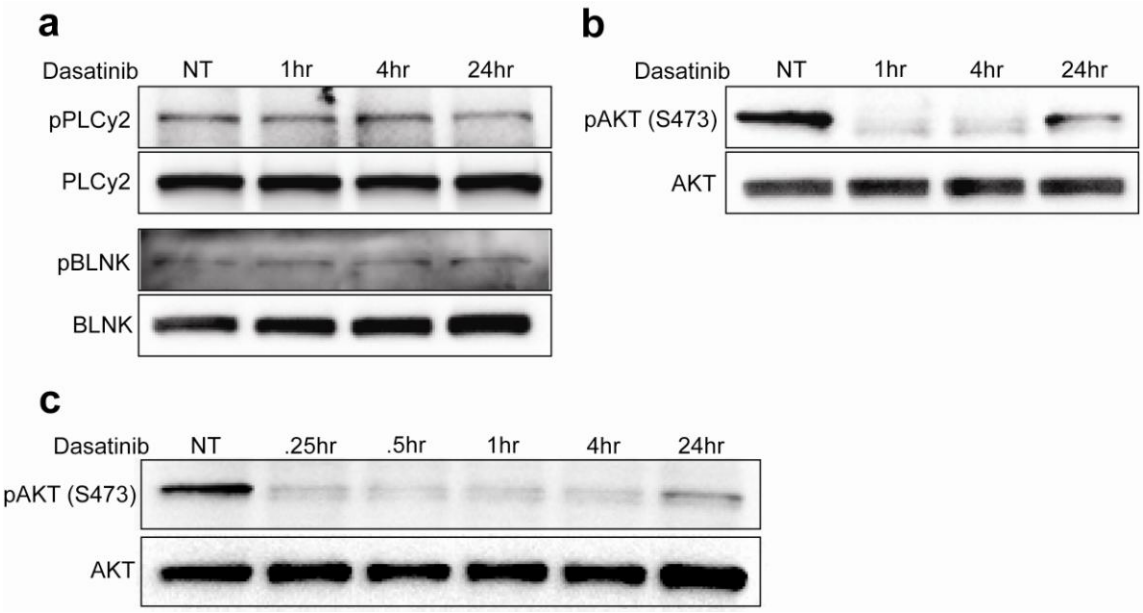


Figure 3.11. Dasatinib inhibits AKT signaling downstream of the preBCR. (A) RCH-ACV cells were treated with dasatinib (50 nM) over a time course and whole cell extracts were subjected to immunoblot analysis for total or phospho-PLCγ2 and BLNK. (**B**) The same cellular lysates were immunoblotted for phospho-AKT (S473) and total AKT. (**C**) RCH-ACV cells were treated with dasatinib (50nM) for an extended time course and whole cell extracts were subjected to immunoblot analysis for phospho-AKT (S473) and total AKT.

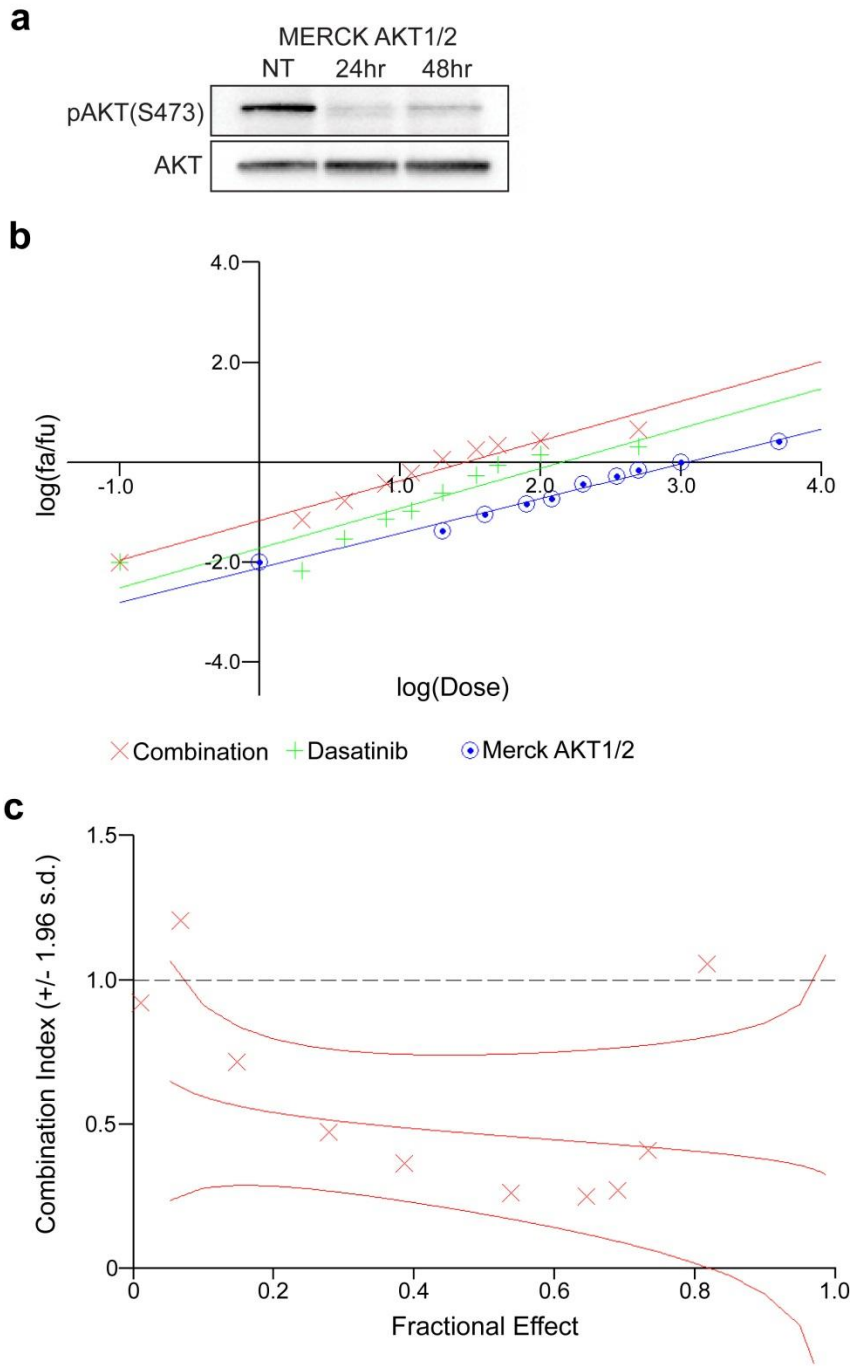


Figure 3.12. The AKT inhibitor MERCK AKT1/2 sustains AKT inhibition and provides a synergistic viability effect with dasatinib. (**A**) RCH-ACV cells were treated with MERCK AKT1/2 (1μM) over a time course and whole cell extracts were subjected to immunoblot analysis for total or phospho-AKT. (**B, C**) RCH-ACV cells were treated with dasatinib and/or MERCK AKT1/2 over a constant ratio dose gradient. Median effect and combination index plotting was used to determine IC50 values for each treatment and calculate synergism . Data points above or below a combination index of 1.0 suggest antagonism or synergy, respectively.

Finally, I analysed FOXO transcription factors activity downstream of AKT. As expected, I see reduced phosphorylation, suggesting activation, of both FOXO1 and FOXO3a (the primary effectors of AKT activation in B-cell development[93]). In addition, I see accumulation of FOXO1 in response to either MERCK AKT1/2 or dasatinib mediated inhibition of AKT (**Figure 3.13a**). In coordination with activation of the FOXO proteins I observe upregulation of the recombination protein RAG1 (Figure **3.13b**).

3.4.5 Dasatinib induces ROR1 upregulation in t(1;19) ALL

The dual sensitivity of t(1;19) ALL to ROR1 loss and pre-BCR inhibition, combined with their synchronous expression profile, suggests a functional connection between the two. Because of the coordinated expression of the pre-BCR along with ROR1 during B-cell development, I hypothesized that pre-BCR assembly and signaling results in regulated expression of ROR1. To test this hypothesis, I performed a dasatinib treatment time-course on the t(1;19) ALL cell line RCH-ACV. To my surprise, I observed ROR1 was actually upregulated, at both the transcript and protein level, in response to dasatinib exposure **(Figure 3.14a,b)**. To ensure this was a direct effect of pre-BCR signaling blockade and subsequent inhibition of AKT, I treated RCH-ACV with the AKT inhibitor MERCK-AKT1/2. As expected, AKT inhibition results in ROR1 protein upregulation similar to dasatinib (**3.14c**).

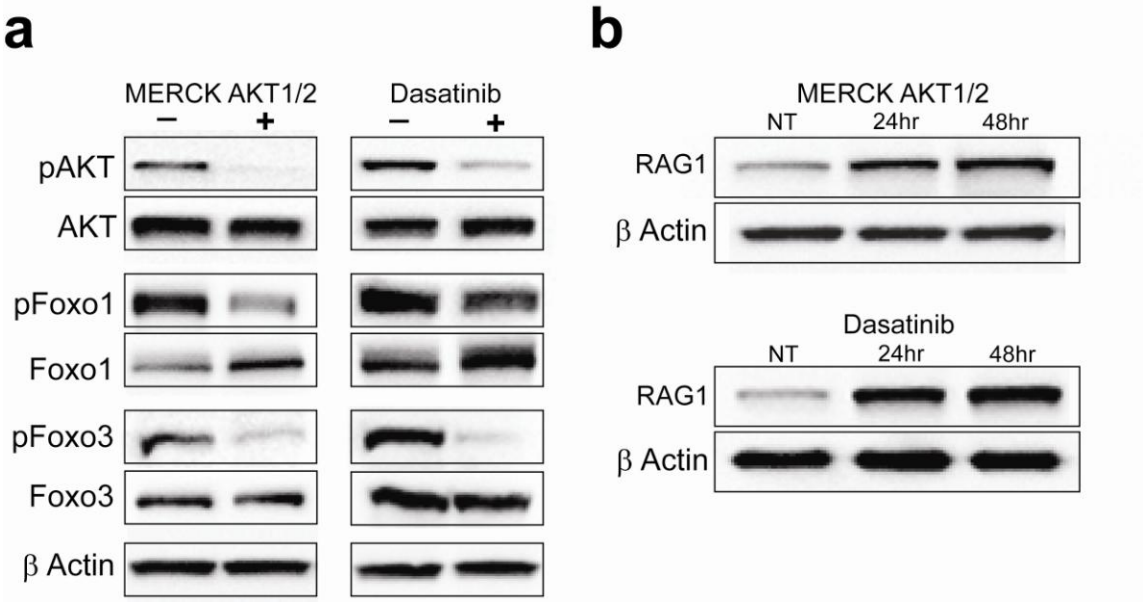


Figure 3.13. AKT inhibition results in Foxo protein activation and RAG1 expression upregulation. (**A**) RCH-ACV cells were treated with MERCK AKT1/2 (1μM) or dasatinib (100nM) for 24 hours and whole cell extracts were subjected to immunoblot analysis for total or phospho-AKT, Foxo1, Foxo3a and β-actin. (**B**) RCH-ACV cells were treated with MERCK AKT1/2 (1μM) or dasatinib (100nM) for 24 or 48 hours and whole cell extracts were subjected to immunoblot analysis for RAG1 and β-actin.

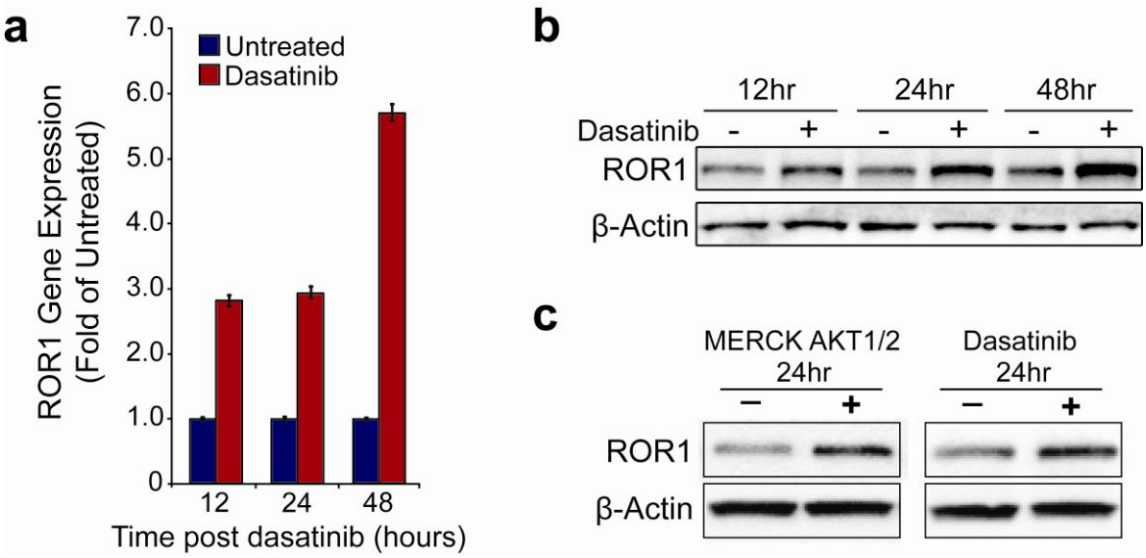


Figure 3.14. Dasatinib treatment induces ROR1 upregulation in t(1;19) ALL. (A) RCH-ACV cells were cultured in the presence of 100nM dasatinib for 12, 24, and 48 hours before RNA was harvested from cell extracts. Quantitative PCR was performed on cDNA with primers specific for ROR1 and GAPDH to determine relative ROR1 mRNA expression levels. Values represent the fold change (normalized to untreated control cells) ± s.e.m. (n = 6). **(B)** RCH-ACV cells were cultured in the presence of 100nM dasatinib for 12, 24, and 48 hours before cells were lysed and subjected to immunoblot analyses with antibodies specific for ROR1 or β-ACTIN to determine relative ROR1 protein expression. (**C**) RCH-ACV cells were cultured in the presence of 100nM dasatinib or 1μM MERCK AKT1/2 for 24 hours before cells were lysed and subjected to immunoblot analyses with antibodies specific for ROR1 or β-ACTIN.

3.4.6 ROR1 and the preBCR drive compensatory signaling pathways

The increase of ROR1 expression after inhibition of the pre-BCR signaling complex led us to hypothesize the existence of counter-balancing pools of signaling proteins that function downstream of ROR1 or the pre-BCR in a mutually compensatory manner to influence cell growth and viability. To evaluate this possibility, I examined protein phosphorylation patterns after treatment of cells with dasatinib or ROR1 siRNA (**Figure 3.15**). While a small number of proteins exhibited no change by either treatment, the majority of proteins showed opposing effects on phosphorylation status after treatment with dasatinib or ROR1 siRNA. In general, proteins could be categorized in the following three groups: 1) phosphorylation decreased by dasatinib but increased or no change by ROR1 siRNA, 2) phosphorylation increased by dasatinib but decreased or no change by ROR1 siRNA, or 3) phosphorylation reduced by both dasatinib and ROR1 siRNA (Figure 4d). Notably, certain SRC family kinases (such as SRC and LYN) showed decreased phosphorylation after dasatinib treatment (as expected), but other SRC-family kinases were unaffected or increased after dasatinib exposure. Of these, phosphorylation of FGR, LCK and YES was reduced after ROR1 silencing. In addition, PTK2B (aka focal adhesion kinase 2; FAK2) phosphorylation was reduced after dasatinib treatment and increased after silencing of ROR1. In contrast, the highly related kinase, PTK2 (aka focal adhesion kinase; FAK) showed the opposite trend where dasatinib exposure led to increased phosphorylation and ROR1 silencing caused a decrease in phosphorylation status. Collectively, one can identify multiple protein homologues that are driven in opposite directions by treatment with dasatinib or ROR1 siRNA (SRC-family kinases, FAKs, MAPKs). Finally, a small number of proteins

showed reduced phosphorylation after both dasatinib and ROR1 siRNA. These include p53, p27, AKT (Serine 473), p70S6 Kinase, RSK1/2/3, STAT1, and STAT4.

Altogether, these data point toward at least three distinct signaling pools in t(1;19) cells: 1) proteins downstream of only the pre-BCR—which are further induced by ROR1 silencing, 2) proteins downstream of only ROR1—which are further induced by pre-BCR inhibition, and 3) effector proteins downstream of both pools 1 and 2. As such, inhibition of only the ROR1- or pre-BCR-specific pools (but not both) should result in a transient decrease of the common downstream effector proteins; however, phosphorylation of these common downstream proteins should be partially rescued once further activation of the counter-balancing pathway has occurred. In fact, my earlier data, examining AKT phosphorylation following dasatinib treatment (**Figure 3.11**) and ROR1 upregulation following AKT inhibition (**Figure 3.14**), support this hypothesis, and show ROR1 upregulation in response to dasatinib treatment corresponds to recovery of AKT phosphorylation (**Figure 3.16a, b**).

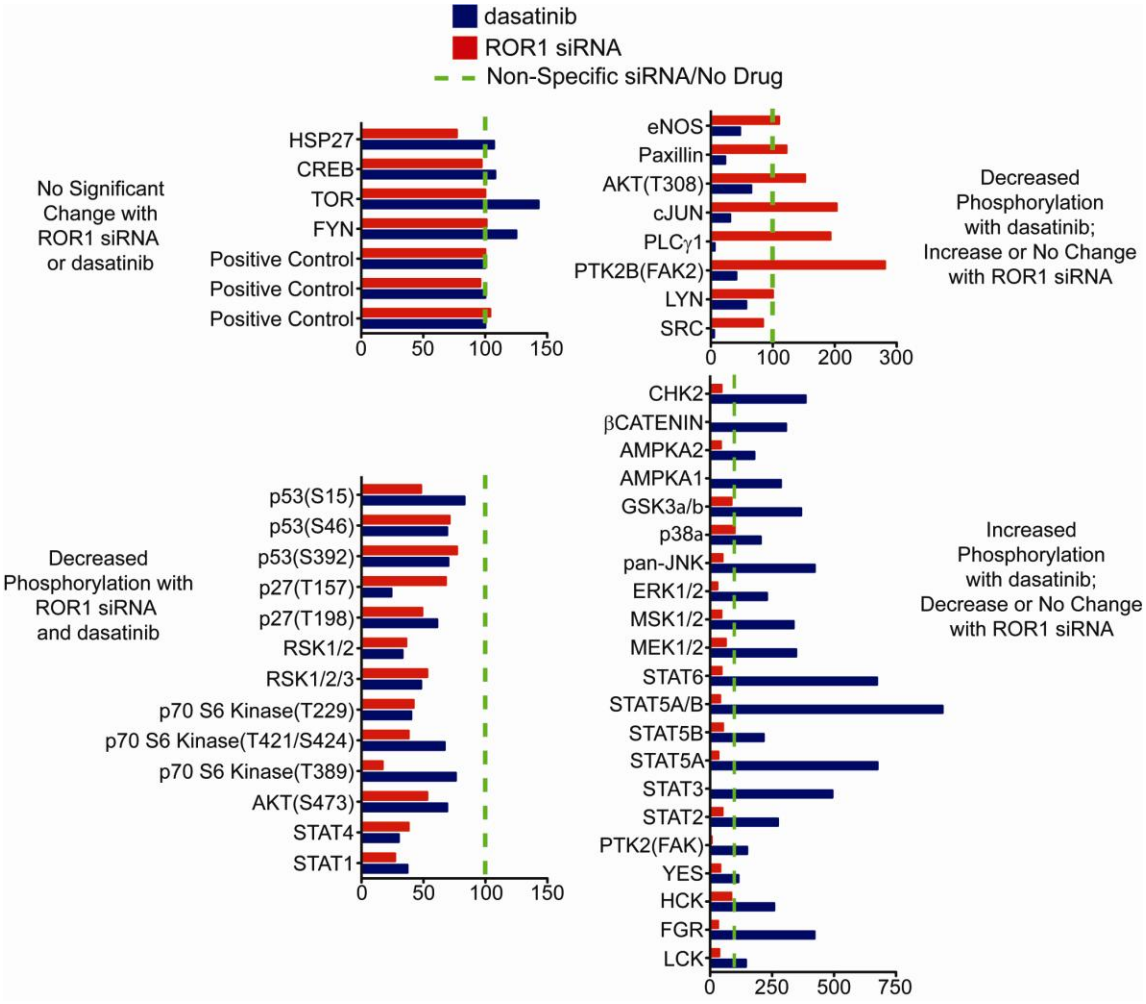


Figure 3.15. ROR1 and the pre-BCR drive compensatory signaling pathways. RCH-ACV cells were treated with dasatinib for 4 hours or ROR1 siRNA for 72 hours and whole cell extracts were analyzed by phospho-proteomics. Phosphorylation status of a total of 46 phospho-proteins was established for dasatinib relative to cells in the absence of any drug and for ROR1 siRNA relative to cells transfected with non-specific siRNA. The phosphorylation level of no drug/non-specific controls is set to 100% in all cases, which is indicated by the green dashed line.

