Regulation and Signaling Mechanisms of ROR1 in B cell Acute Lymphoblastic Leukemia

By

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CERTIFICATE OF APPROVAL

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Abbreviations

4E-BP1	Eukaryotic translation intiation factor 4E binding protein 1
ActD	Actinomycin D
AKT	RAC-alpha serine/threonine protein kinase, Protein kinase B
ALL	Acute lymphoblastic leukemia
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
B cell	Bursa or bone marrow derived cell
BAD	BCL2 associated agonist of cell death
BCA	Bicinchoninic acid assay
BCR-ABL	B cell receptor - Abelson murine leukemia gene fusion
BLK	B lymphocyte kinase
BLNK	B-cell linker
BRD4	Bromodomain containing 4
BSA	Bovine serum albumin
BTK	Bruton tyrosine kinase
CAM-1	Kinase number 8 (CAM-1 is also known as KIN-8)

CAR	Chimeric antigen receptor
CD137	Cluster of differentiation 137
CD19	Cluster of differentiation 19
CDK	Cyclin dependent kinase
CDKN2A/B	Cyclin dependent kinase inhibitor 2A/B
cDNA	Complement deoxyribonucleic acid
CHX	Cycloheximide
CLL	Chronic lymphocytic leukemia
CpG	Cytosine-phosphate-Guanine
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acd
DNMT1	DNA methyltransferase 1
EB-1	E2a-Pbx1 activated gene in pre-B cells 1
EDTA	Ethylene diamine tetraacetic acid
EGFR	Epidermal growth factor receptor
Fab	Fragment antigen binding

FBS	Fetal bovine serum
FoxO	Forkhead box, sub-group O
G9a	Euchromatic histone lysine methyltransferase 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Grb2	Growth factor recpetor bound protein 2
GusB	Glucuronidase beta
HDAC1	Histone deacytlase 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
Igα/β	Immunoglobulin alpha and beta
IL-6	Interleukin 6
ITAM	Immunoreceptor tyrosine activation motif
JNK	Jun amino-terminal kinase
KAT7	Lysine acetyltransferase 7
LRP5/6	Low density lipoporotein receptor-related protein 5/6
LYN	Lck/Yes novel tyrosine kinase
MALAT1	Metastasis associated lung adenocarcinoma transcript 1

MAPK	Mitogen-activated protein kinase
MCL	Mantle cell lymphoma
MDM2	Mouse double minute 2
MEG3	Maternally expressed gene 3
MET	Hepatocyte growth factor receptor (MET is also known as HGFR)
microRNA	Micro ribonucleic acid
MRD	Minimal residual disease
mRNA	messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC1/2	Mammalian target of rapamycin complex 1/2
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenl)-2-(4-sulfophenyl)-2H-tetrazolium
NaCl	Sodium chloride
NFκb	Nuclear factor kappa B
NKX2-1	NK2 homeobox 1
NP-40	Nonidet P40
NTRK	Neurotropic tyrosine kinase
OPTI-MEM	Minimal essential medium

p21	Cyclin dependent kinase inhibitor 1A
p53	Tumor protein of 53 kilodaltons
PBS	Phosphate buffered saline
PBX1	Pre B cell leukemia homeobox 1
PHD	Plant homology domain
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C
PLCγ2	Phospholipase C gamma 2
PML	Promyelocytic leukemia
pre-BCR	pre-B-cell receptor
PTEN	Phosphatase and tensin homolog
PROTAC	Proteolysis targeting chimera
qRT-PCR	Quantitative real-time polymerase chain reaction
R10	RPMI-based cell culture media containing 10% FBS
R20	RPMI-based cell culture media containing 20% FBS
RAG1/2	Recombination activating gene 1/2
RAPTOR	Regulatory associated protein of mammalian target of rapamycin

RAS	Rat sarcoma
RhoH	RAS homolog family member H
Rictor	Rapamycin-insensitive companion of mTOR
RING	Really interesting new gene
RLT	RNA lysis buffer
RNAi	Ribonucleic acid interference
ROR	Receptor tyrosine kinase-like orphan receptor
RPMI	Roswell Park Memorial Institute medium
RUNX1	Runt related transcription factor
S6K1	Ribosomal protein S6 kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide agarose gel electrophoresis
SFK	Src-family kinase
siRNA	Small interfering ribonucleic acid
SLC	Surrogate light chain
SP1	Specificity protein 1
SRA	SET and RING finger associated domain
STAT3	Signal transducer and activator of transcription 3

SYK	Spleen associated tyrosine kinase
t(1;19) pre-B-ALL	Precursor B-cell acute lymphoblastic leukemia with a translocation between chromosomes 1 and 19
TBC1D1	GTPase-activating protein domain containing protein 1
TCF/LEF	T-cell factor/lymphoid enhancer factor
TCF3	Transcription factor 3
TRIM21	Tripartite motif containing 21
Tris-HCl	Tris Hydrochloric acid
TSC1/2	Tuberous sclerosis 1/2
TTD1/2	Tudor-like domain 1/2
UBD	Ubiquitin binding domain
UHRF1	Ubiquitin-like with PHD and RING finger domains 1
UTR	Untranslated region
VpreB	V-set pre-B cell surrogate light chain
Wnt16	Wingless integration site member 16
WNT5A	Wingless integration site member 5A
λ5	Lambda 5

Acknowledgements

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Abstract

Acute lymphocytic leukemia (ALL) is the most common cancer diagnosed in children and adolescents. Despite improved patient survival over the past fifty years, up to 20% of patients do not respond to therapy or develop resistance to treatment. Therefore, it is critical to identify new therapeutic targets to improve patient outcome. Approximately 4% of patients who present with ALL harbor a translocation between chromosomes 1 and 19 (t(1;19) pre-B-ALL). This translocation generates the *TCF3-PBX1* fusion gene product, which arrests normal B cells in an immature developmental stage and maintains constitutively active kinase signaling. Small molecule tyrosine kinase inhibitors, like dasatinib, suppress these signaling pathways, but some leukemic cells can survive by activating rescue mechanisms. In response to dasatinib, the t(1;19) pre-B-ALL subtype exhibits upregulated ROR1 expression as an alternate mechanism to bypass kinase inhibition. ROR1 is required for t(1;19) pre-B-ALL survival, yet it cannot be easily targeted because ROR1 lacks kinase activity. Therefore, I sought to identify mechanisms important for ROR1 expression or signaling to determine additional proteins that may act as surrogate drug targets to inhibit ROR1.