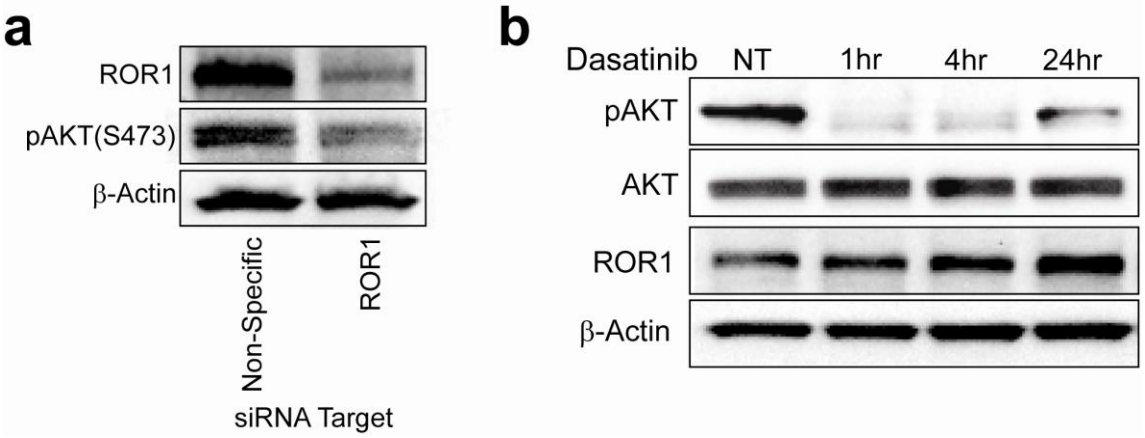


Figure 3.16. Loss of ROR1 impairs AKT signaling and upregulation correlates with AKT signaling recovery. (**A**) RCH-ACV cells were electroporated with non-specific or ROR1-targeting siRNA. After 3 days, cells were lysed and subjected to immunoblot analysis for ROR1, phosho-AKT(S473), or β-ACIN. (**B**) RCH-ACV cells were cultured in the presence of 50nM dasatinib for 0, 1, 4 or 24 hours before cells were lysed and subjected to immunoblot analyses with antibodies specific for phospho-AKT(S473), total AKT, ROR1 or β-ACTIN.

3.4.7 ROR1 dependence and dasatinib sensitivity in B-cell malignancies

Based on the observed upregulation of ROR1 in response to pre-BCR inhibition by dasatinib as well as the counter-balancing signaling pools driven by these two molecules, I hypothesized that ROR1 may partially rescue cell survival after inhibition of the pre-BCR. If this were the case, then targeting of ROR1 in the context of dasatinib exposure would potentiate dasatinib killing of t(1;19) cells. To test this hypothesis, I treated t(1;19) ALL cell lines with ROR1 or non-specific siRNA and subsequently exposed these cells to graded concentrations of dasatinib. As predicted, cells subjected to ROR1 silencing were more sensitive to dasatinib compared with cells treated with non-specific siRNA (**Figure 3.17**). This additive effect suggests that ROR1, indeed, functions as a rescue pathway for cell survival in the context of pre-BCR inhibition, and mitigation of this rescue pathway by alternate means (ROR1 siRNA) can further abrogate cell viability. It also suggests a potential benefit of therapeutic strategies that would simultaneously target both ROR1 (with RNAi or other means) and the pre-BCR (with dasatinib).

Finally, since t(1;19) ALL does not represent the only B-lineage malignancy that is arrested at an intermediate/late stage of B-cell development, I analyzed ROR1 status and dasatinib sensitivity in additional primary cells from B-cell malignancies of intermediate/late stage B-cell development. I examined primary samples from patients diagnosed with the intermediate/late B-cell malignancies, t(17;19) ALL and Burkitt's leukemia, both of which are CD34-negative, express either the pre-BCR or mature-BCR, as indicated by μ heavy-chain (μHC) expression (see **Figure 2.7**). Both the t(17;19) ALL and Burkitt's samples showed high ROR1 expression levels, similar to those

observed in t(1;19) ALL samples (**Figure 2.7**). As expected, these samples were also sensitive to dasatinib treatment, with IC50s comparable to t(1;19) ALL (**Figure 3.18**). These results support the idea that ROR1 expression and dasatinib sensitivity are conserved characteristics of intermediate/mature B-cell malignancies.

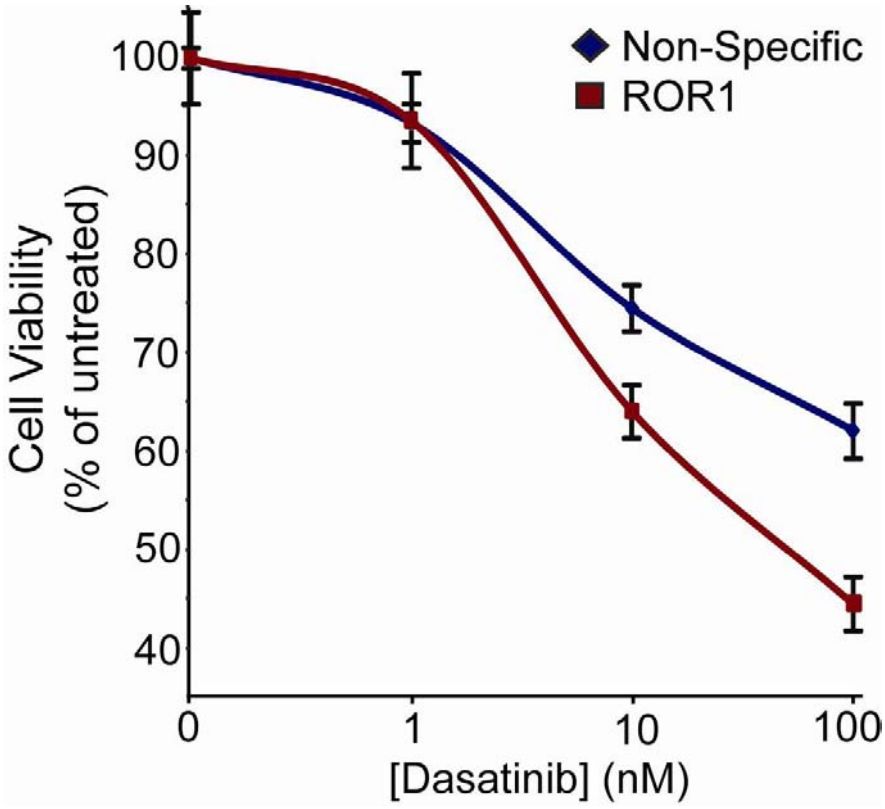


Figure 3.17. ROR1 loss magnifies dasatinib sensitivity in t(1;19) ALL. RCH-ACV cells were electroporated in the presence of non-specific siRNA or siRNA targeting ROR1 and then plated in culture media. After 2 days, graded concentration of dasatinib were added. Cells were allowed to culture an additional 2 days before they were subjected to an MTS assay for measurement of cell viability. Values represent percent mean (normalized to no-drug control wells) ± s.e.m. (n = 6).

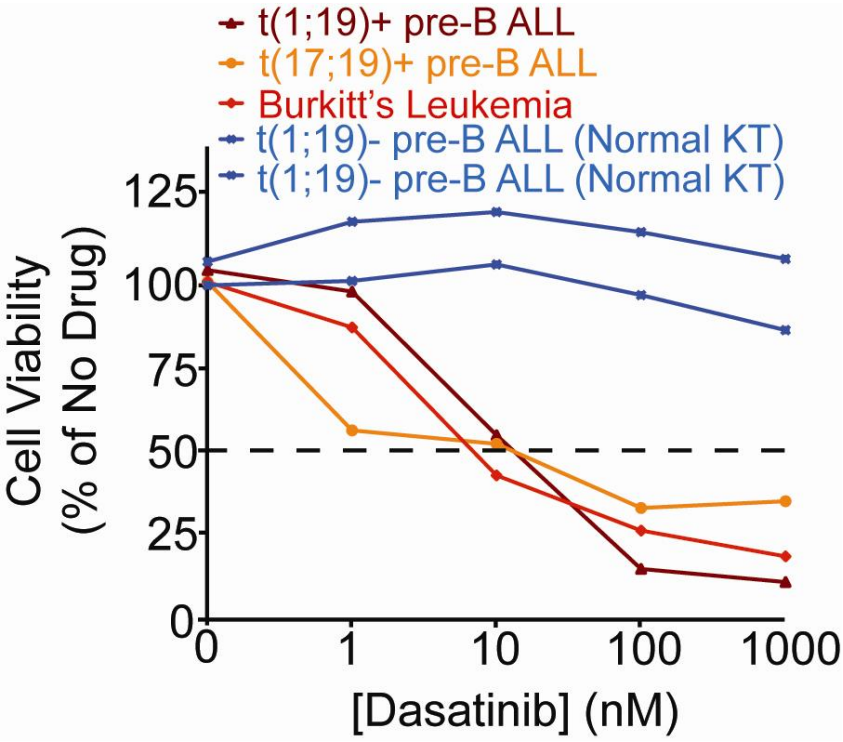


Figure 3.18. Dasatinib sensitivity is conserved in intermediate/mature B cell malignancies expressing ROR1. t(1;19) ALL, t(17;19) ALL, Burkitt's lymphoma, and normal karyotype primary samples were cultured in the presence of graded concentrations of dasatinb. After 3 days, cells were subjected to an MTS assay for measurement of cell viability. Values represent percent mean, normalized to no-drug control wells.

3.5 Discussion

The work outlined in Chapter 3, offers the first insight into the functional role of ROR1 in t(1;19) ALL as well as in normal B-cell development maturation. Taken together with the work described in Chapter 2, these findings support a model in which the 1;19 translocation arrests normal B-cell development at a stage in which both ROR1 and the pre-BCR are expressed and drive counter-balancing signaling pools that are critical for cell viability. Although ROR1 and the pre-BCR complex are not mutated oncogenes, they do represent onco-requisite pathways and so remain viable therapeutic targets for the treatment of t(1;19) ALL. My analyses of phospho-proteomics following inhibition of these molecules further suggests that ROR1 and pre-BCR both drive similar, yet distinct signaling cascades that both culminate in activation of critical cell growth/survival pathways such as AKT kinase. Additionally, these two signaling pools appear to counter-balance one another whereby inhibition of one pool accentuates the other, leading to partial rescue of cell growth/viability. Consequently, inhibition of both pathways leads to a greater reduction of cell growth and viability (**Figure 3.19**). The observation of ROR1 overexpression and dasatinib sensitivity in additional B-cell malignancies, suggests this paradigm is more broadly applicable to B-lineage malignancies that are arrested at an intermediate/late stages of B-cell development.

Similar to Chapter 2, this work identifies ROR1 as a consistent therapeutic target in t(1;19) ALL and other B-cell malignancies, and offers alternative mechanisms for targeting ROR1. I describe in Chapter 2, immunotherapies that utilize chimeric antigen receptor (CAR)-modified T-cells that would target the mere presence of ROR1. Here, I have outlined ROR1 functional dependence in t(1;19) ALL. As such, a therapeutic

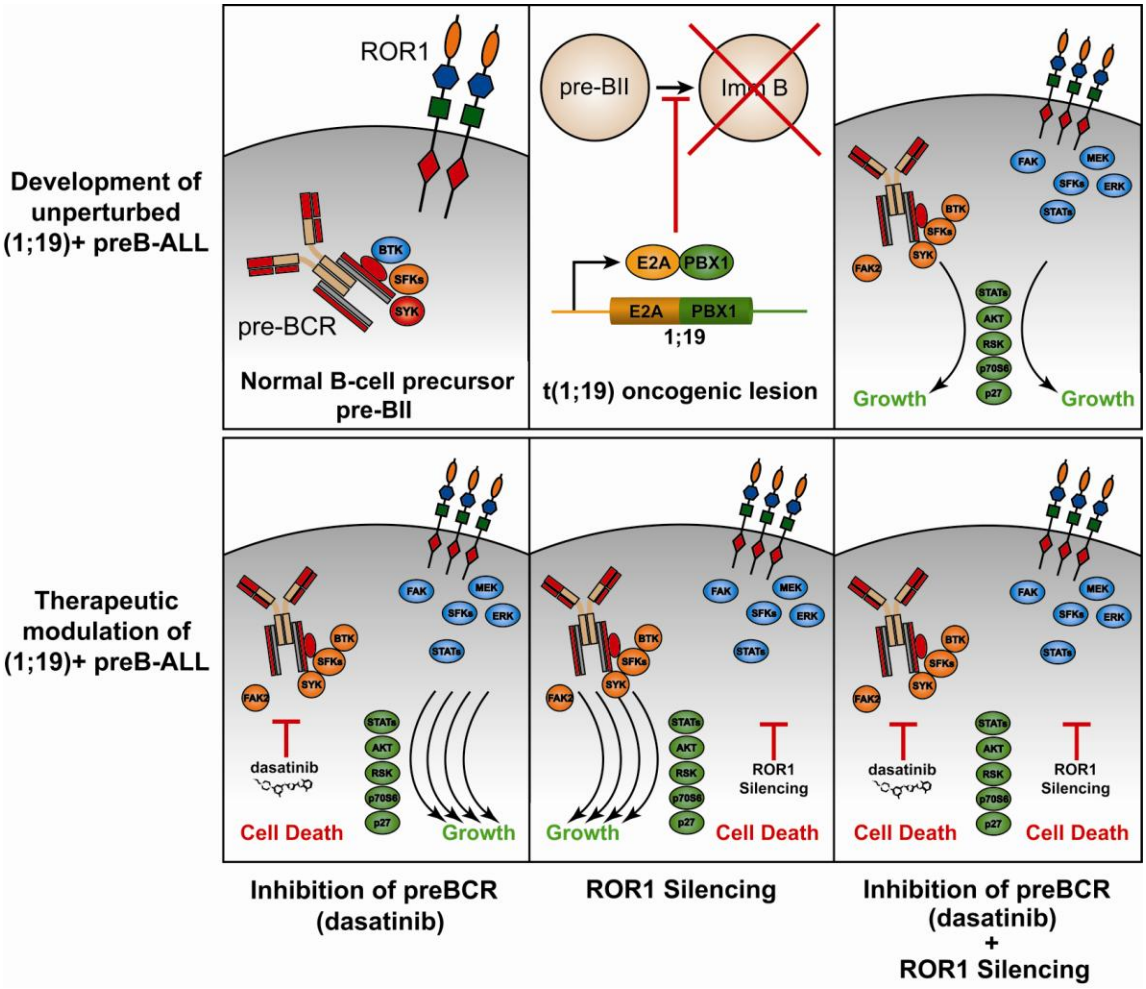


Figure 3.19. Model for development of unperturbed t(1;19) ALL malignancy as well as counter-balancing effects of modulating ROR1 or the pre-BCR. Normal B-cell progenitors naturally upregulate ROR1 expression and signal from the pre-BCR complex at the pre-BII stage of development. The t(1;19) oncogenic lesion generates the E2A-PBX1 chimeric transcription factor that contributes to the developmental arrest of B-cell progenitors at the pre-BII stage of development. Expression of ROR1 and signaling from the pre-BCR complex are critical for cell viability due to activation of homologous, yet distinct pools of signaling proteins that culminate in activation of critical pathways such as the AKT pathway. Modulation of ROR1 or the pre-BCR is not as effective at killing t(1;19) cells as simultaneous antagonism of both pathways, because of the mutually compensatory nature of these two signaling pathways. Abbreviations: (SFK) SRC-family kinases.

method that disrupts ROR1 function could serve as a functional treatment. Unfortunately, the best current option for disrupting receptor tyrosine kinase function is with the use of small molecule tyrosine kinase inhibitors, and ROR1 does not appear to be a functional tyrosine kinase. But, as I clearly show, ROR1 is required for t(1;19) viability, and thus must have a mechanism of action that could potentially be disrupted by a small molecule. Drugs that either disrupt specific interaction required for ROR1 activity, or inhibit downstream mediators of ROR1 activity could both be valuable. ROR1's mechanism of action, however, is still unknown and significant work remains before a full understanding of ROR1's mechanism and function can be utilized for inhibitor development. Still, an immunological based approach could be a more immediate approach to disrupting ROR1 for therapeutic purposes. Here, rather than using a CAR-modified T-cell to target ROR1 cells, one could utilize a monoclonal antibody to target ROR1 expressing cells. Ideally, this method would function in a way similar to Herceptin in breast cancer (discussed in Chapter 1), where circulating antibody would bind ROR1, disrupt ROR1's function, and result in proliferation inhibition and subsequent cell death. In the event that ROR1 turns out to be expressed in additional normal adult tissues and thus precludes the use of ROR1-CAR modified T-cells as a therapy, drugs designed to inhibit ROR1 function (whether antibody or small molecule) would likely remain viable therapeutic options due to the lower expression profile observed at the RNA level and the rapid proliferation rate of acute leukemias sensitive to ROR1 loss. Once again, further study addressing the potential toxicity of targeting ROR1 surface expression and or function will be critical for advancing ROR1-targeting as a viable therapeutic option.