Using an siRNA screen approach, I identified UHRF1 as a candidate regulator of ROR1 and hypothesized that UHRF1 was required for ROR1 expression and t(1;19) pre-B-ALL cell viability. The work presented in this dissertation demonstrates that ROR1 protein levels are maintained by UHRF1, which can be biochemically and

pharmacologically targeted in combination with dasatinib treatment to maximize t(1;19) pre-B-ALL inhibition.

Although ROR1 is required for t(1;19) pre-B-ALL survival and is a compensatory mechanism to rescue leukemic cells from dasatinib treatment, the ROR1 signaling pathway is poorly understood. In this subtype of ALL, ROR1 is required for activation of AKT. AKT is activated through phosphorylation on serine 473 by the multi-subunit protein complex, mTORC2, to promote cell proliferation and survival. Therefore, I hypothesized that ROR1 activates AKT through interactions with mTORC2. Work presented in this dissertation demonstrates that ROR1 interacts with members of two mTOR complexes and that upregulation of ROR1 correlates with an increase in mTORC2 expression in response to dasatinib treatment. These findings establish a framework for a novel mechanism responsible for ROR1-dependent AKT activation in t(1;19) pre-B-ALL.

Collectively, the work described in this dissertation describes the regulation of ROR1 and its mechanism to activate AKT signaling in t(1;19) pre-B-ALL. These findings encourage further characterization of ROR1 oncogenic activity. More importantly, the work from this dissertation offers new insight on designing therapeutic strategies to target ROR1 in t(1;19) pre-B-ALL and other ROR1-expressing cancers.

1 Introduction

In this dissertation, I will describe my research that provides evidence of a post-transcriptional regulatory mechanism responsible for maintaining levels of the receptor tyrosine kinase-like orphan receptor 1 (ROR1) protein. I will also present work that suggests a mechanism of ROR1-dependent activation of RAC-alpha serine/threonine protein kinase (AKT) that is required for leukemic survival. In this introductory chapter, I will discuss the current state of acute lymphocytic leukemia (ALL), which is in need of additional targeted therapies. Then, I will focus on one specific subtype of ALL that has a well-characterized kinase-dependent signaling pathway. Next, I will discuss normal functions of ROR1, which is critical for the survival of several malignancies including ALL, in relation to other kinases. I will also present the current status of small molecule inhibitors and immunomodulatory therapeutics in development to target ROR1 in cancer. I will end the introductory chapter describing the potential of targeting ROR1 indirectly through upstream regulators, one of which I identify in this dissertation: ubiquitin-like with PHD and ring finger domain 1 (UHRF1).

After the introduction, I will describe my work using an siRNA screen to identify candidate regulators of ROR1 in a subset of ALL, including UHRF1. Subsequent experiments demonstrate a critical role of UHRF1 in leukemic cell survival by maintaining levels of ROR1 protein. Furthermore, I will describe work demonstrating that this UHRF1-ROR1 pathway can be targeted by naphthazarin, a known UHRF1

inhibitor. Finally, I will present results that suggest biochemical or pharmacological inhibition of UHRF1 increases the effect of a small molecule kinase inhibitor, dasatinib, against t(1;19) pre-B-ALL.

In Chapter 4, I will summarize the findings of my work investigating the mechanism driving ROR1-dependent activation of AKT, which is required for the survival of t(1;19) pre-B-ALL. Chapter 5 will focus on a discussion of my dissertation work and future directions of these projects. Finally, the Appendix (Chapter 6) briefly describes my contributions to additional publications using high-throughput inhibitor and siRNA screens, which were developed in our lab, to identify critical kinases in two types of solid tumors.

1.1 **New treatment options are needed for acute lymphoblastic leukemia**

1.1.1 *Background*

ALL occurs in at least 25% of all pediatric cancer patients, making it the most common cancer in children (1). ALL is a disease that originates from T or B lymphocytes in the bone marrow that gain the ability to outcompete normal lymphocytes for space and eventually invade the peripheral blood (1). The incidence of pediatric ALL is highest in children between the ages of two to three years old, and most patients present with an advanced stage of ALL in which more than 25% of the bone marrow consists of leukemic cells (1, 2).

In the 1960s, pediatric ALL patients had a five-year survival rate of less than 10%, but the improvement of chemotherapy regimens and the use of targeted therapy, such as imatinib and dasatinib (1, 3), has raised the survival rate over 80% (4, 5). Despite this vast improvement for patient outcome, ALL patients still have a 10-20% risk of having refractory or relapse disease. Relapse after a successful response to chemotherapy is due, in part, to minimal residual disease[1]. Previous studies have shown pediatric ALL patients with minimal residual disease levels greater than 0.01% after initial chemotherapy treatment have a significantly higher risk of relapse, regardless of prognosis (6, 7). Even after successful treatment, young patients are at risk for long-term effects from their therapy, including secondary cancers, chronic medical conditions such as osteonecrosis, and obesity (8).

1.1.2 *A brief overview of normal B cell development*

In 1890, Emil von Behring and Shibasakuro Kitasato first reported that antitoxins against diphtheria and tetanus bacteria were generated by cells with pre-formed antibody receptors (9). These cells were later identified as bursal or bone-marrow derived cells (B cells), which originate in the fetal liver and fetal/adult bone marrow (10-12). B cells develop from common lymphoid progenitor cells and form immature, precursor B cells under the guidance of cytokines and transcription factors, including interleukin-7 and paired box gene 5 (13). These immature, pre B-cells express the pre-B-cell receptor (pre-BCR) that dictates subsequent maturation and will be discussed in

[1] Using flow cytometry, minimal residual disease is defined by the presence of 0.01-0.1% leukemic cells in the bone marrow (6).

detail in Section 1.2 (14, 15). B cell maturation involves the expression and rearrangement of the heavy and light chain immunoglobulin genes, forming the mature B-cell receptor, before final differentiation into antibody-secreting cells through clonal selection in the spleen and lymph nodes (13, 15-18). B cells that are arrested in a developmental stage and fail to fully mature may transform into diseases such as cancer.

1.1.3 *The 1;19 translocation gives rise to precursor B-cell ALL*

The genetic landscape of ALL is well-characterized by a diverse group of genetic mutations and aberrations (19-21). Up to 85% of ALL subtypes have been shown to originate from immature, precursor B cells (22). The majority of B cell ALLs possess chromosomal number abnormalities, and the remaining subtypes are defined by a genetic translocation that generates a fusion product. These genetic aberrations typically lead to developmental arrest of B cells during normal differentiation and dysregulation of intrinsic cell signaling.

Approximately 4% of pediatric B-cell ALL patients harbor a translocation between chromosomes 1 and 19, t(1;19)(q23;p13.3), which results in the gene fusion of the activation domains of *TCF3* to the DNA-binding domain of *PBX1* (t(1;19) pre-B-ALL) (22, 23). This fusion product has two functional consequences. First, TCF3-PBX1 arrests B cells in intermediate, pre-B-cell stages of development and prevents further differentiation (24). Second, PBX1 becomes a strong transcriptional activator and drives aberrant expression of target genes such as *EB-1* and *Wnt16* (25-29). While some targets are upregulated, genes such as *CASPASE 7* and *CDKN2A/B* are downregulated to inhibit

apoptosis and cell cycle inhibition (30). Interestingly, polymorphisms in *CASPASE7* and *14* have been associated with increased risk of childhood leukemia (31). Together, these downstream effects of t(1;19) promote leukemic cell proliferation and survival.

One of the earliest karyotype reports of a 1;19 translocation in a pre B-ALL patient was published in 1984 by Williams and colleagues and was later confirmed by other groups in independent studies of pediatric ALL patients (32-36). Work by Stephen Hunger's group suggested that t(1;19) pre B-ALL was originally indicative of a poor prognosis, as patients were at significantly higher risk to fail therapy and relapse (23, 37). This observation was further supported by Mark P. Kamps's group who demonstrated the oncogenic potential of TCF3-PBX1 *in vitro* and *in vivo* (38). Furthermore, irradiated BALB/c mice injected with NIH-3T3 cells transduced with *TCF3-PBX1* developed acute myeloid leukemia, but this was likely due to secondary mutations, suggesting that other genes may be necessary to induce leukemia in the context of *TCF3-PBX1* (39).

More recently, the improved response rate of patients, who were treated with updated chemotherapy regimens, has re-classified the 1;19 translocation as an intermediate risk factor (40). As such, patient outcome may continue to improve with the development of novel therapeutic agents. For example, dasatinib is a multi-kinase inhibitor and is effective against t(1;19) pre-B-ALL *in vitro* and in xenograft mouse models (24). Additional therapeutic strategies currently in development will be discussed in more detail in section 1.3.7.

1.2 Pre B-cell receptor (pre-BCR) signaling is required for the proliferation, survival, and differentiation of normal B cells

1.2.1 *Pre-BCR formation and activation is required for normal B-cell development and t(1;19) pre-B-ALL*

The 1;19 translocation arrests normal B cell differentiation, and this blockade occurs during the same stages in which the pre-BCR is expressed. Therefore, B cells with the 1;19 translocation possess a constitutively expressed and active pre-BCR. The pre-BCR is a critical signaling complex that drives multiple downstream signaling cascades that work in concert to promote B cell proliferation, differentiation, and survival. As such, the pre-BCR undergoes a complex, well-regulated assembly and activation process.

During the early stages of normal B cell development, immature B cells that have successfully recombined the immunoglobulin heavy chain genes initiate the formation of the pre-BCR and develop into pre B-cells (41). The final pre-BCR complex consists of two μ heavy chains, each covalently bound with a surrogate light chain, and signal transducing subunits immunoglobulin-α and β (Igα and Igβ) (**Figure 1-1**) (42-44). The surrogate light chain is produced from two independent polypeptides, λ5 and VpreB (45, 46), where λ5 is required for the surrogate light chain association with the heavy chain (47). The two signal transducing subunits Igα and Igβ, which are linked by disulfide bonds, contain extracellular immunoglobulin domains and intracellular immunoreceptor tyrosine-based activation motifs (41). Upon pre-BCR activation,