While direct ROR1-targeting may still be far away from clinical use, small molecule kinase inhibitors have proven clinically successful and present a more immediate clinical option for these B-cell malignancies. My studies show dasatinib impairs the viability of t(1;19) ALL primary samples and cell lines at clinically relevant concentrations and that the primary dasatinib targets in this setting are kinases associated with the pre-BCR signaling complex. This explains the lack of sensitivity to dasatinib in the pre-B-cell line REH and other primary ALL samples that lack expression of the pre-BCR due to differentiation arrest at earlier stages of B-lineage development where the pre-BCR complex is not yet expressed. Conversely, I observe high ROR1 expression and dasatinib-sensitivity in primary t(17;19) ALL and Burkitt's lymphoma samples due to developmental arrest at a comparatively mature stage of B-cell differentiation in which the malignant cells do express either the pre- or mature-BCR. This work suggests that expression of the pre-BCR or BCR should be a reliable marker for dasatinib sensitivity; however, it remain unclear how reliable this correlation remains when dealing with mature-BCR-positive malignancies. A good example of the complexity of mature BCR-positive disease is CLL, which is a heterogeneous disease that can originate from both pre- and post-germinal center B-cells and results in some CLLs with and others without hypermutated light-chain[185, 186]. All of these CLL samples, regardless of light-chain hypermutation, express ROR1, yet only approximately 40% respond to dasatinib treatment[187]. Importantly, this response does not correlate with the mutation status of light-chain, and suggests either a muted dependence upon the BCR or increased activation of potential rescue pathways. Work with CLL both supports the relationship between BCR expression and dasatinib sensitivity, while simultaneous

showing it is not a completely conserved phenomenon. Likewise, it is a good example of retained co-expression of ROR1 with the BCR, but again, not entirely conserved sensitivity to dasatinib. As a whole, this work supports expanded study of dasatinib therapy for certain B-cell malignancies.

Although t(1;19) ALL patients generally exhibit 80-85% relapse free survival rates[134], many of these patients must tolerate intensive chemotherapy regimens to achieve remission. Further, there remains a subset of patients with t(1;19) who experience a relapse. Salvage therapies including stem cell transplant remain suboptimal with less than 50% retrieval. Therefore, these patients may benefit with the addition of dasatinib to salvage therapies to improve outcomes. Additionally, standard cytotoxic drugs exhibit toxicities that can give rise to a multitude of long term side effects and secondary malignancies. As such, a gene targeted drug such as dasatinib could offer great benefit to these patients by reducing rates of relapse and allowing patients to be treated with less intensive chemotherapy regimens while still exhibiting similar or more favorable outcomes.

Finally, my work investigating the effect of dasatinib on t(1;19) ALL cell lines may provide some insight into ROR1 expression and function. I show that ROR1 is upregulated in response to inhibition of pre-BCR signaling by dasatinib in t(1;19) ALL cell lines, and the subsequent partial protection from dasatinib sensitivity suggests that ROR1 acts as a rescue pathway for pre-BCR signaling. This observation perhaps provides a clue to the function of ROR1 in these leukemic cells, suggesting that ROR1 and the pre-BCR signaling complex may initiate a shared signaling cascade. Indeed my phospho-proteomic analysis supports this notion since homologous, yet distinct

signaling pools appear to be driven by the pre-BCR and ROR1, which both culminate in activation of critical pathways such as AKT kinase. The finding of a wide variety of phospho-proteins that are positively or negatively regulated following ROR1 silencing (**Figure 3.15**) represents a major advance for ROR1 biology, since no prior studies have demonstrated phosphorylation pattern changes induced by ROR1.

In conclusion, my work has expanded the pool of patients who could potentially benefit from ROR1 targeted therapy and has further suggested a high rate of overlap between the overexpression of ROR1 and sensitivity to the targeted kinase inhibitor, dasatinib. Addition of t(1;19) and t(17;19) ALL as well as Burkitt's leukemia/lymphoma to the growing list of B-cell malignancies that exhibit ROR1 surface expression provides impetus for further study of ROR1 biology and development of ROR1-targeted therapies. Further, while dasatinib does not appear to directly target ROR1, it does effectively reduce the viability of many ROR1-positive B-cell malignancies due to expression of the pre- or mature-BCR in these cells. Hence, my work would suggest further studies that may lead to implementation of dasatinib therapy for B-cell malignancies expressing the pre- or mature-BCR, which could also be supplemented with ROR1-directed therapies for enhanced efficacy.

APPENDIX I

E2A-PBX1, WNT16B, ROR1 and the preBCR

Results

A1.1 E2A-PBX1 overexpression has no effect of ROR1 expression

The product of the 1;19 translocation is the E2A-PBX1 chimeric transcription factor. The conserved expression of ROR1 in t(1;19) ALL made this aberrant transcription factor a convenient and likely source of ROR1's seemingly unique expression profile. To address the question of whether the E2A-PBX1 regulates ROR1 expression I performed experiments wherein I removed E2A-PBX1 from a system expressing ROR1 or added it to a system not expressing ROR1, and assessed ROR1's expression in response.

In the first set of experiments, I treated RCH-ACV with siRNA directed at ROR1, E2A-PBX1 or WNT16B. As WNT16B had previously been shown to be regulated by E2A-PBX1, it served as a valuable positive-control. When knocking down ROR1 or WNT16B, I only saw an expression effect on the direct siRNA target, and no effect on expression of the other or E2A-PBX1. However, when I targeted E2A-PBX1, I see knockdown of E2A-PBX1 and its transcriptional target WNT16B, but no effect of ROR1 (see **Figure 2.6**). The results of this work suggested E2A-PBX1 was not transcriptionally regulating ROR1.

In my second set of experiments, I took E2A-PBX1 cloned from RCH-ACV cells (see **Appendix II: Constructs, Vectors, and Sequences**) into our MIGR1 vector for expression in the murine pro-B cell line BaF3 and pENTR3.2-V5-DEST vector for expression in human pro-B cell line REH and HEK293s. None of these model systems showed evidence of E2A-PBX1 induced ROR1 expression. Surprisingly, these systems also did not show evidence of E2A-PBX1 regulating WNT16B, suggesting that E2A-

PBX1's transcriptional activity is, at least in part, context specific to the t(1;19) ALL background.

Later, a metaanalysis of microarray studies of common pediatric ALL subtypes and normal B lineage precursors (described in Chapter II and Chapter III) revealed ROR1 expression is normally upregulated during B-cell development. This study clearly outlined transcriptional profiles unique to t(1;19) ALL background or normal B-development. The profiles of ROR1 and WNT16B were particularly telling, where ROR1 is observed in both t(1;19) ALL and its normal B-cell equivalent (pre-BII) because its expression is a product of development, WNT16B only appears in t(1;19) ALL because its expression is a result of E2A-PBX1 (**Figure AI.1a**). The WNT/β-CATENIN signaling pathway proved interesting beyond WNT16B. When we compare LEF1 and GSK3β, dueling components of canonical WNT signaling, we see that *LEF1* is upregulated in all ALL subtypes compared to their normal B-cell counterparts, whereas *GSK3β* is downregulated in all ALL backgrounds compared to their normal B-cell counterparts (**Figure AI.1b**). In particular, t(1;19) ALL shows the highest upregulation of LEF1 compared to other ALL subtypes. t(1;19) ALL displays a more than 2-fold increase in LEF1 expression compared to other subtypes, whereas normal pro-B, pre-BI, and pre-BII progenitors expressed roughly similar levels. These results suggest t(1;19) ALL have an increased dependence upon canonical WNT signaling; however, further analysis proved beyond the scope of my thesis.

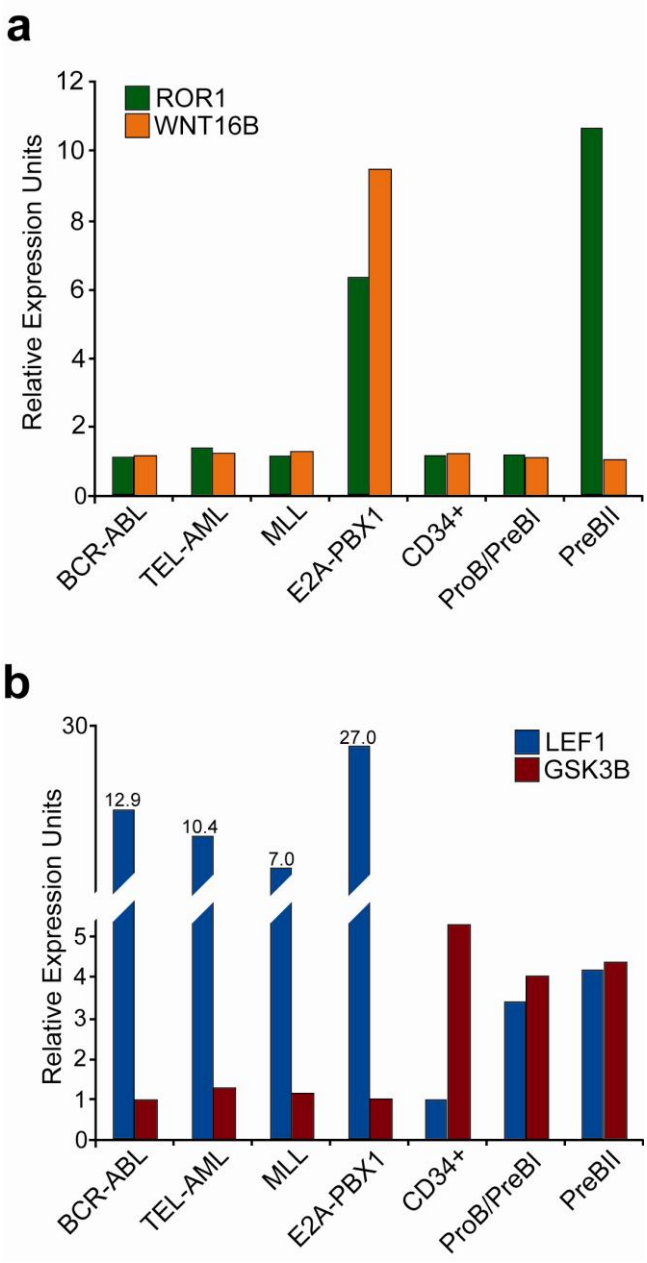


Figure AI.1. Gene expression profiles of t(1;19) ALL. Gene expression microarray data for pediatric ALL patients and normal B-cell progenitor populations were compiled into a meta-analysis. Patients with MLL gene rearrangements, t(9;22) (BCR-ABL), t(12;21) (TEL-AML), or t(1;19) (E2A-PBX1) (n=15 for each subset) and non-malignant B-cell progenitor populations (CD34+ Lin-, pro-B, pre-B, pre-BII large, pre-BII small, and immature B) (n=15 total) were examined and Affymetrix intensity values for (**A**) ROR1 and WNT16B and (**B**) LEF1 and GSK3B are averaged and plotted for each sample set.

In an attempt to address the question of how ROR1 expression is regulated in t(1;19) ALL, I followed up on work performed in the multiple myeloma cell line MM1 and primary CLL samples that described the regulation of ROR1 expression by STAT3[188]. Here, they show that IL6 stimulation in MM1 cells results in Stat3 phosphorylation and ROR1 upregulation, and introduction of STAT3 siRNA abrogates this effect. They continue to show by chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assay (EMSA) that STAT3 directly binds the ROR1 promoter in both MM1 cells and CLL primaries. This work provided the first evidence of ROR1 regulation in a hematopoietic lineage. In an attempt to show ROR1 regulation by STAT3 in t(1;19) ALL I treated cells with siRNA directed at STAT3 as well as treated cells with an inhibitor of STAT3[189]. Both of these experiments failed to alter ROR1 expression at the transcript level. The reason for the differences in my findings is unclear, but there are several potential explanations. First, whereas ROR1 is naturally upregulated in intermediate B-cell precursors, it is not normally expressed in mature B cells. This a major difference between ROR1 expression in t(1;19) ALL and CLL, as t(1;19) ALL represents an intermediate B-cell malignancy and CLL a mature B-cell disease. As a result, ROR1 regulation may be different in t(1;19) ALL compared to CLL. Second, the authors of this work look at ROR1 regulation in response to stimulation, rather than basal expression. Perhaps, STAT3 functions to augment basal ROR1 levels in response to a stimulus. This is a possibility that will need to be explored further. Finally, there is the possibility of investigator error. The extensive work showing STAT3 interaction with the ROR1 promoter in this study is instigated by the dual observation of ROR1 upregulation and STAT3 phosphorylation following IL6

stimulation; however, the time-course of this observation is highly surprising, and shows maximal ROR1 protein upregulation only 18 minutes after IL6 stimulation. This rapid increase in total protein is shocking for a transcription mediated response[190].

In an alternative approach to understand ROR1 regulation, I have initiated a study of the ROR1 promoter that could provide the first evidence of how ROR1 is regulated in t(1;19) ALL. This work is being done in collaboration with Dr. Joshi Alumkal at OHSU. In this study we are comparing histone modifications in the ROR1 promoter region of two pediatric ALL cell lines expressing ROR1 (RCH-ACV and Kasumi-2) and two pediatric ALL cell lines that do not express ROR1 (REH and MHH-cALL2). Preliminary data from this work looks promising; however, it remains at a nascent stage and still requires significant follow-up.

A1.2 There is no evidence of ROR1 and WNT16B interaction

Conserved expression of ROR1 in the t(1;19) ALL background, known to uniquely overexpress the WNT ligand WNT16B, made for the very temping and intriguing hypothesis that WNT16B serves as a novel ligand for ROR1 in this leukemia. Because ROR1 possesses an extracellular frizzled domain, it has generated much interest as a potential WNT ligand receptor. However, beyond a contrived co-IP experiment using an epitope tagged WNT5A and a soluble, secreted version of the ROR1 extracellular domain[66], no evidence of ROR1 participating in a canonical or non-canonical WNT signaling cascade exists.

To test my hypothesis, I attempted to IP WNT16B with ROR1 or ROR1 with WNT16B. My initial attempts began without an antibody capable of IPing endogenous ROR1. As such, I generated an epitope-tagged ROR1 construct that could be expressed

in HEK293 cells, along with WNT16B, and then IPed. ROR1-V5 (see Appendix II: Vectors, Constructs, and Sequences) allowed for transient expression in HEK293 and efficient IP. Immunoprecipitates from ROR1-V5 transfected cells showed no evidence of WNT16B enrichment when compared to ROR1 control or no-V5 antibody control (**Figure AI.2a**). Likewise, when incubated with conditioned media from RCH-ACV, ROR1-V5 IPs from HEK293s showed no evidence of WNT16B/ROR1 interaction.

Another approach, targeting endogenous protein expression, used WNT16B or ROR1-specific antibody to IP their target protein and look for co-IP of the other. Initial experiments targeted WNT16B, as I had an antibody that successfully IPed and blotted WNT16B, but our ROR1 antibody (Cell Signaling, 4102) only worked for blotting. IP of WNT16B from RCH-ACV cells showed no evidence of association with ROR1 **(Figure AI2b)**. Later, I acquired a second ROR1 antibody that worked for endogenous IP (R&D Systems, AF2000). IP of ROR1 and blotting for co-IPed WNT16B yielded the same results.

In a final attempt to show some evidence of direct ROR1/WNT16B interaction, I sought to show depletion of WNT16B from RCH-ACV conditioned media following incubation with HEK293 cells that overexpressed ROR1. Here, I compared WNT16B levels in conditioned media to that same media following incubation with HEK293 cells either empty-vector control transfected or ROR1 transfected. Western blotting of the conditioned media following incubation showed no evidence of ROR1-mediated WNT16B depletion **(Figure AI.2c)**.

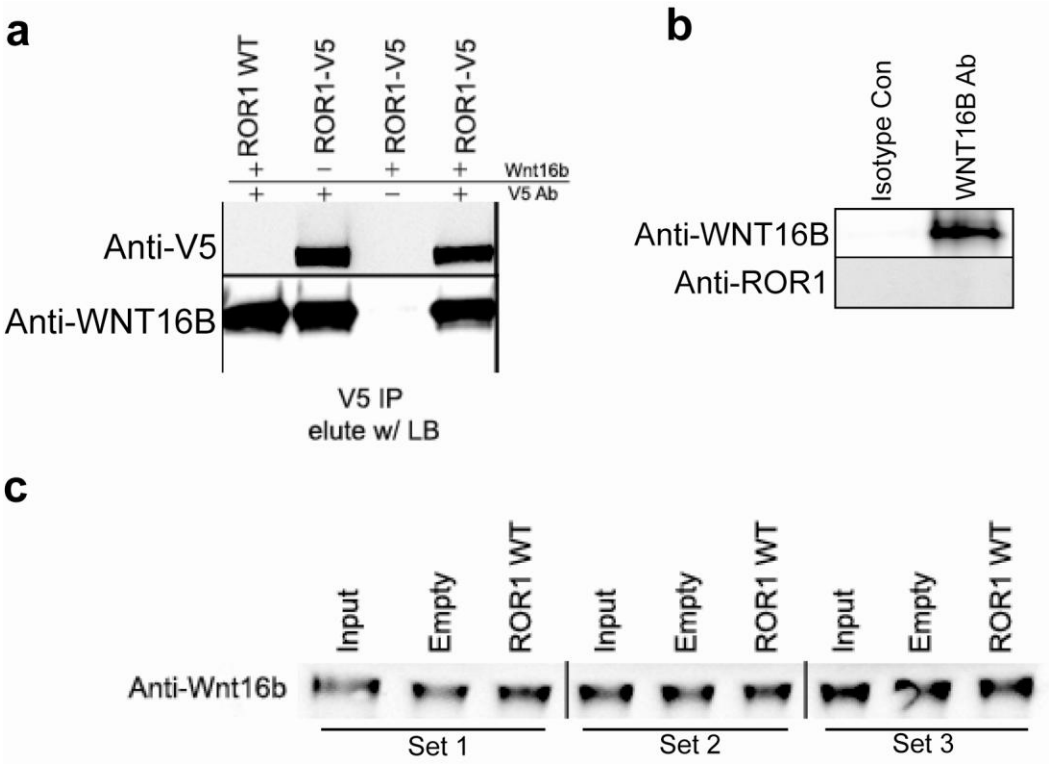


Figure AI.2. There is no evidence of direct WNT16B/ROR1 interaction. (A) HEK293 cells were transiently transfected with ROR1-V5 and/or WNT16B and ROR1-V5 was immunoprecipitated using anti-V5 antibody. **(B)** Endogenous WNT16B, expressed in RCH-ACV, was immunoprecipitated using WNT16B specific antibody and immunoprecipitates were blotted for WNT16B and ROR1. **(C)** HEK293 cells were transfected with ROR1 and incubated with conditioned media collected from RCH-ACV cells. Media was then collected and subjected to western blot for WNT16B.