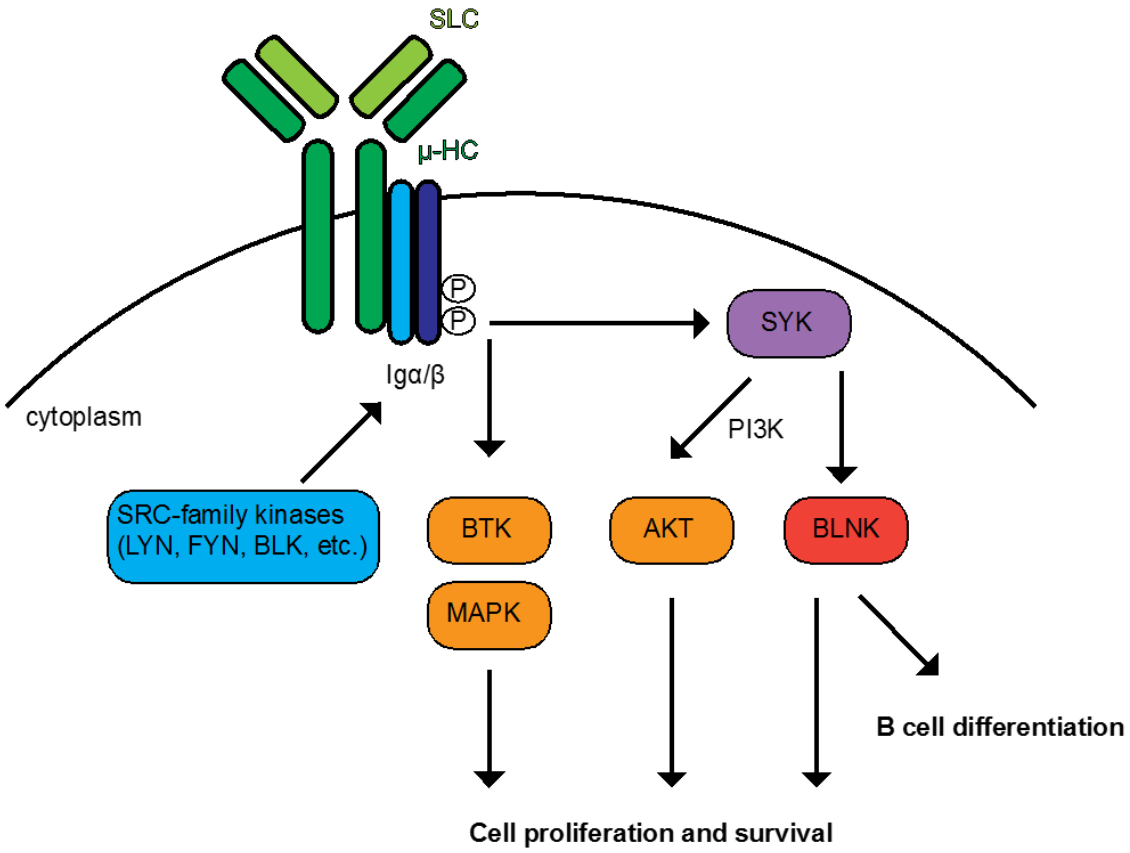

Figure 1-1 pre-BCR signaling promotes B-cell differentiation, proliferation, and survival.

The pre B-cell receptor (pre-BCR) is composed of the surrogate light chains (SLC), heavy chains (μ-HC), and two signaling subunits (Igα and β). After pre-BCR assembly and activation, phosphorylation of Igα/β by SRC-family kinases activates downstream kinase signaling pathways, including SYK and AKT, to induce B cell proliferation and survival. Concomitantly, effectors such as BLNK induce RAG1/2-dependent light chain rearrangement to synthesize the mature BCR and promote B cell differentiation. Adapted from Bicocca *et al* (2012) (24).

phosphorylation of these activation motifs trigger recruitment of SH2-containing signaling proteins to initiate intracellular signaling (42, 48, 49) (**Figure 1-1**).

Although the structure and assembly of the pre-BCR are well-understood, the mechanistic details of pre-BCR activation continue to be debated in the field. For instance, galectin-1 is a bone marrow stromal lectin that may be secreted to bind $\lambda 5$ to induce pre-BCR clustering (50, 51). However, galectin-1 deficient mice do not exhibit significant impairment of B cell development, suggesting that galectin-1 is not sufficient for pre-BCR activation (52). In contrast, there are multiple reports of a ligand-independent process of pre-BCR activation through spontaneous formation and aggregation of pre-BCR complexes (53, 54). Moreover, calcium signaling—an indicator of pre-BCR activity—induces pre-BCR internalization and B cell differentiation (55). While the details behind activation of the pre-BCR are still under debate, one can appreciate the complexity of these mechanisms that may dictate subsequent signaling and B cell differentiation.

1.2.2 *Pre-BCR activation leads to multiple downstream kinase signaling pathways*

The pre-BCR is required for several kinase signaling cascades critical for B cell proliferation and differentiation including phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), Bruton tyrosine kinase (BTK), and mitogen-activated protein kinase (MAPK) (56) (**Figure 1-1**). After the pre-BCR is assembled and activated on the cell surface of B cells, intracellular immunoreceptor tyrosine-based activation motifs on the Igα and Igβ subunits are phosphorylated on tyrosine residues by several SRC family

kinases including Lyn and Blk (48, 57). These SRC family kinases also phosphorylate spleen associated tyrosine kinase (SYK), which is required for subsequent B-cell development (58-60).

SYK activation stimulates B cells to undergo an intense proliferative stage by activating PI3K (61-64) (**Figure 1-1**). PI3K activates AKT, which is a known oncogene in many cancers (65). AKT inactivates cell cycle inhibitors while promoting cell growth and proliferation (66, 67). AKT also facilitates B cell survival by phosphorylating and inactivating the pro-apoptotic factor BAD and several FoxO transcription factor family members that function as tumor suppressors (68-70).

In addition to AKT-dependent cell proliferation, SYK induces B cell differentiation by phosphorylating B-cell linker (BLNK)[2], which downregulates PI3K activity (71). BLNK also recruits additional proteins such as PLCγ2, BTK, and GRB2 to the plasma membrane to promote calcium-influx and activation of nuclear factor kappa B (NFκB) and RAS signaling (72-76). BLNK induces expression of the transcription factor Aiolos, which downregulates λ5 to stop surrogate light chain expression while promoting RAG1/2-dependent gene recombination (77, 78). Successful RAG1/2-dependent light chain rearrangement replaces the pre-BCR surrogate light chain to form a mature BCR and therefore, promotes progression through the intermediate pre B-cell stages (79) (**Figure 1-1**).

[2] Also known as SLP-65.

When pre-BCR signaling is constitutively active in t(1;19) pre-B-ALL, leukemic cells are able to outcompete normal B cells and colonize the bone marrow. However, these leukemic cells proliferate using the same effector kinases—many of which are targetable with small molecule inhibitors—that are utilized by normal cells. Dasatinib, for example, inhibits many of the kinases directly mediated by the pre-BCR, reducing the survival of t(1;19) pre-B-ALL and chronic lymphocytic leukemia (CLL) (24, 80). The response, unfortunately, is not durable due to rescue mechanisms that allow leukemic cells to survive single-agent kinase inhibition. In t(1;19) pre-B-ALL, we have demonstrated one such mechanism in which the protein ROR1 is able to rescue AKT signaling in response to dasatinib (24).

1.3 ROR1 is a poorly understood pseudokinase that is required for normal B cell development and t(1;19) pre-B-ALL

1.3.1 *The ROR family of receptor tyrosine kinases is required for normal development*

The ROR protein family is comprised of ROR1 and ROR2 and was identified through a PCR screen to find tyrosine kinases similar to the neurotrophic tropomyosin tyrosine kinase receptors that regulate neuronal development (81). Sharing 58% amino acid overall identity, ROR1 and ROR2 are single-pass transmembrane receptors that are highly conserved among vertebrates and invertebrates (82-87). Both receptors share a similar overall structure: an extracellular domain made up of immunoglobulin, cysteine-rich, and Kringle motifs and an intracellular region consisting of a tyrosine kinase, two

serine/threonine-rich, and serine-rich domains (88). Despite sharing 68% identity in the protein kinase domain, only ROR2 was initially shown to have kinase activity (81).

Some of the earliest characterization studies of the ROR protein family were in mouse models that revealed differential expression patterns and functions during embryonic development. Both *ROR* genes are expressed in developing neural tissue starting at stage E8 and peaking by stage E13 (86, 89). *ROR1* expression is restricted to parts of the nervous system and developing lens epithelium, while *ROR2* is ubiquitously expressed (86, 89). Knockout mouse models correlate with these patterns: Both *ROR1*$^{-/-}$ and *ROR2*$^{-/-}$ knockout mice die within 24 hours after birth due to heart defects and respiratory failure (90). *ROR2*$^{-/-}$ mice exhibit dwarfism and multiple skeletal defects, a phenotype that is enhanced in a *ROR1*/*ROR2* double knockout mouse (90, 91). In contrast, *ROR1*$^{-/-}$ mice do not have a severe prenatal phenotype, suggesting some compensation by ROR2[3] (90). Nevertheless, the neuronal, skeletal, and respiratory effects of *ROR1*$^{-/-}$ and *ROR2*$^{-/-}$ knockout mice illustrate the requirement for the ROR protein family during normal development.

Unlike embryonic development, adult tissue expression of *ROR1/2* in mice is much less common. *ROR1* expression is still detectable in postnatal and adult mouse heart and brain, whereas *ROR2* is no longer expressed (86). Consistent with murine studies showing a downregulation of *ROR1* expression in adult cells, human *ROR1* is

[3] A later study suggests that the ROR1$^{-/-}$ knockout mouse exhibits growth retardation and reduced life expectancy but can survive until at least weaning (92).

expressed in few adult tissues, including pancreas and adipocytes[4] (81, 94). In lymphocytes, ROR1 protein has been detected in immature B cells during intermediate stages of development before it is downregulated in mature B cells (24, 94). Furthermore, the ROR1 protein is highly expressed on the surface of many immature and mature B cell leukemias (discussed in more detail in section 1.3.3) (24, 94).

The ROR protein family is highly conserved among species and functional studies in these model organisms have provided insight into ROR1/2 signaling. For instance, one of the well-studied structural components of ROR1/2 is the extracellular cysteine-rich domain that is commonly found in Frizzled-related proteins (88). The cysteine-rich domain in the *X. laevis* ortholog of ROR2 has been shown to physically interact with Wnt5a to inhibit convergent-extension via c-jun N-terminal kinase signaling and planar cell polarity pathways (95, 96). Furthermore, *Wnt5a*$^{-/-}$ and *ROR2*$^{-/-}$ knockout mice share very similar phenotypes, both exhibiting cardiac, respiratory, and muscular developmental defects (97). Moreover, the *C. elegans* ROR1/2 homolog, CAM-1, is required for cell motility and asymmetric cell division through inhibition of the Wnt homolog EGL-20 (82, 98). These studies—and those that characterize the *ROR1/2* $^{-/-}$ knockout mice—suggest a role for the ROR protein family during development and Wnt signaling that may be conserved in humans.

[4] A recent study, however, suggests that ROR1 may also be expressed in additional adult tissues, including the parathyroid, esophagus, and stomach (93).

1.3.2 *Activation of ROR1 is not fully understood*

Like ROR2, Wnt5a has been suspected to bind and activate ROR1. Fukuda and colleagues proposed that independent of LRP5/6, the co-receptor for Wnt receptors, Wnt5a binds ROR1 to activate NFκB signaling in CLL (99). However, the authors make observations based on overexpressed ligands and receptors, an experimental approach that inherently comes with the risk of misrepresenting the endogenous biology by using ectopically expressed proteins. The authors also note that co-expression of *Wnt5a* and *ROR1* fails to activate expression of TCF/LEF, a Wnt-signaling-dependent transcription factor (99). In spite of this, expression of the extracellular domain of ROR1 was able to bind Wnt5a *in vitro* (99). However, the potential role of ROR2, which is known to bind Wnt5a, in Wnt-dependent activation of ROR1 was not discussed. In fact, the same group and others eventually reported that ROR1-ROR2 heterodimers are critical in the formation of normal neuronal synapses and leukemogenesis (100, 101). While ROR1 activation in CLL may still be under debate, the ROR1 ligand in ALL has yet to be identified.