A1.3 Exogenous ROR1 expression does not confer viability advantages

Because t(1;19) ALL cells lines, xenographs and a primary sample showed dependence upon ROR1 expression, I examined whether exogenous overexpression in model cell lines would confer a viability advantage. To test this, I used to a retroviral expression system to stably overexpress ROR1 in the BaF3 cell line. BaF3 cells are a murine proB cell line that depend upon IL3 media supplementation for viability. The dependence upon IL3 can be negated by addition of an oncogene that activates pathways normally activated by IL3. This model system has been used extensively in the characterization of leukemic oncogenes such as BCR-ABL[191-193]. Overexpression of ROR1 in this cell background had no effect on cell outgrowth following IL3 removal (**Figure AI.3b**). Similarly, ROR1 overexpressing BaF3 cells had no significant change in growth kinetic compared to empty vector control when grown in complete media (**Figure AI.3a**) or reduced IL3 media (data not shown). Likewise, stable ROR1 overexpression in the human erythroleukemia cell line TF1 (which is dependent upon GM-CSF conditioned media[194]) yielded results similar to those observed in BaF3 cells (data not shown). Finally, I analyzed the phosphorylation state of critical signaling pathways following stable overexpression of ROR1 in the human proB cell line REH. In accordance with previous studies, exogenous ROR1 overexpression had no effect on phosphorylation pathways identified as altered by the loss of ROR1 in RCH-ACV cells (data not shown). Together, these studies suggest ROR1 functions in a context specific manner, likely depending upon co-expression of partner/effector proteins found in intermediate B cell progenitors.

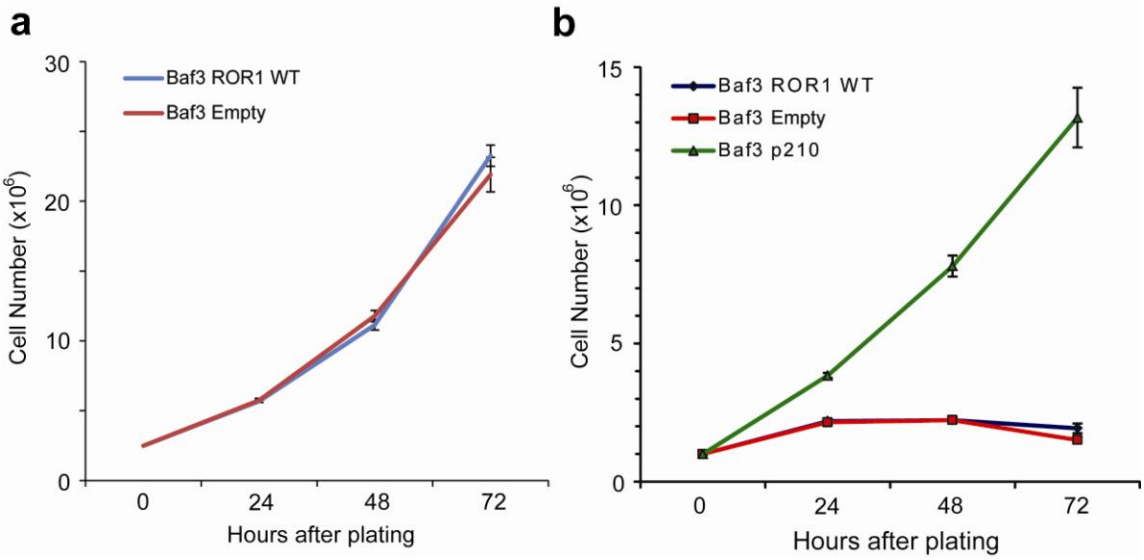


Figure AI.3. ROR1 overexpression in BaF3 cells has no effect on cell growth. BaF3 cells stably overexpressing ROR1 were grown in complete media (**A**) or IL3-depleted media (**B**) and cells counts were performed over 72 hours. For comparison, BaF3 Empty and BaF3 p210 (BCR-ABL) were used as controls.

A1.4 ROR1 participation in canonical/noncanonical WNT signaling

The extracellular frizzled domain possessed by ROR1 and ROR2 has implicated them in canonical/noncanonical WNT signaling. As described in Chapter 2, there is extensive evidence of ROR2 mediating WNT signaling in in vitro and in vivo models. Likewise, there is evidence of ROR1 interacting with WNT5a and mediating noncanonical WNT signaling. To investigate the possibility of ROR1 participating in WNT signaling, I performed western-blotting of β-CATENIN following ROR1 knockdown as well as investigated LEF/TCF activation in response to ROR1 loss and to stimulation with WNT ligand.

Initial experiments investigating BETA-CATENIN levels following ROR1 knockdown yielded interesting results, showing coordinate loss of β-CATENIN in response to ROR1 knockdown and viability effect (**Figure AI.4a**). However, it was not clear from these results whether the effect on β-CATENIN levels was a direct response to ROR1 loss or an indirect result of viability loss.

To address this question I utilized a LEF/TCF reporter construct that results in GFP expression in response to β-CATENIN mediated LEF/TCF transcription activation. Using a transient expression system in HEK293 cells, I performed simultaneous transfection of the reporter, ROR1, Δ6-7 ROR1 (which lacks its frizzle domain) and different WNT ligands. As HEK293s lack constitutive LEF/TCF activity, this system allowed me to explore whether introduction of ROR1 constructs could activate LEF/TCF activity in the absence of ligand. Also, I was able to evaluate whether ROR1 constructs would enhance or abrogate LEF/TCF activity in the presence of WNT ligand. I utilized several approaches to stimulate the system with ligand. WNT3a conditioned

media, collect from murine LWnt3a cells, resulted in a robust activation of the LEF/TCF reporter; however, the addition of ROR1 to the system had no effect on this activation. WNT16b condition media, collected from RCH-ACV cells, did not result in activation of the LEF/TCF reporter and the addition of ROR1 did not change this result.

Because earlier experimental results suggested ROR1 may be functioning in a context-specific manner, I generated a stable LEF/TCF reporter RCH-ACV cell lines. Unlike HEK293 cells, RCH-ACV cells have baseline LEF/TCF activity and as a result I could test whether this basal activity was accentuated or abrogated in response to ROR1 siRNA knockdown. Likewise, I could evaluate basal LEF/TCF activity in response to WNT ligand or drug addition. Again, I did not observe any consistent change in reporter activity following modulation of ROR1 expression. However, much to my surprise, I do see consistent abrogation of LEF/TCF reporter activity in response to dasatinib treatment (**Figure AI.4b**). 24 hour dasatinib treatment results in a dose dependent reduction of reporter activity, suggesting a potential connection between pre-BCR signaling and canonical WNT-signaling activation.

Interestingly, my phospho-proteome profiling experiment described in Chapter 3 also identified β-CATENIN as being modulated downstream of both ROR1 and AKT (**Figure 3.15**). The proteome profiler showed a significant increase in β-CATENIN-phosphorylation in response to dasatinib treatment. This observation correlates with my work using the LEF/TCF reporter in RCH-ACV cells, as phosphorylation of β-CATENIN results in its degradation and subsequent abrogation of LEF/TCF activation. However, after knocking down ROR1, the phospho-proteome profiler shows a decrease in β-CATENIN phosphorylation. This result seems to counter my observation in **Figure**

AI.4a, but a closer look at the proteome profiler results show the phosphorylation of β-CATENIN completely disappears. While it is possible this is a real result, I anticipate it is more likely a result of β-CATENIN loss resulting from the viability effect of ROR1 loss.

As a whole, there does appear to be an important role for the canonical WNT/β-CATENIN signaling pathway in t(1;19) ALL. However, my results suggest that it is dasatinib that disrupts this signaling pathway, likely due to inhibition of the pre-BCR, and ROR1 loss has not resulted in a consistent effect on WNT/β-CATENIN signaling.

A1.5 Rescuing dasatinib sensitivity in t(1;19) ALL

In Chapter 3, I show dasatinib treatment consistently impairs t(1;19) ALL viability and inhibits pre-BCR activation and signaling. I also show that loss of Igα and Igβ, necessary components of the pre-BCR, impairs t(1;19) ALL viability, showing the importance of the pre-BCR in t(1;19) ALL. I hypothesized that dasatinib's mechanism of action was via inhibition of the critical SRC-family kinases required for activation of the pre-BCR and, possibly, by inhibition of BTK. The SRC-family of tyrosine kinases as well as TEC-family kinases, of which BTK is a member, are known targets of dasatinib (**Figure 3.2**). In an attempt to prove these dasatinib targets as the viability dependent target in t(1;19) ALL, I made dasatinib resistant mutants of SRC, LYN and BTK and stably overexpressed them in RCH-ACV cells. It is known that multiple SRC-family kinases play a redundant role in activation of the pre-BCR, as knockout mouse models require loss of Lyn, Fyn, and Blk to disrupt pre-BCR activation (discussed in detail in Chapter 3). However, I hypothesize that overexpression of a single dasatinib-resistant SRC-kinase should be able to rescue what would otherwise be the loss of all

three kinases in the presence of dasatinib. I successfully generated dasatinib-resistant SRC, LYN and BTK mutants utilizing previously described gatekeeper residue mutation in SRC and BTK, and using homology modeling to make an equivalent mutation in LYN (see: Appendix II; Constructs, Vectors and Sequences)[195, 196]. The BTK and LYN mutants were successfully stably overexpressed in RCH-ACV cells and showed insensitivity to dasatinib treatment (**Figure AI.5a**). Unfortunately, neither of these mutant successfully rescued dasatinib sensitivity in these cells (**Figure AI.5b**).

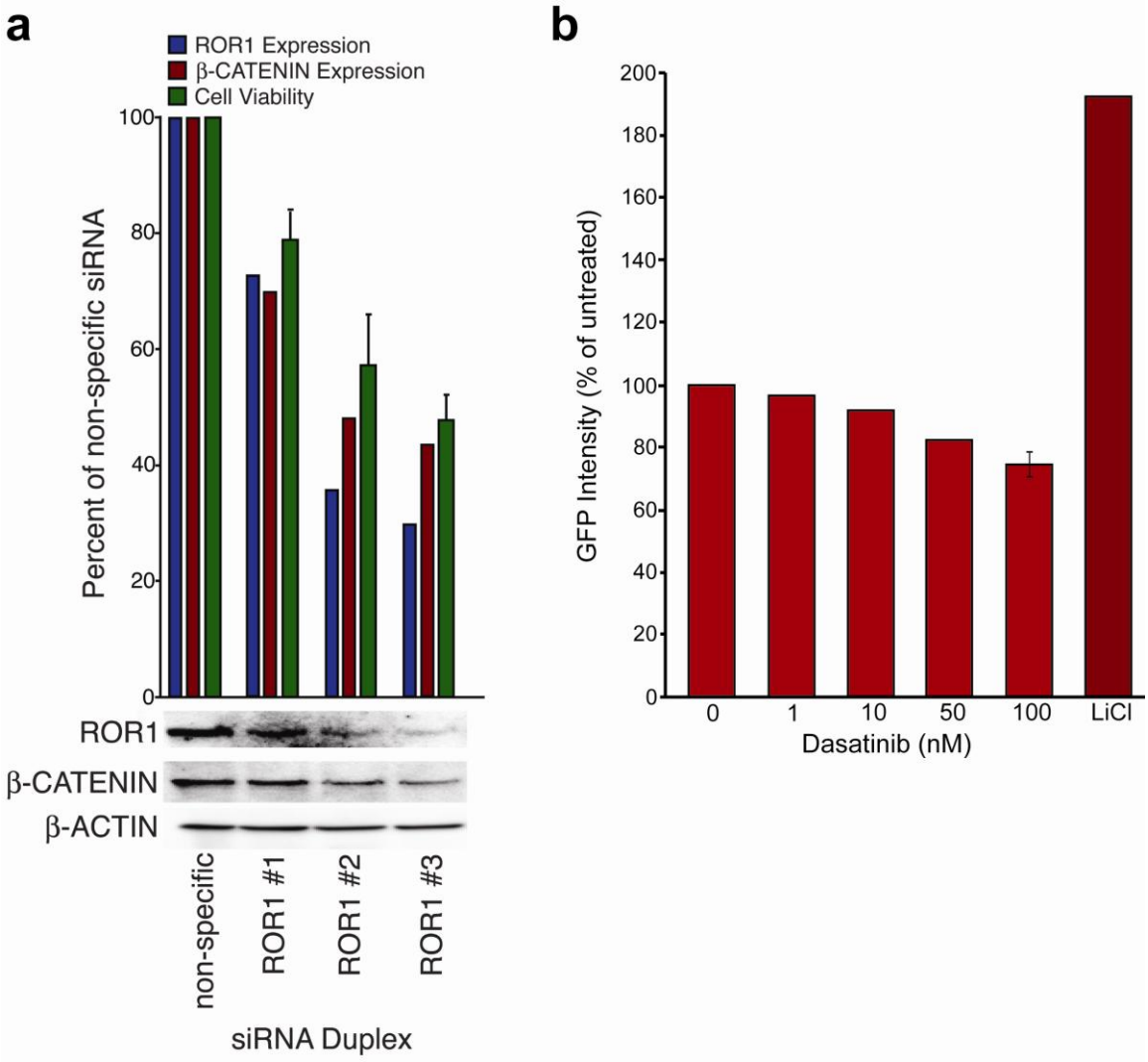


Figure AI.4. ROR1 and WNT signaling. (**A**) RCH-ACV cells were electroporated in the presence of an individual duplex of non-specific siRNA or 3 individual duplexes of siRNA targeting different portions of ROR1 and cells were plated into culture media. After 3 days, cells were lysed and subjected to immunoblot with antibodies specific for ROR1, BETA-CATENIN or β-ACTIN. After 4 days, parallel cultures of cells were subjected to an MTS assay to measure cell viability. Values represent percent mean (normalized to non-specific control wells) ± s.e.m. (n = 6). (**B**) Transgenic RCH-ACV cells possessing a Lef/TCF reporter construct were treated with graded concentrations of dasatinib or LiCl (5mM) for 24 hours prior to GFP analysis by flow cytometry. Values represent percent mean (normalized to untreated) ± s.e.m. (n = 3). Experimental replicates are only available for the 100nM dasatinib dosage.

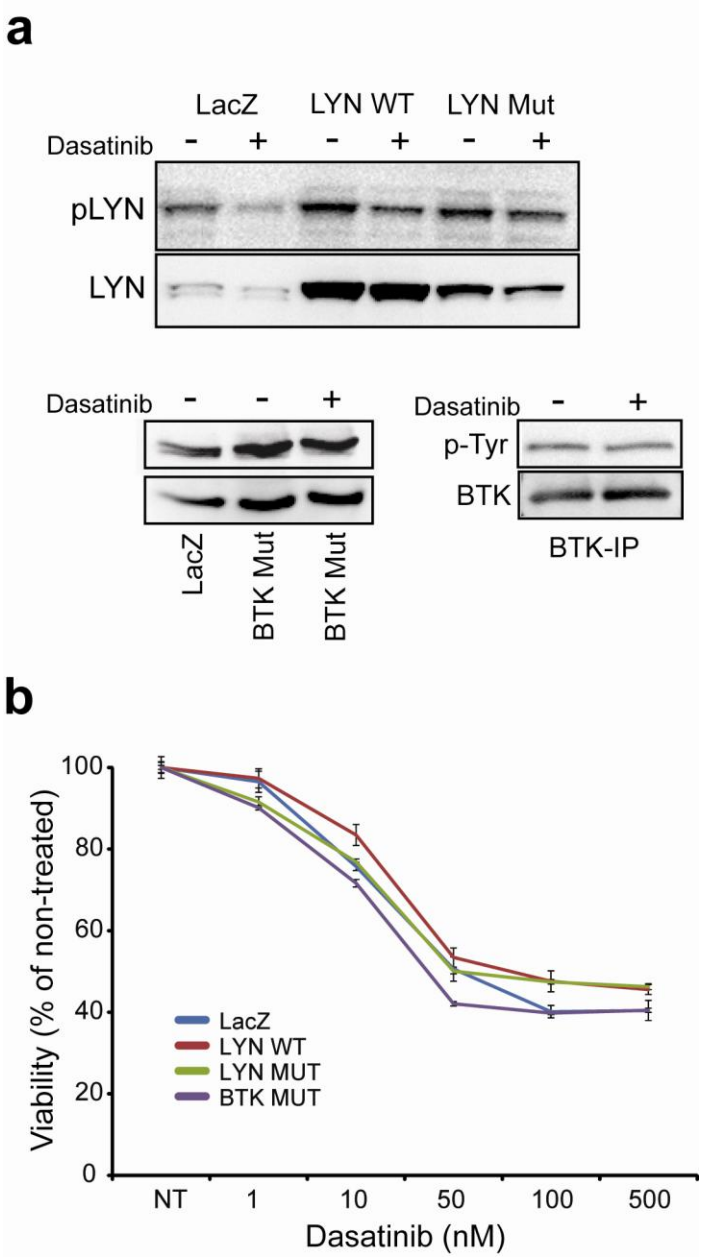


Figure AI.5. Dasatinib-insensitive LYN and BTK do not rescue t(1;19) ALL dasatinib-sensitivity. (**A**) RCH-ACV cells stably expressing LYN WT, LYN Mut or BTK Mut were cultured in the presence of 100nM dasatinib for 1 hour before cells were lysed and subjected to immunoblot analyses with antibodies specific for phospho- or total-LYN, or total-BTK. BTK Mut-positive cells were incubated in the presence of dastinib (100nM) for 1 hour before cells were lysed and BTK was immunoprecipitated and immunoblot analysis of phospho-tyrosine or total-BTK was performed. (**B**) RCH-ACV cells stably expressing LacZ-control, LYN WT, LYN Mut, or BTK Mut were incubated in graded concentration of dasatinib for 72hr before the an MTS reagent was added to assess viability. Values respesent percent mean (normalized to untreated) ± s.e.m. (n = 5).

DISCUSSION

Conserved expression of ROR1 in the t(1;19) ALL background made the E2A-PBX1 fusion product an enticing driver of ROR1 expression. However, reports of consistent ROR1 expression in CLL, another B-cell malignancy, but one that does not possess the E2A-PBX1 transcription factor, suggested there was likely another reason for ROR1's expression profile in t(1;19) ALL. Microarray analysis of ROR1 expression in common ALL backgrounds as well as normal B-cell progenitors showed, for the first time, that ROR1 is expressed in normal B-cell progenitor populations, pre-BII large and small. Perhaps not surprising, this stage of development (pre-BII small) happens to be the stage of developmental arrest observed in t(1;19) ALL. This observation suggests ROR1's expression in the t(1;19) ALL background is a result of the leukemia's state of differentiation arrest. siRNA knockdown experiments, showing E2A-PBX1 knockdown had no effect on ROR1 expression but does impair expression of known transcription targets, support this idea. Together, these results strongly support a model in which ROR1 is naturally upregulated during B-cell development and generation of the E2A-PBX1 oncogene acts to arrest B-cell differentiation at a stage in which ROR1 is expressed and functionally important.