1.3.3 *ROR1 and ROR2 mutations are associated with human disease*

Despite the overlap between *ROR1* and *ROR2* expression patterns and potential ligands, there is growing evidence to support unique roles for ROR1 and ROR2 in human disease. Mutations in *ROR2* manifest into one of two genetic disorders, both of which are reminiscent of the *ROR2*$^{-/-}$ knockout mouse phenotype. Individuals with

homozygous mutations in *ROR2*[5] develop Robinow syndrome, an autosomal recessive genetic disease that gives rise to morphological and skeletal defects (102, 103). Heterozygous mutations in *ROR2* that flank the tyrosine kinase domain cause the autosomal dominant disorder Brachydactyly B, resulting in shortened fingers and toes (104, 105). In contrast, ROR1 has not been implicated in genetic disease until recently, when a *ROR1* variant (R736T) was associated with autosomal recessive deafness. This variant is located in the tyrosine kinase domain[6] and is thought to be an inactivating mutation since it fails to activate NFκB signaling (106).

Compared to genetic disorders, the ROR protein family has been more extensively studied in cancer. ROR2, for instance, is upregulated in many types of solid tumors, including renal cell carcinoma and osteosarcoma, and it can function as a tumor promoter or suppressor depending on the cancer subtype (107). Similarly, ROR1 is associated with more aggressive disease and upregulated in many types of solid tumors and hematologic malignancies (108, 109).

ROR1 has been shown to be highly expressed in a variety of solid tumors (93, 109, 110). In breast cancer, for example, ROR1 promotes epithelial-to-mesenchymal transition, activates AKT signaling, and is suspected to be a prognostic factor of triple negative breast cancer (110-112). Studies in melanoma also suggest a role for ROR1 in AKT activation as well as upregulation of the mesenchymal markers N-cadherin and vimentin, both of which are associated with epithelial-to-mesenchymal transition that

[5] presumably loss of function mutations
[6] Position 736 corresponds to an arginine that is conserved in all tyrosine kinases (88).

leads to cancer metastases (113, 114). Furthermore, *ROR1* has been reported to be expressed by cancer stem cells in glioblastoma and may be necessary for their invasive and migratory activity (115). *ROR1* expression is also correlated with poor clinical outcome and promotes cell migration and invasion of ovarian cancer (116, 117). Interestingly, ROR1 function in ovarian cancer may be associated with ROR2, as a *ROR1/2* double knockdown leads to a stronger anti-tumor effect (117).

Some of the earliest work characterizing the expression and tumor-promoting function of ROR1 was in hematologic malignancies. In the late 2000s, several studies reported that ROR1 protein levels are higher in CLL compared to normal cells and required for leukemic survival through NFκB signaling (99, 118-122), suggesting its potential as a therapeutic target. Treatment strategies that target ROR1 are in development and will be discussed in more detail in section 1.3.7.

Once high ROR1 expression was reported in CLL, more groups noted *ROR1* expression and oncogenic activity in other hematologic malignancies. Indeed, non-hodgkin lymphomas, acute myeloid leukemias, and hairy cell leukemias exhibit high levels of ROR1 (94, 122, 123). In B cell ALLs, *ROR1* is highly expressed and is also required for leukemic cell survival through cross-talk with the pre-BCR and its direct and indirect effector kinases, including AKT (24, 122, 124, 125) (**Figure 1-2**).

1.3.4 *ROR1 is a pseudokinase that lacks intrinsic kinase activity*

ROR2—but not ROR1—was shown to have intrinsic kinase activity (81) and yet Wnt5a was indicated as a potential activator of ROR1 (99). Contrary to this, multiple

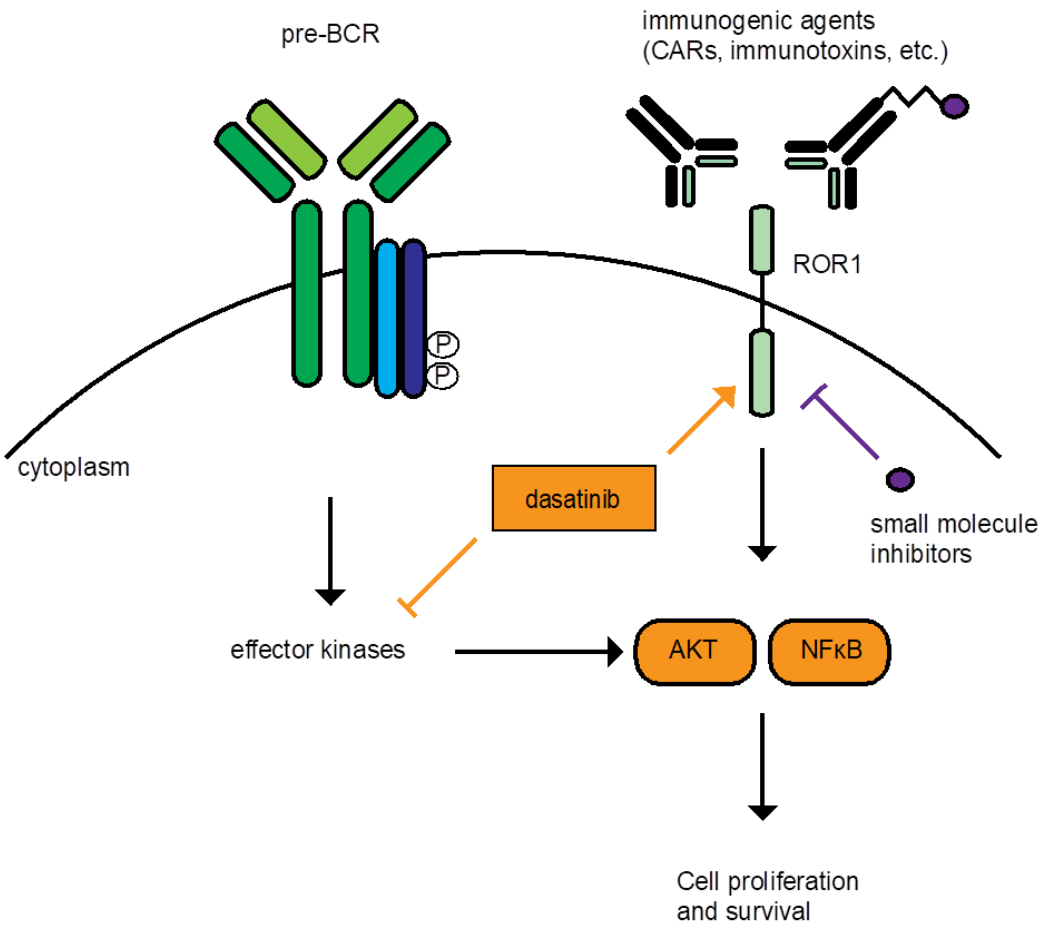

Figure 1-2 ROR1 cross-talks with the pre-BCR and can be targeted by multiple strategies.

Previous work shows that ROR1 cross-talks with the pre-BCR, as demonstrated through dasatinib-dependent upregulation of ROR1 (24). As a result, ROR1 aids in the rescue of pre-BCR downstream signaling pathways, including AKT and NFκB to resist dasatinib treatment. Currently, multiple groups are investigating the potential of targeting ROR1 through various immunogenic agents and small molecules. Adapted from Bicocca *et al* (2012) (24).

groups, including ours, have demonstrated a lack of phosphorylation on the ROR1 kinase domain (24, 126, 127). Gentile *et al* (2011) has reported that the proline-rich, but not the kinase, domain on ROR1 is phosphorylated to promote cell survival (126). Furthermore, the authors analyzed the kinase domain of ROR1 and noted that residues in the glycine-rich loop, catalytic loop, and activation segment within the kinase domain contain mutated residues compared to known, functional kinases (126). Moreover, Bainbridge *et al* mutated aspartate 482, which is in the glycine-rich loop, but kinase activity was not affected, suggesting that changing one key residue is not sufficient to restore activity (128). Another study further demonstrated that ROR1 is a pseudokinase by using thermal shift assays to detect nucleotide (ligand) binding[7], and we have also described a lack of kinase activity for ROR1 in t(1;19) pre-B-ALL (24, 127). Although ROR1 kinase activity may still be under debate, it is clear that ROR1, like many kinases, is important for cancer survival and is a potential therapeutic target.

1.3.5 *ROR1 may act as a scaffold to mediate downstream signaling*

ROR1 is a pseudokinase that lacks intrinsic kinase activity in t(1;19) pre-B-ALL (Section 1.3.4), making drug design and studies of signaling mechanisms challenging. However, pseudokinases may act as scaffolds to activate intrinsic cell signaling by recruiting functional kinases and other binding partners (129). In lung cancer, ROR1 acts as a scaffold to recruit and stabilize cavin-1 and caveolin-1, structural components of caveolae (130). Other candidate ROR1 binding partners have been identified in t(1;19)

[7] Or a lack there of, in the case of ROR1.

pre-B-ALL, including TRIM21 and TBC1D1 (24). Interestingly, TRIM21 is an E3 ubiquitin ligase that is required for NFκB signaling upon exposure to adenoviruses (131). In addition, the GTPase-activating protein-domain containing protein TBC1D1 is an AKT substrate that cross-talks with the nutrient-responsive mammalian target of rapamycin (mTOR) pathway (132). These studies suggest that ROR1 may interact with a variety of proteins, including kinases, to initiate cell proliferation and survival pathways, such as NFκB and AKT. ROR1 is required for AKT signaling in t(1;19) pre-B-ALL—especially in response to pre-BCR inhibition—yet the details of how this occurs is unknown. In Chapter 4, I present data that indicate a potential interaction between ROR1 and the mTOR complexes, mTORC1 and mTORC2, to activate AKT signaling.

1.3.6 *The success and challenges of designing small molecules to inhibit kinase activity in leukemia*

Kinases are a dynamic and diverse group of proteins that regulate many cellular processes, including gene expression, cell division, and cell death. Their roles in cell proliferation and survival have been exploited in human diseases such as cancer (133). Fortunately, the mechanisms by which kinases exert their effects have undergone intense investigation, leading to the development of therapeutic agents that significantly improve patient survival. Perhaps one of the most famous examples is imatinib, a small-molecule inhibitor that blocks the ATP-binding pocket of ABL1 kinase (134, 135). ABL1 is commonly fused to BCR to form an oncogenic fusion product BCR-ABL1—the driver of chronic myeloid leukemia (136, 137). Since its approval in 2001, imatinib (and its

derivatives) has significantly improved the survival of patients who present with chronic myeloid leukemia, thereby encouraging research to understand and design inhibitors against other kinases.

Although there has been success in designing small molecule inhibitors (138, 139), there are still challenges in using them as single agents. Especially in the case of ATP competitors, such as imatinib and dasatinib, lack of selectivity may occur due to multiple kinases sharing conserved residues within the ATP-binding pocket (140). Drug resistance through upregulation of alternate pathways or secondary mutations is also common (24, 140, 141). Therefore, it is important to consider inhibitors as single agents and in combination with other therapeutic agents. Dasatinib, for instance, is effective against t(1;19) pre-B-ALL, but this response can be enhanced when it is combined with silencing of *ROR1* expression by siRNA (24). ROR1 inhibitors, however, have yet to be successfully translated into the clinic.

1.3.7 *ROR1 targeting strategies have potential efficacy but have yet to be approved for patient treatment*

Since ROR1 lacks kinase activity in t(1;19) pre-B-ALL, designing drugs that directly target this pseudokinase is challenging, yet its high expression in both solid tumors and leukemia makes it a potential therapeutic target. Indeed, multiple groups have investigated small molecule inhibitor strategies to target ROR1-expressing cancers (**Figure 1-2**). In t(1;19) pre-B-ALL, dasatinib kills cells by inhibiting many of the kinases downstream of the pre-BCR, but a subset survive treatment due to a ROR1-mediated

rescue mechanism (24). Additionally, Kancera developed KAN0439834, a drug that is described to be a ROR1-specific inhibitor and has been reported to induce apoptosis in CLL (142). While this reagent does not affect levels of phosphorylated BTK, a critical kinase in CLL and directly associated with the pre-BCR, levels of non-direct BTK targets are reduced with KAN0439834, suggesting potential non-specific effects (142). Since specific inhibition of ROR1 has not yielded the ideal therapeutic response yet, alternative approaches are needed to exploit ROR1 expression and optimize the response in ROR1-dependent cancers (**Figure 1-2**).