Interrogation of WNT16B and WNT-signaling, in general, proved to be far more difficult than initially thought. Issues with WNT16B stem from a general lack of basic knowledge about this proposed ligand. At the time of my work, WNT16B had not been classified as a canonical or noncanonical WNT and has no described function. As such, I never really knew if I was utilizing an active WNT ligand when performing assays that required transfection of WNT16B or incubation in a conditioned media. This difficult

problem with WNT ligands is best demonstrated by my experience with the well characterized canonical WNT ligand WNT3a. A well-established protocol for generating functional WNT3a ligand exists in which murine L cells stably expressing human WNT3a are used to make conditioned media[197]. WNT3a conditioned media generated according to this protocol generates a robust response in the Lef/TCF reporter-based assay (as described above); however, when the same WNT3a expression vector is transfected into HEK293s, it fails to generate functional WNT3a. While the HEK293s make WNT3a protein that is detectable in whole-cell lysates and culture media by western-blot, it is not an active, mature protein and fails to activate the Lef/TCF reporter. In an attempt to address this potential problem we performed most experiments looking at ROR1/WNT16B interaction with sources of WNT16B that were not limited to overexpression in HEK293s. The conditioned media of t(1;19) ALL cell lines, RCH, Kasumi-2, and 697 all show levels of WNT16B detectable by western-blot. This conditioned media was used in IP experiments looking for direct interactions as well as in pathway activation experiments and yielded similar results to WNT16B generated by transient transfections.

Despite these difficulties, the role of WNT16B in t(1;19) ALL remains an interesting question. A recent study, showing zebrafish WNT16 regulates a signaling network required for hematopoietic stem cell specification, and is the first work to describe WNT16 as a non-canonical WNT and connect it to Notch signaling. In addition, this is first work to functionally link WNT16 to hematopoiesis and reinforces interest in WNT16B's role in t(1;19) ALL leukemogenesis[198]. It is clear that WNT16B's expression is a product of the E2A-PBX1 transcription factor and is unique

to this ALL background. ROR1 remains an enticing potential receptor for this ligand but more work must be done to understand the basic functions of each of these two proteins before we will know if and how they work together. The fact that ROR1 is expressed in normal B cell progenitors that do not express WNT16B certainly suggests ROR1 function is not dependent upon WNT16B as a ligand. However, the possibility of WNT16B being expressed by stromal cells and interacting with ROR1 on B-cell progenitors in a paracrine manner is intriguing idea and would suggest that the acquired expression of WNT16B following the 1;19 translocation could allow for autocrine activation of the same pathway in circulating leukemic blasts. The metaanalysis comparing common B-ALL subtypes and normal B cell progenitors provides many interesting clues as to what signaling pathways are important in ALL. Again, a number of differentially regulated genes pointed toward the canonical WNT/β-CATENIN pathway as being important in t(1;19) ALL. The negative regulator of the canonical pathway GSK3β is downregulated in t(1;19) ALL, while the downstream transcription factor LEF is substantially upregulated. This expression profile, again, makes canonical WNT/β-CATENIN signaling a very tempting target for further exploration.

While my attempts at directly linking ROR1 to WNT-signaling failed to generate any definitely conclusions, my fortuitous inclusion of dasatinib as a drug-treatment control resulted in the interesting observation that 24 hour dasatinib incubation results in a dose dependent abrogation of LEF/TCF reporter activity. This discovery brings up some interesting questions about the drivers of canonical WNT signaling in t(1;19) ALL and definitely warrants future study.

My attempt to rescue dasatinib sensitivity in t(1;19) ALL failed with single dasatinib-resistant mutants of what I felt were the most likely viability-dependent targets of dasatinib. There are many potential explanations as to why these rescue experiment were unsuccessful, but the most likely reason is that reconstitution of a single enzyme in this system is not sufficient to rescue the multiple targets of dasatinib. I have yet to co-express multiple dasatinib-resistant kinases and it is likely that I am not completely rescuing pre-BCR signaling. While the SRC-kinases are thought to have redundant roles in activating the pre-BCR, this work is based upon B-cell development analysis in murine models, and it is highly likely that different t(1;19) ALL patients samples and cell lines could be preferentially dependent upon specific SRC-kinase expression. In a similar manner, the viability effect could be distributed between SRC-kinase inhibition and BTK inhibition and I have not co-expressed the two protein constructs. In addition, SRC-kinases likely play important roles in the cell outside of activating the pre-BCR. I see in my proteome-profiler experiment (**Figure 3.15**), that SRC is strongly inhibited. While SRC is not typically associated with the pre-BCR, it has been shown to activate PLCγ1[199], and I also see inhibition of PLCγ1 in the proteome-profile. It is not clear whether inhibition of PLCγ1 has a viability effect on t(1;19) ALL or if inhibition of this pathway exacerbates the viability effect of pre-BCR inhibition, but this is a possibility I will have to investigate. Likewise, my constant-ration multidrug synergy testing experiment, utilizing dasatinib and MERCK AKT1/2, showed moderate synergy between the two drug. If, in fact, there is a synergistic effect between dasatinib and MERCK AKT1/2, this would suggest dasatinib's (or potentially MERCK AKT1/2's) viability effect is not exclusively mediated by AKT inhibition. If this is true, then

perhaps this additional viability effect is mediate by SRC/PLCγ1 or another kinase target of dasatinib (**Figure 3.2**).

The viability dependence of t(1;19) ALL to ROR1 loss and dasatinib treatment acts as a valuable assay for interrogating these pathways. The use of a lentiviral system to stably overexpress mutant proteins will allow us to further understand the mechanism of these viability dependent pathways. Future work on t(1;19) ALL will utilize this system to selectively disrupt ROR1 signaling and selectively rescue dasatinib sensitivity. For instance, stable overexpression of an AKT construct that is perpetually associated with the plasma membrane, and thus constitutively active and independent of PI3K, should maintain negative-control of Foxo proteins and, theoretically rescue the dasatinib induced viability effect mediated by inhibition of the pre-BCR. However, if dasatinib has targets outside the pre-BCR and AKT, I should see only a partial viability rescue. In addition to conclusively showing off-target viability effects of dasatinib, this system should help us identify those effects by eliminating contamination of the complex downstream targets of AKT signaling. Likewise, knockdown of ROR1 results in decreased AKT phosphorylation at the S473 (PDK2) site and upregulation at the T308 (PDK1) site (**Figure 3.15, 3.16**). This suggests ROR1 loss is not disrupting PI3K (which disrupts both sites, as seen with pre-BCR inhibition) and is instead just disrupting PDK2 activity. Use of a constitutively active AKT construct could help test this hypothesis, as it should rescue the pre-BCR inhibition phenotype, but have no effect on the ROR1 knockdown phenotype. In a similar manner, ROR1 mutants can be used to interrogate ROR1 function in t(1;19) ALL. I am currently in the process of generating a panel of ROR1 mutants that will allow us to understand the critical portions of the

ROR1 protein required to mediate its function in t(1;19) ALL. Using the 3'-UTR siRNA system described in **Figure 3.4**, I can stably overexpress ROR1 mutants in RCH-ACV cells and then knockdown viability dependent expression of endogenous ROR1. The presence of a functional exogenous ROR1 will rescue these siRNA treated cells, whereas exogenous expression of a non-functional ROR1 should not rescue these cells. This system will allow us to first interrogate entire region of ROR1, followed by specific amino-acid mutations and will constitute the first biochemical understanding of ROR1 function in lymphoid biology.

APPENDIX II

ADDITIONAL MATERIALS AND METHODS

AND CONTRIBUTIONS

Constructs, Vectors and Sequences

ROR1

The *ROR1* (homo sapiens receptor tyrosine kinase-like orphan receptor 1) gene open-reading frame was purchased from GeneCopoeia, ORFEXPRESS-Shuttle Clone GC-A0499. This construct is generated from accession number NM_005012. The ORFEXPRESS-Shuttle Clone was then used for mutagenesis and Gateway recombination into destination vectors.

Mutants: *Δ6-7 ROR1, Δ7 ROR1, Δ6-8 ROR1, Δ9 CSS ROR1, ROR1-V5*

Methods: For *ROR1* exon loss mutants, PCR products were generated using primers specific to the 1st and 9th exon of *ROR1*. Products were resolved by agarose gel electrophoresis and bands were extracted using the Montage DNA Gel Extraction Kit (Millipore). Purified PCR products were then cloned into pENTR/D-TOPO (Invitrogen). Cloned ROR1 PCR products and the ORFEXPRESS-Shuttle Clone ROR1 vectors were then digested with BglII and BlpI. Restriction digest products were then resolved and purified as described above. The digested PCR product and the digested ORFEXPRESS-Shuttle Clone ROR1 vector were then dephosphorylated and combined for a ligation reaction (Roche). For ROR1-V5, site-directed mutagenesis was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). The ORFEXPRESS-Shuttle Clone ROR1 vector served as the template and the TAG stop codon included in the vector backbone was mutated to an alanine (GCG).

Sequences: See **Figure 2.3** for sequence detail.

BCL6

The *BCL6* (human B-cell CLL/lymphoma 6) gene open-reading frame was purchased from GeneCopoeia, ORFEXPRESS-Shuttle Clone GC-F0729. This construct is generated from accession number NM_001706. The ORFEXPRESS-Shuttle Clone was then used for mutagenesis and Gateway recombination into destination vectors.

Mutants: none

BTK

The *BTK* (human Bruton agammaglobulinemia tyrosine kinase) gene open-reading frame was purchased from GeneCopoeia, ORFEXPRESS-Shuttle Clone GC-A0534. This construct is generated from accession number NM_000061. The ORFEXPRESS-Shuttle Clone was then used for mutagenesis and Gateway recombination into destination vectors.

Mutants: T474I (dasatinib resistant; gatekeeper mutant)

Methods: Site-directed mutagenesis for BTK (T474I) was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). The ORFEXPRESS-Shuttle Clone BTK vector served as the template.

Sequence:

mRNA WT 1583-TGC/ACC/AAG/CAG/CGC/CCC/ATC/TTC/ATC/ATC/ACT/GAG/TAC/ATG/GCC/AAT
Protein WT 464-C T K Q R P I F I I T E Y M A N
mRNA Mut 1583-TGC/ACC/AAG/CAG/CGC/CCC/ATC/TTC/ATC/ATC/ATT/GAG/TAC/ATG/GCC/AAT
Protein Mut 464-C T K Q R P I F I I I E Y M A N

LYN

The *LYN* (human v-yes-1 Yamaguchi sarcoma viral related oncogene homolog) gene open-reading frame was purchased from GeneCopoeia, ORFEXPRESS-Shuttle Clone GC-G0143. This construct is generated from accession number NM_002350. The ORFEXPRESS-Shuttle Clone was then used for mutagenesis and Gateway recombination into destination vectors.

Mutants: T319I (dasatinib resistant; gatekeeper mutant)

Methods: Site-directed mutagenesis for LYN (T319I) was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). The ORFEXPRESS-Shuttle Clone *LYN* vector served as the template.

Sequence:

mRNA WT 1213-AGG/GAG/GAG/CCC/ATT/TAC/ATC/ATC/ACC/GAG/TAC/ATG/GCC/AAG

Protein WT 311- R E E P I Y I I T E Y M A K

mRNA Mut 1213-AGG/GAG/GAG/CCC/ATT/TAC/ATC/ATC/ATC/GAG/TAC/ATG/GCC/AAG

Protein Mut 311- R E E P I Y I I I E Y M A K

E2A-PBX1

The *E2A-PBX1* fusion gene product open-reading frame was amplified from the RCH-ACV cell line using pENTR Directional TOPO Cloning Kit (Invitrogen). The 2491bp E2A-PBX1 product was cloned into pENTR/D-TOPO. The pENTR/D-TOPO clone was then used for mutagenesis and Gateway recombination into destination vectors.

Mutants: None

WNT16B

The *WNT16B* gene open-reading frame was amplified from the RCH-ACV cell line using pENTR Directional TOPO Cloning Kit (Invitrogen). The 1120bp WNT16B product was cloned into pENTR/D-TOPO. The pENTR/D-TOPO clone was then used for mutagenesis and Gateway recombination into destination vectors.

Mutants: None

Destination Vectors

See **Appendix Figure AII** for vector maps.

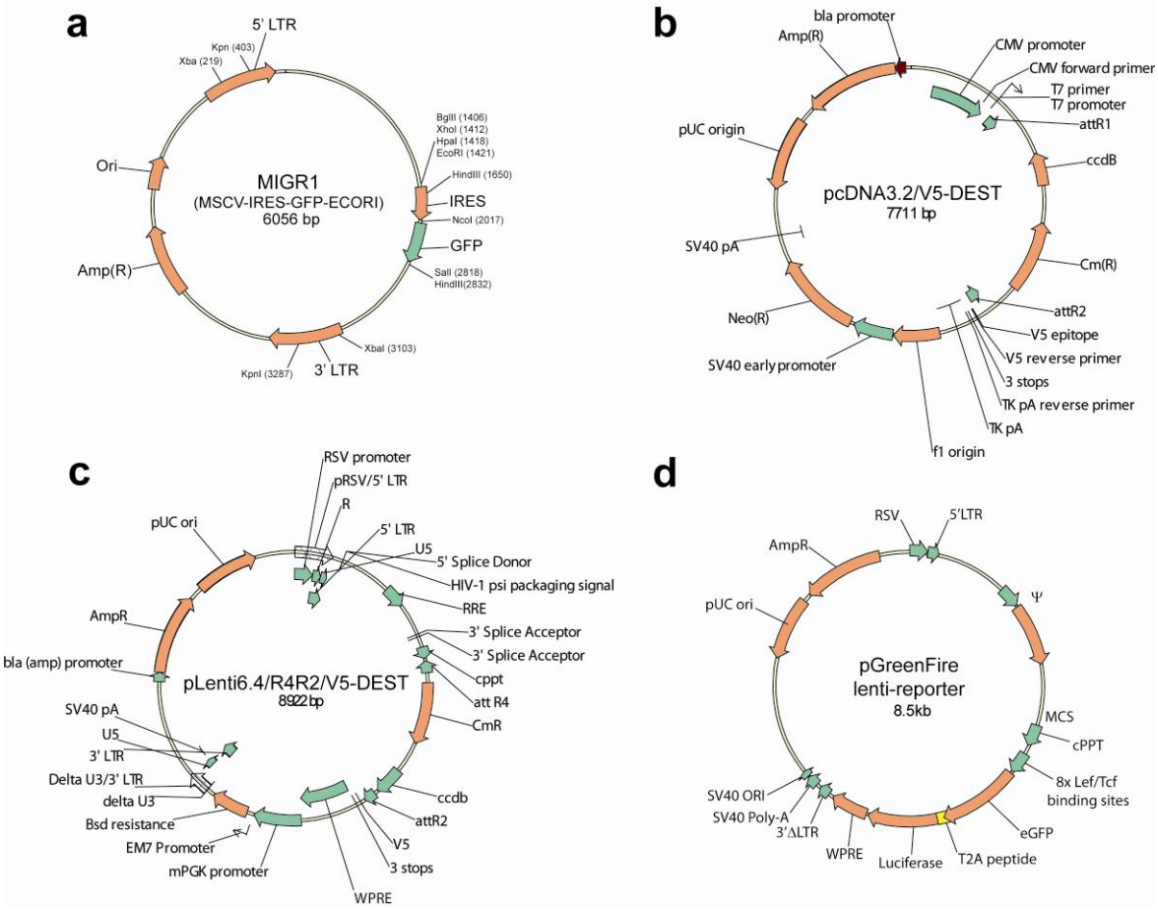


Figure AII.1. Destination Vectors. (A) MSCV-IRES-GFP ECOR1 (MIGR1) was constructed by Juli Miller (Pear Lab). The humanized GFP contains red shift (64; Phe-Leu, 65; Ser-Thr) and solubility mutations (163; Val-Ala) and contains an extra Ala at position 2 for Kozak site. This was excised as an 800bp EcoR1 fragment that was blunted with Klenow and cloned into pBSKII. This was removed as an 800bp NcoI-SalI fragment that was ligated in a 3-way reaction with EcoRI-SalI cleaved MSCV2.2 (~5kb) and 592 bp EcoR1-Nco1 fragment from pCITE (IRES from EMCV). **(B)** pcDNA3.2/V5-DEST is commercially available from Invitrogen. It possesses a C-terminal V-5 epitope tag and contains the neomycin resistance gene for selection of stable cell lines using G418. **(C)** pLenti6.4/R4R2/V5-DEST is commercially available from Invitrogen and is the destination vector of the ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit. This vector allows for lentiviral-based high-level expression of a target gene from any promoter of choice. It possesses a C-terminal V5 epitope tag and contains a blastacidin resistance gene for stable selection. For stable expression experiment in t(1;19) ALL cell lines, the EF1α promoter provided with the kit was always used. (**D**) pGreenFire (pGF) lenti-reporter was constructed by Kim-Hien Dao. Figures adapted from Vector NTI (Invitrogen).

Primers

ROR1

qRT-PCR:
Forward (F4): ACC GCA CCG TCT ATA TGG AGT CT
Details: Nt (950-972), Tm (59.4)
Reverse (R3): GCA TAG TGG CAC AGG GAA GG
Details: Nt (1091), Tm (58.3)

RT-PCR
Forward (F6): GGC TGC TGC CCA AGA AAC AGA
Details: Nt (477-497), Exon1/2, Tm (60.5)
Forward (F4): ACC GCA CCG TCT ATA TGG AGT CT
Details: Nt (950-972), Exon 5, Tm (59.4)
Reverse (R4): ATG TGC AGA AAA TCT CCG TGG TCC A
Details: Nt (2159-2183), Exon 9, Tm (60.6)

Mutagenesis:
TAG KO ORFEXPRESS
Forward: AGA GCT ACC TCT TTC TCT TCG GTC CTG GGA GG
Reverse: CCG CAC TCG AGC GAC AGT TCT GCA GAA ATC ATA GAT
Y Kinase Domain Deletion (del 813-1634)
Forward: AGA GCT ACC TCT TTC TCT TCG GTC CTG GGA GG
Reverse: CCT CCC AGG ACC GAA GAG AAA GAG GTA GCT CT

E2A-PBX1

qRT-PCR:
Forward (F1): ACC AGC CTC ATG CAC AAC CAC
Details: NT (1396-1416); Tm (60.5)
Reverse (R2): GCA TGT TGT CCA GCC GCA TC
Details: NT (1530-1549); Tm (59.7)

WNT16b

qRT-PCR:
Forward (F2): CCT ACG GAG CCC AAG GAA ACT G
Details: NT (328-349)
Reverse (R5): CAC TCT GTC ATG TTG CCT GCA CT
Details: NT (675-697)

CONTRIBUTIONS

Vincent T. Bicocca (VTB)[1], Bill H. Chang (BHC)[1], Marc M. Loriaux (MML)[1], Markus Muschen (MM)[2], Brian J. Druker (BJD)[1,3], and Jeffrey W. Tyner (JWT)[1].

1. Knight Cancer Institute, Oregon Health & Science University, Portland, OR.

2. Department of Laboratory Medicine, University of California, San Francisco, CA.

3. Howard Hughes Medical Institute

VTB designed and performed research, analyzed data, and assembled figures; BHC, MML, and BJD provided expert advice and reagents; MM analyzed data and provided expert advice and reagents; JWT designed and performed research, analyzed data, and assembled figures. Individual contributions are detailed below.

List of Figures

1.1 Experiment performed and figure constructed by JWT

2.1 Figure constructed by VTB.

2.2 Figure constructed by VTB.

2.3 Experiments performed and figures constructed by VTB.

2.4 Samples acquired by BHC and MML. Experiment performed and figure constructed by VTB.

2.5 Analysis performed by MM. Figure constructed by VTB.

2.6 Experiments performed and figures constructed by VTB.

2.7 Experiments performed and figures constructed by VTB.

3.1 Figure constructed by VTB.

3.2 Figure constructed by VTB

3.3 Experiments performed and figures constructed by VTB and JWT.

3.4 Experiments performed and figures constructed by VTB and JWT.

3.5 Xenographs by MM. Experiments performed and figures constructed by VTB and JWT.

3.6 Samples acquired by BHC and MML. Experiments performed and figures constructed by VTB and JWT.

3.7 Experiments performed and figures constructed by VTB.

3.8 Analysis performed by MM. Figure constructed by VTB.

3.9 Experiments performed and figures constructed by VTB.

3.10 Experiments performed and figures constructed by VTB.

3.11 Experiments performed and figures constructed by VTB.

3.12 Experiments performed and figures constructed by VTB.

3.13 Experiments performed and figures constructed by VTB.

3.14 Experiments performed and figures constructed by VTB.

3.15 Experiments performed and figures constructed by VTB and JWT.

3.16 Experiments performed and figures constructed by VTB.

3.17 Experiments performed and figures constructed by VTB.

3.18 Experiments performed and figures constructed by VTB and JWT.

3.19 Figure constructed by VTB.

AI.1 Analysis performed by MM. Figure constructed by VTB.

AI.2 Experiments performed and figures constructed by VTB.

AI.3 Experiments performed and figures constructed by VTB.

Bibliography

1. WHO Statistical Information System, *Number and rates of registered dealths, U.S.o.A.* World Health Organization, 2005.

2. American Cancer Society, *Cancer Facts and Figures 2008*, 2008, American Cancer Society: Atlanta, GA.

3. The Merck Manuals Online Medical Library, *Hematology and Oncology*, 2008.

4. Swerdlow, S.H., E. Campo, N.L. Harris, E.S. Jaffe, S.A. Pileri, H. Stein, J. Thiele, and J.W. Vardiman, *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.* Lyon, France: International Agency for Research on Cancer, 2008. **4th ed.**

5. Kersey, J.H., *Fifty years of studies of the biology and therapy of childhood leukemia.* Blood, 1997. **90**(11): p. 4243-51.

6. Greaves, M.F. and J. Wiemels, *Origins of chromosome translocations in childhood leukaemia.* Nat Rev Cancer, 2003. **3**(9): p. 639-49.

7. Lengauer, C., *How do tumors make ends meet?* Proc Natl Acad Sci U S A, 2001. **98**(22): p. 12331-3.

8. Look, A.T., *Oncogenic transcription factors in the human acute leukemias.* Science, 1997. **278**(5340): p. 1059-64.

9. Children's Oncology Group, *Classification of Newly Diagnosed Acute Lymphoblastic Leukemia (ALL).* CureSearch Children's Oncology Group, 2010(AALL08B1).

10. Smith, M.A., L.A. Gloeckler-Ries, J.G. Gurney, and J.A. Ross, *SEER Pediatric Monograph. Leukemia.* National Cancer Institute, 1999.

11. Children's Oncology Group, *Long-Term Follow-Up Guidelines for Survivors of Childhood, Adolescent, and Young Adult Cancers.* 2008. **Version 3.0**.

12. National Cancer Institute, *Late Effects of Treatment for Childhood Cancer (PDQ®).* 2010.

13. Krause, D.S. and R.A. Van Etten, *Tyrosine kinases as targets for cancer therapy.* N Engl J Med, 2005. **353**(2): p. 172-87.

14. Rowley, J.D., *Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining.* Nature, 1973. **243**(5405): p. 290-3.

15. Heisterkamp, N., K. Stam, J. Groffen, A. De Klein, and G.C. Grosveld, *Structural organization of the bcr gene and its role in the Ph' translocation.* Nature, 1985. **315**: p. 758-761.

16. de Klein, A., A.G. van Kessel, G.C. Grosveld, C.R. Bartram, A. Hagemeijer, D. Bootsma, N.K. Spurr, N. Heisterkamp, J. Groffen, and J.R. Stephenson, *A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia.* Nature, 1982. **300**(5894): p. 765-7.

17. Daley, G.Q., R.A. Van Etten, and D. Baltimore, *Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome.* Science, 1990. **247**(4944): p. 824-30.

18. Druker, B.J., M. Talpaz, D.J. Resta, B. Peng, E. Buchdunger, J.M. Ford, N.B. Lydon, H. Kantarjian, R. Capdeville, S. Ohno-Jones, and C.L. Sawyers, *Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia.* N Engl J Med, 2001. **344**(14): p. 1031-7.

19. Forman, S.J., M.R. O'Donnell, A.P. Nademanee, D.S. Snyder, P.J. Bierman, G.M. Schmidt, J.L. Fahey, A.S. Stein, P.M. Parker, and K.G. Blume, *Bone marrow transplantation for patients with Philadelphia chromosome-positive acute lymphoblastic leukemia.* Blood, 1987. **70**(2): p. 587-8.

20. Cools, J., D.J. DeAngelo, J. Gotlib, E.H. Stover, R.D. Legare, J. Cortes, J. Kutok, J. Clark, I. Galinsky, J.D. Griffin, N.C. Cross, A. Tefferi, J. Malone, R. Alam, S.L. Schrier, J. Schmid, M. Rose, P. Vandenberghe, G. Verhoef, M. Boogaerts, I. Wlodarska, H. Kantarjian, P. Marynen, S.E. Coutre, R. Stone, and D.G. Gilliland, *A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome.* N Engl J Med, 2003. **348**(13): p. 1201-14.

21. Golub, T.R., G.F. Barker, M. Lovett, and D.G. Gilliland, *Fusion of PDGF receptor beta to a novel ets-like gene, tel, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation.* Cell, 1994. **77**(2): p. 307-16.

22. de Silva, C.M. and R. Reid, *Gastrointestinal stromal tumors (GIST): C-kit mutations, CD117 expression, differential diagnosis and targeted cancer therapy with Imatinib.* Pathol Oncol Res, 2003. **9**(1): p. 13-9.

23. Lynch, T.J., D.W. Bell, R. Sordella, S. Gurubhagavatula, R.A. Okimoto, B.W. Brannigan, P.L. Harris, S.M. Haserlat, J.G. Supko, F.G. Haluska, D.N. Louis, D.C. Christiani, J. Settleman, and D.A. Haber, *Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib.* N Engl J Med, 2004. **350**(21): p. 2129-39.

24. Yarden, Y. and M.X. Sliwkowski, *Untangling the ErbB signalling network.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 127-37.

25. Joensuu, H., P.J. Roberts, M. Sarlomo-Rikala, L.C. Andersson, P. Tervahartiala, D. Tuveson, S. Silberman, R. Capdeville, S. Dimitrijevic, B. Druker, and G.D. Demetri, *Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor.* N Engl J Med, 2001. **344**(14): p. 1052-6.

26. Motzer, R.J., T.E. Hutson, P. Tomczak, M.D. Michaelson, R.M. Bukowski, O. Rixe, S. Oudard, S. Negrier, C. Szczylik, S.T. Kim, I. Chen, P.W. Bycott, C.M. Baum, and R.A. Figlin, *Sunitinib versus interferon alfa in metastatic renal-cell carcinoma.* N Engl J Med, 2007. **356**(2): p. 115-24.

27. Schittenhelm, M.M., S. Shiraga, A. Schroeder, A.S. Corbin, D. Griffith, F.Y. Lee, C. Bokemeyer, M.W. Deininger, B.J. Druker, and M.C. Heinrich, *Dasatinib (BMS-354825), a dual SRC/ABL kinase inhibitor, inhibits the kinase activity of wild-type, juxtamembrane, and activation loop mutant KIT isoforms associated with human malignancies.* Cancer Res, 2006. **66**(1): p. 473-81.

28. Shah, N.P., C. Tran, F.Y. Lee, P. Chen, D. Norris, and C.L. Sawyers, *Overriding imatinib resistance with a novel ABL kinase inhibitor.* Science, 2004. **305**(5682): p. 399-401.

29. Geyer, C.E., J. Forster, D. Lindquist, S. Chan, C.G. Romieu, T. Pienkowski, A. Jagiello-Gruszfeld, J. Crown, A. Chan, B. Kaufman, D. Skarlos, M. Campone, N. Davidson, M. Berger, C. Oliva, S.D. Rubin, S. Stein, and D. Cameron, *Lapatinib plus capecitabine for HER2-positive advanced breast cancer.* N Engl J Med, 2006. **355**(26): p. 2733-43.

30. Shepherd, F.A., J. Rodrigues Pereira, T. Ciuleanu, E.H. Tan, V. Hirsh, S. Thongprasert, D. Campos, S. Maoleekoonpiroj, M. Smylie, R. Martins, M. van Kooten, M. Dediu, B. Findlay, D. Tu, D. Johnston, A. Bezjak, G. Clark, P. Santabarbara, and L. Seymour, *Erlotinib in previously treated non-small-cell lung cancer.* N Engl J Med, 2005. **353**(2): p. 123-32.

31. Bange, J., E. Zwick, and A. Ullrich, *Molecular targets for breast cancer therapy and prevention.* Nat Med, 2001. **7**(5): p. 548-52.

32. Yu, D. and M.C. Hung, *Overexpression of ErbB2 in cancer and ErbB2-targeting strategies.* Oncogene, 2000. **19**(53): p. 6115-21.

33. Hudis, C.A., *Trastuzumab - Mechanism of Action and Use in Clinical Practive.* N Engl J Med, 2007. **357**: p. 39-51.

34. National Cancer Institute, *Toward Personalized Cancer Care.* 2009.

35. Hayden, E.C., *Personalized cancer therapy gets closer.* Nature, 2009. **458**: p. 131-132.

36. Schultz, K.R., W.P. Bowman, A. Aledo, W.B. Slayton, H. Sather, M. Devidas, C. Wang, S.M. Davies, P.S. Gaynon, M. Trigg, R. Rutledge, L. Burden, D. Jorstad, A. Carroll, N.A. Heerema, N. Winick, M.J. Borowitz, S.P. Hunger, W.L. Carroll, and B. Camitta, *Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study.* J Clin Oncol, 2009. **27**(31): p. 5175-81.

37. Doyle, J.J., A.I. Neugut, and J.S. Jacobson, *Chemotherapy and cardiotoxicity in older breast cancer patients: a population-based study.* J Clin Oncol, 2005. **23**: p. 8597-605.

38. Extermann, M., *Measurement and impact of comorbidity in older cancer patients.* Crit Rev Oncol Hematol, 2000. **35**: p. 181-200.

39. Greenman, C., P. Stephens, R. Smith, G.L. Dalgliesh, C. Hunter, G. Bignell, H. Davies, J. Teague, A. Butler, C. Stevens, S. Edkins, S. O'Meara, I. Vastrik, E.E. Schmidt, T. Avis, S. Barthorpe, G. Bhamra, G. Buck, B. Choudhury, J. Clements, J. Cole, E. Dicks, S. Forbes, K. Gray, K. Halliday, R. Harrison, K. Hills, J. Hinton, A. Jenkinson, D. Jones, A. Menzies, T. Mironenko, J. Perry, K. Raine, D. Richardson, R. Shepherd, A. Small, C. Tofts, J. Varian, T. Webb, S. West, S. Widaa, A. Yates, D.P. Cahill, D.N. Louis, P. Goldstraw, A.G. Nicholson, F. Brasseur, L. Looijenga, B.L. Weber, Y.E. Chiew, A. DeFazio, M.F. Greaves, A.R. Green, P. Campbell, E. Birney, D.F. Easton, G. Chenevix-Trench, M.H. Tan, S.K. Khoo, B.T. Teh, S.T. Yuen, S.Y. Leung, R. Wooster, P.A. Futreal, and M.R. Stratton, *Patterns of somatic mutation in human cancer genomes.* Nature, 2007. **446**(7132): p. 153-8.

40. Sjoblom, T., S. Jones, L.D. Wood, D.W. Parsons, J. Lin, T.D. Barber, D. Mandelker, R.J. Leary, J. Ptak, N. Silliman, S. Szabo, P. Buckhaults, C. Farrell, P. Meeh, S.D. Markowitz, J. Willis, D. Dawson, J.K. Willson, A.F. Gazdar, J. Hartigan, L. Wu, C. Liu, G. Parmigiani, B.H. Park, K.E. Bachman, N. Papadopoulos, B. Vogelstein, K.W. Kinzler, and V.E. Velculescu, *The consensus coding sequences of human breast and colorectal cancers.* Science, 2006. **314**(5797): p. 268-74.

41. Walters, D.K., V.L. Goss, E.P. Stoffregen, T.L. Gu, K. Lee, J. Nardone, L. McGreevey, M.C. Heinrich, M.W. Deininger, R. Polakiewicz, and B.J. Druker, *Phosphoproteomic analysis of AML cell lines identifies leukemic oncogenes.* Leuk Res, 2006. **30**(9): p. 1097-104.

42. Mello, C.C. and D. Conte, Jr., *Revealing the world of RNA interference.* Nature, 2004. **431**(7006): p. 338-42.

43. Tyner, J.W., D.K. Walters, S.G. Willis, M. Luttropp, J. Oost, M. Loriaux, H. Erickson, A.S. Corbin, T. O'Hare, M.C. Heinrich, M.W. Deininger, and B.J. Druker, *RNAi screening of the tyrosine kinome identifies therapeutic targets in acute myeloid leukemia.* Blood, 2008. **111**(4): p. 2238-45.

44. Masiakowski, P. and R.D. Carroll, *A novel family of cell surface receptors with tyrosine kinase-like domain.* J Biol Chem, 1992. **267**(36): p. 26181-90.

45. Oishi, I., S. Takeuchi, R. Hashimoto, A. Nagabukuro, T. Ueda, Z.J. Liu, T. Hatta, S. Akira, Y. Matsuda, H. Yamamura, H. Otani, and Y. Minami, *Spatio-temporally regulated expression of receptor tyrosine kinases, mRor1, mRor2, during mouse development: implications in development and function of the nervous system.* Genes Cells, 1999. **4**(1): p. 41-56.

46. Gentile, A., L. Lazzari, S. Benvenuti, L. Trusolino, and P.M. Comoglio, *Ror1 is a pseudokinase that is crucial for met-driven tumorigenesis.* Cancer Res, 2011. **71**(8): p. 3132-41.

47. Pawson, T., *Protein modules and signalling networks.* Nature, 1995. **373**: p. 573-580.

48. Sudol, M., *The WW module competes with the SH3 domain?* Trends Biochem Sci, 1996. **21**: p. 161-163.

49. Songyang, Z., S.E. Shoelson, J. McGlade, P. Olivier, T. Pawson, X.R. Bustelo, M. Barbacid, H. Sabe, H. Hanafusa, and T. Yi, *Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, and Vav.* Mol Cell Biol, 1994. **14**(4): p. 2777-2785.

50. Matsuda, T., H. Suzuki, I. Oishi, S. Kani, Y. Kuroda, T. Komori, A. Sasaki, K. Watanabe, and Y. Minami, *The receptor tyrosine kinase Ror2 associates with the melanoma-associated antigen (MAGE) family protein Dlxin-1 and regulates its intracellular distribution.* J Biol Chem, 2003. **278**: p. 29057-64.

51. Green, J.L., T. Inoue, and P.W. Sternberg, *The C. elegans ROR receptor tyrosine kinase, CAM-1, non-autonomously inhibits the Wnt pathway* Development, 2007. **134**: p. 4053-4062.

52. Kim, C. and W.C. Forrester, *Functional analysis of the domains of the C elegans Ror receptor tyrosine kinase CAM-1.* Dev Biol, 2003. **15**(264): p. 376-90.

53. Green, J.L., T. Inoue, and P.W. Sternberg, *Opposing Wnt pathways orient cell polarity during organogenesis.* Cell, 2008. **134**(4): p. 646-56.

54. Bhanot, P., M. Brink, C.H. Samos, J.C. Hsieh, Y. Wang, J.P. Macke, D. Andrew, J. Nathans, and R. Nusse, *A new member of the frizzled family from Drosophila functions as a Wingless receptor.* Nature, 1996. **382**(6588): p. 225-30.

55. Reya, T. and H. Clevers, *Wnt signalling in stem cells and cancer.* Nature, 2005. **434**(7035): p. 843-50.

56. Murdoch, B., K. Chadwick, M. Martin, F. Shojaei, K.V. Shah, L. Gallacher, R.T. Moon, and M. Bhatia, *Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3422-7.

57. Van Den Berg, D.J., A.K. Sharma, E. Bruno, and R. Hoffman, *Role of members of the Wnt gene family in human hematopoiesis.* Blood, 1998. **92**(9): p. 3189-202.

58. Billiard, J., D.S. Way, L.M. Seestaller-Wehr, R.A. Moran, A. Mangine, and P.V. Bodine, *The orphan receptor tyrosine kinase Ror2 modulates canonical Wnt signaling in osteoblastic cells.* Mol. Endocrinol., 2005. **19**: p. 90-101.

59. Hikasa, H., M. Shibata, I. Hiratani, and M. Taira, *The Xenopus receptor tyrosine kinase Xror2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling.* Development, 2002. **129**: p. 5227-5239.

60. Oishi, I., H. Suzuki, N. Onishi, R. Takada, S. Kani, B. Ohkawara, I. Koshida, K. Suzuki, G. Yamada, G.C. Schwabe, S. Mundlos, H. Shibuya, S. Takada, and Y. Minami, *The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway.* Genes Cells, 2003. **8**(7): p. 645-54.

61. Mikels, A.J. and R. Nusse, *Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context.* PLoS Biol, 2006. **4**(4): p. e115.

62. Yoda, A., I. Oishi, and Y. Minami, *Expression and function of the Ror-family receptor tyrosine kinases during development: lessons from genetic analyses of nematodes, mice, and humans.* J Recept Signal Transduct Res, 2003. **23**(1): p. 1-15.

63. Yamaguchi, T.P., A. Bradley, A.P. McMahon, and S. Jones, *A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo.* Development, 1999. **126**(6): p. 1211-23.

64. Nomi, M., I. Oishi, S. Kani, H. Suzuki, T. Matsuda, A. Yoda, M. Kitamura, K. Itoh, S. Takeuchi, K. Takeda, S. Akira, M. Ikeya, S. Takada, and Y. Minami, *Loss of mRor1 enhances the heart and skeletal abnormalities in mRor2-deficient*

mice: redundant and pleiotropic functions of mRor1 and mRor2 receptor tyrosine kinases. Mol Cell Biol, 2001. **21**(24): p. 8329-35.

65. Takeuchi, S., K. Takeda, I. Oishi, M. Nomi, M. Ikeya, K. Itoh, S. Tamura, T. Ueda, T. Hatta, H. Otani, T. Terashima, S. Takada, H. Yamamura, S. Akira, and Y. Minami, *Mouse Ror2 receptor tyrosine kinase is required for the heart development and limb formation.* Genes Cells, 2000. **5**(1): p. 71-8.

66. Fukuda, T., L. Chen, T. Endo, L. Tang, D. Lu, J.E. Castro, G.F. Widhopf, 2nd, L.Z. Rassenti, M.J. Cantwell, C.E. Prussak, D.A. Carson, and T.J. Kipps, *Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 3047-52.

67. Paganoni, S., J. Bernstein, and A. Ferreira, *Ror1-Ror2 complexes modulate synapse formation in hippocampal neurons.* Neuroscience, 2010. **165**(4): p. 1261-74.

68. Sato, A., H. Yamamoto, H. Sakane, H. Koyama, and A. Kikuchi, *Wnt5a regulates distinct signalling pathways by binding to Frizzled2.* EMBO J, 2010. **29**(1): p. 41-54.

69. Grumolato, L., G. Liu, P. Mong, R. Mudbhary, R. Biswas, R. Arroyave, S. Vijayakumar, A.N. Economides, and S.A. Aaronson, *Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors.* Genes Dev, 2010. **24**(22): p. 2517-30.

70. Baskar, S., K.Y. Kwong, T. Hofer, J.M. Levy, M.G. Kennedy, E. Lee, L.M. Staudt, W.H. Wilson, A. Wiestner, and C. Rader, *Unique cell surface expression of receptor tyrosine kinase ROR1 in human B-cell chronic lymphocytic leukemia.* Clin Cancer Res, 2008. **14**(2): p. 396-404.

71. Daneshmanesh, A.H., E. Mikaelsson, M. Jeddi-Tehrani, A.A. Bayat, R. Ghods, M. Ostadkarampour, M. Akhondi, S. Lagercrantz, C. Larsson, A. Osterborg, F. Shokri, H. Mellstedt, and H. Rabbani, *Ror1, a cell surface receptor tyrosine kinase is expressed in chronic lymphocytic leukemia and may serve as a putative target for therapy.* Int J Cancer, 2008. **123**(5): p. 1190-5.

72. Shabani, M., H. Asgarian-Omran, M. Jeddi-Tehrani, P. Vossough, M. Faranoush, R.A. Sharifian, G.R. Toughe, M. Kordmahin, J. Khoshnoodi, A. Roohi, N. Tavoosi, H. Mellstedt, H. Rabbani, and F. Shokri, *Overexpression of orphan receptor tyrosine kinase Ror1 as a putative tumor-associated antigen in Iranian patients with acute lymphoblastic leukemia.* Tumour Biol, 2007. **28**(6): p. 318-26.

73. Shabani, M., H. Asgarian-Omran, P. Vossough, R.A. Sharifian, M. Faranoush, S. Ghragozlou, J. Khoshnoodi, A. Roohi, M. Jeddi-Tehrani, H. Mellstedt, H. Rabbani, and F. Shokri, *Expression profile of orphan receptor tyrosine kinase (ROR1) and Wilms' tumor gene 1 (WT1) in different subsets of B-cell acute lymphoblastic leukemia.* Leuk Lymphoma, 2008. **49**(7): p. 1360-7.

74. Broome, H.E., L.Z. Rassenti, H.Y. Wang, L.M. Meyer, and T.J. Kipps, *ROR1 is expressed on hematogones (non-neoplastic human B-lymphocyte precursors) and a minority of precursor-B acute lymphoblastic leukemia.* Leuk Res, 2011. **35**(10): p. 1390-4.

75. Al-Shawi, R., S.V. Ashton, C. Underwood, and J.P. Simons, *Expression of the Ror1 and Ror2 receptor tyrosine kinase genes during mouse development.* Dev Genes Evol, 2001. **211**(4): p. 161-71.

76. Nomi, M., I. Oishi, S. Kani, H. Suzuki, T. Matsuda, A. Yoda, M. Kitamura, K. Ito, S. Takeuchi, K. Takeda, S. Akira, M. Ikeya, S. Takada, and Y. Minami, *Loss of mRor1 enhances the heart and skeletal abnormalities in mRor2-deficient mice: redundant and pleiotropic functions of mRor1 and mRor2 receptor tyrosine kinases.* Mol Cell Biol, 2001. **21**(24): p. 8329-35.

77. Reddy, U.R., S. Phatak, and D. Pleasure, *Human neural tissues express a truncated Ror1 receptor tyrosine kinase, lacking both extracellular and transmembrane domains.* Oncogene, 1996. **13**(7): p. 1555-9.

78. Hudecek, M., T.M. Schmitt, S. Baskar, M.T. Lupo-Stanghellini, T. Nishida, T.N. Yamamoto, M. Bleakley, C.J. Turtle, W.C. Chang, H.A. Greisman, B. Wood, D.G. Maloney, M.C. Jensen, C. Rader, and S.R. Riddell, *The B-cell tumor-associated antigen ROR1 can be targeted with T cells modified to express a ROR1-specific chimeric antigen receptor.* Blood, 2010. **116**(22): p. 4532-41.

79. Monroe, J.G., *ITAM-mediated tonic signalling through pre-BCR and BCR complexes.* Nature Reviews Immunology, 2006. **6**.

80. Herzog, S., M. Reth, and H. Jumaa, *Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling.* Nature Reviews Immunology, 2009. **9**: p. 195-205.

81. Lieber, M.R., *The mechanism of V(D)J recombination: a balance of diversity, specificity and stability.* Cell, 1992. **70**: p. 873-876.

82. Atl, F.W., T.K. Blackwell, and G.D. Vancopoulos, *Development of the primary antibody repertoire.* Science, 1987. **238**: p. 1079-1087.

83. Oettinger, M.A., D.G. Schatz, C. Gorka, and D. Baltimore, *RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination.* Science, 1990. **248**(4962): p. 1517-23.

84. Schatz, D.G., M.A. Oettinger, and D. Baltimore, *The V(D)J recombination activating gene, RAG-1.* Cell, 1989. **59**(6): p. 1035-48.

85. Lieber, M.R., Y. Ma, U. Pannicke, and K. Schwarz, *Mechanism and regulation of human non-homologous DNA end-joining.* Nature Rev Mol Cell Biol, 2003. **4**(712-720).

86. Nishimoto, N., H. Kubagawa, T. Ohno, G.L. Gartland, A.K. Stankovic, and M.D. Cooper, *Normal pre-B cells express a receptor complex of mu heavy chains and surrogate light-chain proteins.* Proc Natl Acad Sci USA, 1991. **88**(14): p. 6284-8.

87. Gong, S. and M.C. Nussenzweig, *Regulation of an early developmental checkpoint in the B cell pathway by Ig beta.* Science, 1996. **272**(5260): p. 411-4.

88. Reth, M., E. Petrac, P. Wiese, L. Lobel, and F.W. Alt, *Activation of V kappa gene rearrangement in pre-B cells follows the expression of membrane-bound immunoglobulin heavy chains.* EMBO J, 1987. **6**(11): p. 3299-305.

89. Pelanda, R., U. Braun, E. Hobeika, M.C. Nussenzweig, and M. Reth, *B cell progenitors are arrested in maturation but have intact VDJ recombination in the absence of Ig-alpha and Ig-beta.* Immunol, 2002. **169**(865-872).

90. Melchers, F., *The pre-B-cell receptor: selector of fitting immunoglobulin heavy chains for the B-cell repertoire.* Nature Rev Immunol, 2005. **5**: p. 578-584.

91. Parker, M.J., S. Licence, L. Erlandsson, G.R. Galler, L. Chakalova, C.S. Osborne, G. Morgan, P. Fraser, H. Jumaa, T.H. Winkler, J. Skok, and I.L. Martensson, *The pre-B-cell receptor induces silencing of VpreB and lambda5 transcription.* EMBO J, 2005. **24**(22): p. 3895-905.

92. Johnson, K., T. Hashimshony, C.M. Sawai, J.M. Pongubala, J.A. Skok, I. Aifantis, and H. Singh, *Regulation of immunoglobulin light-chain recombination by the transcription factor IRF-4 and the attenuation of interleukin-7 signaling.* Immunity, 2008. **28**(3): p. 335-345.

93. Herzog, S., E. Hug, S. Meixlsperger, J. Paik, R.A. DePinho, M. Reth, and H. Jumaa, *SLP-65 regulates immunoglobulin light chain gene recombination through the PI(3)K-PKB-Foxo pathway.* Nat Immunol, 2008. **9**: p. 623-631.

94. Lu, R., K.L. Medina, D.W. Lancki, and H. Singh, *IRF-4,8 orchestrate the pre-B-to-B transition in lymphocyte development.* Genes Dev, 2003. **17**(14): p. 1703-8.

95. Rolink, A.G., E. ten Boekel, T. Yamagami, R. Ceredig, J. Andersson, and F. Melchers, *B cell development in the mouse from early progenitors to mature B cells.* Immunol Lett, 1999. **68**(1): p. 89-93.

96. Jumaa, H., R.W. Hendriks, and M. Reth, *B cell signaling and tumorigenesis.* Annu Rev Immunol, 2005. **23**: p. 415-45.

97. Williams, D.L., A.T. Look, S.L. Melvin, P.K. Roberson, G. Dahl, T. Flake, and S. Stass, *New chromosomal translocations correlate with specific immunophenotypes of childhood acute lymphoblastic leukemia.* Cell, 1984. **36**(1): p. 101-9.

98. Borkhardt, A., C. Wuchter, S. Viehmann, S. Pils, A. Teigler-Schlegel, M. Stanulla, M. Zimmermann, W.D. Ludwig, G. Janka-Schaub, M. Schrappe, and J. Harbott, *Infant acute lymphoblastic leukemia - combined cytogenetic, immunophenotypical and molecular analysis of 77 cases.* Leukemia, 2002. **16**(9): p. 1685-90.

99. Romana, S.P., M. Mauchauffe, M. Le Coniat, I. Chumakov, D. Le Paslier, R. Berger, and O.A. Bernard, *The t(12;21) of acute lymphoblastic leukemia results in a tel-AML1 gene fusion.* Blood, 1995. **85**(12): p. 3662-3670.

100. Ludwig, W.D., H. Rieder, C.R. Bartram, B. Heinze, S. Schwartz, W. Gassmann, H. Loffler, D. Hossfeld, G. Heil, S. Handt, A. Heyll, H. Diedrich, K. Fischer, A. Weiss, B. Volkers, U. Aydemir, C. Fonatsch, N. Gokbuget, E. Thiel, and D. Hoelzer, *Immunophenotypic and Genotypic Features, Clinical Characteristics, and Treatment Outcome of Adult Pro-B Acute Lymphoblastic Leukemia: Results of the German Multicenter Trials GMALL 03/87 and 04/89* Blood, 1998. **92**(6): p. 1898-1909.

101. Bene, M.C., G. Castoldi, W. Knapp, W.D. Ludwid, E. Matutes, A. Orfao, and M.B. van't Veer, *Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL).* Leukemia, 1995. **9**(10): p. 1783-6.

102. Hunger, S.P., *Chromosomal translocations involving the E2A gene in acute lymphoblastic leukemia: clinical features and molecular pathogenesis.* Blood, 1996. **87**(4): p. 1211-24.

103. Hunger, S.P., N. Galili, A.J. Carroll, W.M. Crist, M.P. Link, and M.L. Cleary, *The t(1;19)(q23;p13) results in consistent fusion of E2A and PBX1 coding sequences in acute lymphoblastic leukemias.* Blood, 1991. **77**(4): p. 687-93.

104. Massari, M.E., P.A. Jennings, and C. Murre, *The AD1 transactivation domain of E2A contains a highly conserved helix which is required for its activity in both*

Saccharomyces cerevisiae and mammalian cells. Mol Cell Biol, 1996. **16**(1): p. 121-9.

105. Aronheim, A., R. Shiran, A. Rosen, and M.D. Walker, *The E2A gene product contains two separable and functionally distinct transcription activation domains.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 8063-7.

106. Zhuang, Y., P. Soriano, and H. Weintraub, *The helix-loop-helix gene E2A is required for B cell formation.* Cell, 1994. **79**(5): p. 875-84.

107. Bain, G., E.C. Maandag, D.J. Izon, D. Amsen, A.M. Kruisbeek, B.C. Weintraub, I. Krop, M.S. Schlissel, A.J. Feeney, M. van Roon, M. van der Valk, H.P.J. te Riele, A. Berns, and C. Murre, *E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements.* Cell, 1994. **79**(5): p. 885-92.

108. Bain, G., W.J. Romanow, K. Albers, W.L. Harvran, and C. Murre, *Positive and negative regulation of V(D)J recombination by the E2A proteins.* J Exp Med, 1999. **189**(2): p. 289-300.

109. Romanow, W.J., A.W. Langerak, P. Goebel, I.L. Wolvers-Tettero, J.J. Van Dongen, A.J. Feeney, and C. Murre, *E2A and EBF act in synergy with the V(D)J recombinase to generate a diverse immunoglobulin repertoire in nonlymphoid cells.* Mol Cell, 2000. **5**(2): p. 343-53.

110. Lee, B.L. and C. Murre, *Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12.* J Exp Med, 1998. **188**(4): p. 699-713.

111. Hauser, J., A. Wallenius, N. Sveshnikova, J. Saarikettu, and T. Grundstrom, *Calmodulin inhibition of E2A stops expression of surrogate light chains of the pre-B-cell receptor and CD19.* Mol Immunol, 2010. **47**(5): p. 1031-8.

112. Monica, K., N. Galili, J. Nourse, D. Saltman, and M.L. Cleary, *PBX2 and PBX3, new homeobox genes with extensive homology to the human proto-oncogene PBX1.* Mol Cell Biol, 1991. **11**(12): p. 6149-57.

113. Chang, C.P., I. de Vivo, and M.L. Cleary, *The Hox cooperativity motif of the chimeric oncoprotein E2a-Pbx1 is necessary and sufficient for oncogenesis.* Mol Cell Biol, 1997. **17**(1): p. 81-8.

114. Peltenburg, L.T. and C. Murre, *Specific residues in the Pbx homeodomain differentially modulate the DNA-binding activity of Hox and Engrailed proteins.* Development, 1997. **124**(5): p. 1089-98.

115. Van Dongen, J.J., E.A. Macintyre, J.A. Gabert, E. Delabesse, V. Rossi, G. Saglio, E. Gottardi, A. Rambaldi, G. Dotti, F. Griesinger, F. Parreira, P. Gameiro, M.G. Diaz, M. Malec, A.W. Langerak, J.F. San Miguel, and A. Biondi, *Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia.* Leukemia, 1999. **13**(12): p. 1901-28.

116. Aspland, S.E., H.H. Bendall, and C. Murre, *The role of E2A-PBX1 in leukemogenesis.* Oncogene, 2001. **20**(40): p. 5708-17.

117. Massari, M.E., P.A. Grant, M.G. Pray-Grant, S.L. Berger, J.L. Workman, and C. Murre, *A conserved motif present in a class of helix-loop-helix proteins activates transcription by direct recruitment of the SAGA complex.* Mol Cell, 1999. **4**(1): p. 63-73.

118. Qui, Y., A.K. Sharma, and R. Stein, *p300 mediates transcriptional stimulation by the basic helix-loop-helix activators of the insulin gene.* Mol Cell Biol, 1998. **18**(5): p. 2957-64.

119. Monica, K., D.L. LeBrun, D.A. Dedera, R. Brown, and M.L. Cleary, *Transformation properties of the E2a-Pbx1 chimeric oncoprotein: fusion with E2a is essential but the Pbx1 homeodomain is dispensable.* Mol cell Biol, 1994. **14**(8304-14).

120. Calvo, K.R., P. Knoepfler, S. McGrath, and M.P. Kamps, *An inhibitory switch derepressed by pbx, hox, and Meis/Prep1 partners regulates DNA-binding by pbx1 and E2a-pbx1 and is dispensable for myeloid immortalization by E2a-pbx1*. Oncogene, 1999. **18**(56): p. 8033-43.

121. Kamps, M.P., D.D. Wright, and Q. Lu, *DNA-binding by oncoprotein E2a-Pbx1 is important for blocking differentiation but dispensable for fibroblast transformation.* Oncogene, 1996. **12**(1): p. 19-30.

122. Lu, Q. and M.P. Kamps, *Selective repression of transcriptional activators by Pbx1 does not require the homeodomain.* Proc Natl Acad Sci U S A, 1996. **93**(1): p. 470-4.

123. Lu, Q. and M.P. Kamps, *Heterodimerization of Hox proteins with Pbx1 and oncoprotein E2a-Pbx1 generates unique DNA-binding specifities at nucleotides predicted to contact the N-terminal arm of the Hox homeodomain--demonstration of Hox-dependent targeting of E2a-Pbx1 in vivo.* Oncogene, 1997. **14**(1): p. 75-83.

124. Aspland, S.E. and R.A. White, *Nucleocytoplasmic localisation of extradenticle protein is spatially regulated throughout development in Drosophila.* Development, 1997. **124**(3): p. 741-7.

125. Abu-Shaar, M., H.D. Ryoo, and R.S. Mann, *Control of the nuclear localization of Extradenticle by competing nuclear import and export signals.* Genes Dev, 1999. **13**(8): p. 935-45.

126. LeBrun, D.P., B.P. Matthews, B.J. Feldman, and M.L. Cleary, *The chimeric oncoproteins E2A-PBX1 and E2A-HLF are concentrated within spherical nuclear domains.* Oncogene, 1997. **15**(17): p. 2059-67.

127. Kamps, M.P. and D. Baltimore, *E2A-Pbx1, the t(1;19) translocation protein of human pre-B-cell acute lymphocytic leukemia, causes acute myeloid leukemia in mice.* Mol Cell Biol, 1993. **13**(1): p. 351-7.

128. Dedera, D.A., E.K. Waller, D.P. LeBrun, A. Sen-Majumdar, M.E. Stevens, G.S. Barsh, and M.L. Cleary, *Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice.* Cell, 1993. **74**(5): p. 833-43.

129. Kamps, M.P., A.T. Look, and D. Baltimore, *The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials.* Genes Dev, 1991. **5**(3): p. 358-68.

130. Bijl, J., M. Sauvageau, A. Thompson, and G. Sauvageau, *High incidence of proviral integrations in the Hoxa locus in a new model of E2a-PBX1-induced B-cell leukemia.* Genes Dev, 2005. **19**(2): p. 224-33.

131. Smith, K.S., S.K. Chanda, M. Lingbeek, D.T. Ross, D. Botstein, M. van Lohuizen, and M.L. Cleary, *Bmi-1 regulation of INK4A-ARF is a downstream requirement for transformation of hematopoietic progenitors by E2a-Pbx1.* Molecular Cell, 2003. **12**(2): p. 393-400.

132. Fu, X., S. McGrath, M. Pasillas, S. Nakazawa, and M.P. Kamps, *EB-1, a tyrosine kinase signal transduction gene, is transcriptionally activated in the t(1;19) subset of pre-B ALL, which express oncoprotein E2a-Pbx1.* Oncogene, 1999. **18**(35): p. 4920-9.

133. McWhirter, J.R., S.T. Neuteboom, E.V. Wancewicz, B.P. Monia, J.R. Downing, and C. Murre, *Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia.* Proc Natl Acad Sci U S A, 1999. **96**(20): p. 11464-9.

134. Jeha, S., D. Pei, S.C. Raimondi, M. Onciu, D. Campana, C. Cheng, J.T. Sandlund, R.C. Ribeiro, J.E. Rubnitz, S.C. Howard, J.R. Downing, W.E. Evans,

M.V. Relling, and C.H. Pui, *Increased risk for CNS relapse in pre-B cell leukemia with the t(1;19)/TCF3-PBX1.* Leukemia, 2009. **23**(8): p. 1406-9.

135. Trageser, D., I. Iacobucci, R. Nahar, C. Duy, G. von Levetzow, L. Klemm, E. Park, W. Schuh, T. Gruber, S. Herzog, Y.M. Kim, W.K. Hofmann, A. Li, C.T. Storlazzi, H.M. Jäck, J. Groffen, G. Martinelli, N. Heisterkamp, H. Jumaa, and M. Müschen, *Pre-B cell receptor-mediated cell cycle arrest in Philadelphia chromosome-positive acute lymphoblastic leukemia requires IKAROS function.* J Exp Med, 2009. **206**(8): p. 1739-53.

136. Tyner, J.W., M.W. Deininger, M.M. Loriaux, B.H. Chang, J.R. Gotlib, S.G. Willis, H. Erickson, T. Kovacsovics, T. O'Hare, M.C. Heinrich, and B.J. Druker, *RNAi screen for rapid therapeutic target identification in leukemia patients.* Proc Natl Acad Sci U S A, 2009. **106**(21): p. 8695-700.

137. Kim, E., A. Goren, and G. Ast, *Insights into the connection between cancer and alternative splicing.* Trends Genet, 2008. **24**: p. 7-10.

138. Reese, M.G., F.H. Eeckman, D. Kulp, and D. Haussler, *Improved splice site detection in Genie.* J Comput Biol, 1997. **4**(3): p. 311-23.

139. Brunak, S., J. Engelbrecht, and S. Knudsen, *Prediction of human mRNA donor and acceptor sites from the DNA sequence.* J Mol Biol, 1991. **220**(1): p. 49-65.

140. Hebsgaard, S.M., P.G. Korning, N. Tolstrup, J. Engelbrecht, P. Rouze, and S. Brunak, *Splice site prediction in Arabidopsis thaliana pre-mRNA by combining local and global sequence information.* Nucleic Acids Res, 1996. **24**(17): p. 3439-52.

141. Burge, C. and S. Karlin, *Prediction of complete gene structures in human genomic DNA.* J Mol Biol, 1997. **268**(1): p. 78-94.

142. Barna, G., R. Mihalik, B. Timar, J. Tombol, Z. Csende, A. Sebestyen, C. Bodor, B. Csernus, L. Reiniger, I. Petak, and A. Matolcsy, *ROR1 expression is not a unique marker of CLL.* Hematol Oncol, 2011.

143. Jensen, M.C., L. Popplewell, L.J. Cooper, D. DiGiusto, M. Kalos, J.R. Ostberg, and S.J. Forman, *Antitransgene rejection responses contribute to attenuated persistence of adoptively transferred CD20/CD19-specific chimeric antigen receptor redirected T cells in humans.* Biol Blood Marrow Transplant, 2010. **16**(9): p. 1245-56.

144. Till, B.G., M.C. Jensen, J. Wang, E.Y. Chen, B.L. Wood, H.A. Greisman, X. Qian, S.E. James, A. Raubitschek, S.J. Forman, A.K. Gopal, J.M. Pagel, C.G. Lindgren, P.D. Greenberg, S.R. Riddell, and O.W. Press, *Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma*

using genetically modified autologous CD20-specific T cells. Blood, 2008. **112**(6): p. 2261-71.

145. Porter, D.L., B.L. Levine, M. Kalos, A. Bagg, and C.H. June, *Chimeric Antigen Receptor–Modified T Cells in Chronic Lymphoid Leukemia.* N Engl J Med, 2011. **365**: p. 725-733.

146. Benschop, R.J. and J.C. Cambier, *B cell development: signal transduction by antigen receptors and their surrogates.* Curr Opin Immunol, 1999. **11**(2): p. 143-51.

147. Meffre, E., R. Casellas, and M.C. Nussenzweig, *Antibody regulation of B cell development.* Nat Immunol, 2000. **1**(5): p. 379-85.

148. Saijo, K., C. Schmedt, I.H. Su, H. Karasuyama, C.A. Lowell, M. Reth, T. Adachi, A. Patke, A. Santana, and A. Tarakhovsky, *Essential role of Src-family protein tyrosine kinases in NF-kappaB activation during B cell development.* Nat Immunol, 2003. **4**(3): p. 274-9.

149. Rowley, R.B., A.L. Burkhardt, H.G. Chao, G.R. Matsueda, and J.B. Bolen, *Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Ig alpha/Ig beta immunoreceptor tyrosine activation motif binding and autophosphorylation.* J Biol Chem, 1995. **270**(19): p. 11590-4.

150. Rolink, A., D. Haasner, F. Melchers, and J. Andersson, *The surrogate light chain in mouse B-cell development.* Int Rev Immunol, 1996. **13**(4): p. 341-56.

151. Geier, J.K. and M.S. Schlissel, *Pre-BCR signals and the control of Ig gene rearrangements.* Semin Immunol, 2006. **18**(1): p. 31-9.

152. Papavasiliou, F., M. Jankovic, H. Suh, and M.C. Nussenzweig, *The cytoplasmic domains of immunoglobulin (Ig) alpha and Ig beta can independently induce the precursor B cell transition and allelic exclusion.* J Exp Med, 1995. **182**(5): p. 1389-94.

153. Teh, Y.M. and M.S. Neuberger, *The immunoglobulin (Ig)alpha and Igbeta cytoplasmic domains are independently sufficient to signal B cell maturation and activation in transgenic mice.* J Exp Med, 1997. **185**(10): p. 1753-8.

154. Thomas, M.L. and E.L. Brown, *Positive and negative regulation of Src-family membrane kinases by CD45.* Immunol Today, 1999. **20**(9): p. 406-11.

155. Schmitz, R., G. Baumann, and H. Gram, *Catalytic specificity of phosphotyrosine kinases Blk, Lyn, c-Src and Syk as assessed by phage display.* J Mol Biol, 1996. **260**(5): p. 664-7.

156. Futterer, K., J. Wong, R.A. Grucza, A.C. Chan, and G. Waksman, *Structural basis for Syk tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide.* J Mol Biol, 1998. **281**(3): p. 523-37.

157. Grucza, R.A., K. Futterer, A.C. Chan, and G. Waksman, *Thermodynamic study of the binding of the tandem-SH2 domain of the Syk kinase to a dually phosphorylated ITAM peptide: evidence for two conformers.* Biochemistry, 1999. **38**: p. 5024-5033.

158. Aiba, Y., M. Kameyama, T. Yamazaki, T.F. Tedder, and T. Kurosaki, *Regulation of B-cell development by BCAP and CD19 through their binding to phosphoinositide 3-kinase.* Blood, 2008. **111**(3): p. 1497-503.

159. Okkenhaug, K. and B. Vanhaesebroeck, *PI3K in lymphocyte development, differentiation and activation.* Nature Reviews Immunology, 2003. **3**: p. 317-330.

160. Vanhaesebroeck, B. and D.R. Alessi, *The PI3K-PDK1 connection: more than just a road to PKB.* Biochem J., 2000. **346**(3): p. 561-76.

161. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: navigating downstream.* Cell, 2007. **129**(7): p. 1261-74.

162. Amin, R.H. and M.S. Schlissel, *Foxo1 directly regulates the transcription of recombination-activating genes during B cell development.* Nat Immunol, 2008. **9**: p. 613-622.

163. Accili, D. and K.C. Arden, *FoxOs at the crossroads of cellular metabolism, differentiation, and transformation.* Cell, 2004. **117**(4): p. 421-6.

164. Brunet, A., A. Bonni, M.J. Zigmond, M.Z. Lin, P. Juo, L.S. Ju, M.J. Anderson, K.C. Arden, J. Blenis, and M.E. Greenberg, *Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.* Cell, 1999. **96**(6): p. 857-68.

165. Su, Y.W., Y. Zhang, J. Schweikert, G.A. Koretzky, M. Reth, and J. Wienands, *Interaction of SLP adaptors with the SH2 domain of Tec family kinases.* Eur J Immunol, 1999. **29**(11): p. 3702-11.

166. Chiu, C.W., M. Dalton, M. Ishiai, T. Kurosaki, and A.C. Chan, *BLNK: molecular scaffolding through 'cis'-mediated organization of signaling proteins.* EMBO J, 2002. **21**(23): p. 6461-72.

167. Schlissel, M.S., *Regulation of activation and recombination of the murine Igkappa locus.* Immunol Rev, 2004. **200**: p. 215-223.

168. Muljo, S.A. and M.S. Schlissel, *A small molecule Abl kinase inhibitor induces differentiation of Abelson virus−transformed pre-B cell lines.* Nature Immunology, 2002. **4**(31-37).

169. Ma, S., A. Turetsky, L. Trinh, and R. Lu, *IFN regulatory factor 4 and 8 promote Ig light chain kappa locus activation in pre-B cell development.* J Immunol, 2006. **177**(11): p. 7898-904.

170. Jumaa, H., L. Bossaller, K. Portugal, B. Storch, M. Lotz, A. Flemming, M. Schrappe, V. Postila, P. Riikonen, J. Pelkonen, C.M. Niemeyer, and M. Reth, *Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukaemia.* Nature, 2003. **423**(6938): p. 452-6.

171. Druker, B.J., *Imatinib as a paradigm of targeted therapies.* Adv Cancer Res, 2004. **91**: p. 1-30.

172. Shah, N.P., J.M. Nicoll, B. Nagar, M.E. Gorre, R.L. Paquette, J. Kuriyan, and C.L. Sawyers, *Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia.* Cancer Cell, 2002. **2**(2): p. 117-125.

173. Druker, B.J., *Circumventing Resistance to Kinase-Inhibitor Therapy.* N Engl J Med, 2006. **354**(24): p. 2594-5.

174. Weisberg, E., P.W. Manley, W. Breitenstein, J. Brüggen, S.W. Cowan-Jacob, A. Ray, B. Huntly, D. Fabbro, G. Fendrich, E. Hall-Meyers, A.L. Kung, J. Mestan, G.Q. Daley, L. Callahan, L. Catley, C. Cavazza, M. Azam, D. Neuberg, R.D. Wright, D.G. Gilliland, and J.D. Griffin, *Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl.* Cancer cell, 2005. **7**(2): p. 129-41.

175. La Rosee, P., A.S. Corbin, E.P. Stoffregen, M.W. Deininger, and B.J. Druker, *Activity of the Bcr-Abl kinase inhibitor PD180970 against clinically relevant Bcr-Abl isoforms that cause resistance to imatinib mesylate (Gleevec, STI571).* Cancer Res, 2002. **62**: p. 7149-53.

176. Shah, N.P., C. Tran, F.Y. Lee, P. Chen, D. Norris, and C.L. Sawyers, *Overriding imatinib resistance with a novel ABL kinase inhibitor.* Science, 2004. **305**: p. 399-401.

177. Talpas, M., N.P. Neil, H. Kantarjian, N. Donato, J.M. Nicoll, R.L. Paquette, J. Cortes, S.G. O'Brien, C. Nicaise, E. Bleickardt, M.A. Blackwood-Chirchir, V. Iyer, T.T. Chen, F. Huang, A.P. Decillis, and C.L. Sawyers, *Dasatinib in Imatinib-Resistant Philadelphia Chromosome–Positive Leukemias.* N Engl J Med, 2006. **354**: p. 2531-2541.

178. Karaman, M.W., S. Herrgard, D.K. Treiber, P. Gallant, C.E. Atteridge, B.T. Campbell, K.W. Chan, P. Ciceri, M.I. Davis, P.T. Edeen, R. Faraoni, M. Floyd, J.P. Hunt, D.J. Lockhart, Z.V. Milanov, M.J. Morrison, G. Pallares, H.K. Patel, S. Pritchard, L.M. Wodicka, and P.P. Zarrinkar, *A quantitative analysis of kinase inhibitor selectivity.* Nat Biotechnol, 2008. **26**(1): p. 127-32.

179. Hantschel, O., U. Rix, U. Schmidt, T. Burckstummer, M. Kneidinger, G. Schutze, J. Colinge, K.L. Bennett, W. Ellmeier, P. Valent, and G. Superti-Furga, *The Btk tyrosine kinase is a major target of the Bcr-Abl inhibitor dasatinib.* Proc Natl Acad Sci U S A, 2007. **104**(33): p. 13283-8.

180. Hantschel, O., U. Rix, and G. Superti-Furga, *Target spectrum of the BCR-ABL inhibitors imatinib, nilotinib and dasatinib.* Leuk Lymphoma, 2008. **49**(4): p. 615-9.

181. Hsueh, R.C. and R.H. Scheuermann, *Tyrosine kinase activation in the decision between growth, differentiation, and death responses initiated from the B cell antigen receptor.* Adv Immunol, 2000. **75**: p. 283-316.

182. Steel, G.G. and M.J. Peckham, *Exploitable mechanisms in combined radiotherapy-chemotherapy: the concept of additivity.* Int J Radiat Oncol Biol Phys, 1979. **5**(1): p. 85-91.

183. Chou, T.C., *Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies.* Phasmacological Reviews, 2006. **58**(3): p. 621-81.

184. Chou, T.C. and P. Talalay, *Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors.* Adv Enzyme Regul, 1984. **22**: p. 27-55.

185. Hamblin, T.J., Z. Davis, A. Gardiner, D.G. Oscier, and F.K. Stevenson, *Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia.* Blood, 1999. **94**: p. 1848-54.

186. Oscier, D.G., A. Thompsett, D. Zhu, and F.K. Stevenson, *Differential rates of somatic hypermutation in V(H) genes among subsets of chronic lymphocytic leukemia defined by chromosomal abnormalities.* Blood, 89. **89**: p. 4153-60.

187. Tyner, J.W., S. Spurgeon, L.B. Fletcher, W. Yang, T. Kovacsovics, B.J. Druker, and M.M. Loriaux, *A Small-Molecule Inhibitor Screen rapidly Identifies Therapeutic Targets and Individualized Therapeutic Strategies in Patients with Acute and Chronic Leukemias.* Blood (ASH Annual Meeting Abstracts), 2010. **116**(21): p. 1136.

188. Li, P., D. Harris, Z. Liu, J. Liu, M. Keating, and Z. Estrov, *Stat3 activates the receptor tyrosine kinase like orphan receptor-1 gene in chronic lymphocytic leukemia cells.* PloS One, 2010. **5**(7): p. e11859.

189. Siddiquee, K., G. Zhang, W.C. Guida, M.A. Blaskovich, B. Greedy, H.R. Lawrence, M.L. Yip, R. Jove, M.M. McLaughlin, N.J. Lawrence, S.M. Sebti, and J. Turkson, *Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity.* Proc Natl Acad Sci USA, 2007. **104**(18): p. 7391-6.

190. Singh, J. and R.A. Padgett, *Rates of in situ transcription and splicing in large human genes.* Nature Structural & Molecular Biology, 2009. **16**: p. 1128-1133.

191. Canitrot, Y., D. Lautier, G. Laurent, M. Frechet, A. Ahmed, A.G. Turhan, B. Salles, C. Cazaux, and J.S. Hoffman, *Mutator phenotype of BCR - ABL transfected Ba/F3 cell lines and its association with enhanced expression of DNA polymerase beta.* Oncogene, 1999. **18**(17): p. 2676-2680.

192. Parada, Y., L. Banerji, J. Glassford, N.C. Lea, M. Collado, C. Rivas, J.L. Lewis, M.Y. Gordon, N.S.B. Thomas, and E.W.F. Lam, *BCR-ABL and Interleukin 3 Promote Haematopoietic Cell Proliferation and Survival through Modulation of Cyclin D2 and p27Kip1 Expression.* J Biol Chem, 2001. **276**: p. 23572-80.

193. O'Hare T, P.R., Stoffregen EP, Keats JA, Abdullah OM, Moseson EM, Rivera VM, Tang H, Metcalf CA, Bohacek RS, Wang Y, Sundaramoorthi R, Shakespeare WC, Dalgarno D, Clackson T, Sawyer TK, Deininger MW, Druker BJ., *Inhibition of wild-type and mutant Bcr-Abl by AP23464, a potent Inhibition of wild-type and mutant Bcr-Abl by AP23464, a potent.* Blood, 2004. **104**: p. 2532-39.

194. Kitamura, T., T. Tange, T. Terasawa, S. Chiba, T. Kuwaka, K. Miyagawa, Y.F. Piao, K. Miyazono, A. Urabe, and F. Takaku, *Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin.* J Cell Physiol, 1989. **140**(2): p. 323-34.

195. Hantschel O, R.U., Schmidt U, Bürckstümmer T, Kneidinger M, Schütze G, Colinge J, Bennett KL, Ellmeier W, Valent P, Superti-Furga G., *The Btk tyrosine kinase is a major target of the Bcr-Abl inhibitor dasatinib.* Proc Natl Acad Sci USA, 2007. **104**(33): p. 13283-8.

196. Du, J., P. Bernasconi, K.R. Clauser, D.R. Mani, S.P. Finn, R. Beroukhim, M. Burns, B. Julian, X.P. Peng, H. Hieronymus, R.L. Maglathlin, T.A. Lewis, L.M. Liau, P. Nghiemphu, I.K. Mellinghoff, D.N. Louis, M. Loda, S.A. Carr, A.L. Kung, and T.R. Golub, *Bead-based profiling of tyrosine kinase phosphorylation identifies SRC as a potential target for glioblastoma therapy.* Nat Biotechnol, 2009. **27**(1): p. 77-83.

197. Willert, K., J.D. Brown, E. Danenberg, A.W. Duncan, I.L. Weissman, T. Reya, J.R. Yates, and R. Nusse, *Wnt proteins are lipid-modified and can act as stem cell growth factors.* Nature, 2003. **423**(6938): p. 448-52.

198. Clements, W.K., A.D. Kim, K.G. Ong, N.D. Moore, N.D. Lawson, and D. Traver, *A somitic Wnt16/Notch pathway specifies haematopoietic stem cells.* Nature, 2011. **474**: p. 220-224.

199. Poulin, B., F. Sekiya, and S.G. Rhee, *Intramolecular interaction between phosphorylated tyrosine-783 and the C-terminal Src homology 2 domain activates phospholipase C-g1*. Proc Natl Acad Sci USA, 2005. **102**: p. 4276-81.