Unlike small molecules, utilizing ROR1 for antibody-based therapeutic reagents has led to more promising results. For example, Baskar *et al* (2012) generated antibodies against ROR1, one of which was subsequently conjugated to an exotoxin to make an immunotoxin. Both the antibodies and the immunotoxin selectively bound CLL and mantle cell leukemia primary patient sample cells and cell lines. Furthermore, the mantle cell leukemia cell line HBL-2 underwent apoptosis in response to the immunotoxin (143). The ROR1 antibody from Baskar *et al* (2012) was also used to target lipid nanoparticles carrying the anti-tumor sphingosine analogue derivative OSU-2S to primary CLL cells and reduce leukemic burden in a transgenic mouse model (144, 145) (**Figure 1-2**).

Other high affinity antibodies and chimeric antigen receptors that recognize the extracellular domain of ROR1 have been produced by multiple groups (94, 108, 146). In fact, the humanized ROR1 antibody UC-961 (also known as Cirmtuzimab), has been

successful in Phase I clinical trials to treat CLL patients (147). Furthermore, chimeric antigen receptor-T cell therapies have demonstrated exquisite potency in various hematologic malignancies. For instance, CTL109, which consists of CD19 and the T-cell co-stimulatory molecule CD137, was originally engineered in Carl June's lab to treat CLL and has also shown high efficacy in ALL (148-150). Chimeric antigen receptor-T cells have also been designed against ROR1 and have shown efficacy against CLL (94, 146), but these have yet to be evaluated in ALL (**Figure 1-2**).

Although there has been recent success in developing ROR1-targeting agents, these potential therapeutics have not been fully tested in combination with standard chemotherapy, nor have they been utilized in the clinic. Therefore, there are still opportunities to further understand both regulation upstream and functional effects downstream of ROR1 to determine alternative targets that can be exploited to inhibit ROR1. Our group has shown t(1;19) pre-B-ALL is especially sensitive to pre-BCR effector kinase inhibition using dasatinib (24). Inhibition of the pre-BCR, however, is unsustainable due to an upregulation of ROR1 to rescue kinase signaling. We have demonstrated that this compensatory mechanism can be inhibited when *ROR1* expression is suppressed in combination with dasatinib (24). Collectively, these results establish a rationale for targeting ROR1 in combination with other known mechanisms critical for leukemic cell survival.

1.3.8 *Regulatory mechanisms of ROR1 are not well-understood*

Although ROR1 has been investigated as a potential drug target, therapeutics in development are mostly limited to immunogenic strategies. With the success of imatinib, there is an opportunity to identify small molecule inhibitors that could inhibit ROR1. Since ROR1 kinase activity is not detectable in t(1;19) pre-B-ALL (24), targeting ROR1 with small molecules is challenging. However, other proteins that are important in modulating *ROR1* expression and stability in leukemic B cells could become surrogate targets to inhibit ROR1. Therefore, I sought to understand mechanisms of ROR1 regulation in t(1;19) pre-B-ALL.

Currently, few studies have been published describing regulation of *ROR1* expression. For example, microRNAs miR-27b-3b and miR-382 have been shown to post-transcriptionally silence *ROR1* expression in gastric and ovarian cancer, respectively (151, 152). In contrast to transcript repression, the transcription factors STAT3 and NKX2-1 have been shown to promote *ROR1* expression. STAT3, for instance, directly binds the *ROR1* promoter to activate transcription in the multiple myeloma cell line MM1 in an interleukin-6-dependent manner (153). Similarly, NKX2-1 binds the *ROR1* promoter in lung adenocarcinoma cells to induce *ROR1* expression and promote EGFR signaling (154). Despite these discoveries, inhibition of these mechanisms has yet to be thoroughly investigated. To suppress ROR1-dependent survival mechanisms, it is imperative to identify proteins that mediate ROR1 levels in t(1;19) pre-B-ALL. Targeting *ROR1* expression and stability may prove to be a useful strategy to combat its

upregulation in t(1;19) pre-B-ALL treated with other small molecules, such as dasatinib. In this dissertation, I use an siRNA screen approach to identify candidate regulators of ROR1 expression, including Ubiquitin-like with PHD and Ring Finger domain 1 (UHRF1).

1.4 UHRF1 maintains genetic methylation and regulates gene expression in proliferating cells

In 2000, Hopfner *et al* identified UHRF1 as a regulator of the alpha isoform of Topoisomerase II in normal and cancerous proliferating cells (155). This group also determined that UHRF1 expression is highest during the G1/S phase during the cell cycle, indicative of its role in cell proliferation (156, 157). In normal tissues, *UHRF1* is expressed in the thymus, bone marrow, and liver, suggesting a role for UHRF1 in hematopoiesis (155, 158). Indeed, *UHRF1* is upregulated during S phase in hematopoietic stem cells and expressed in both germinal center and CD19+ peripheral blood B cells (159).

UHRF1 has functions as a nuclear chromatin modifying protein and as an E3 ubiquitin ligase (**Figure 1-3**) (158, 160). Its role in the nucleus has been well-studied; it functions to maintain hemi-methylated DNA through interactions with histone modifications, DNA, and DNA-associated proteins (158). Specifically, the PHD and two Tudor-like domains recognize tri-methylated Lysine 9 on histone H3, promoting the maintenance of heterochromatin (161, 162). After the SRA domain of UHRF1 recognizes methylated CpG sites, UHRF1 facilitates DNA-binding through interactions with

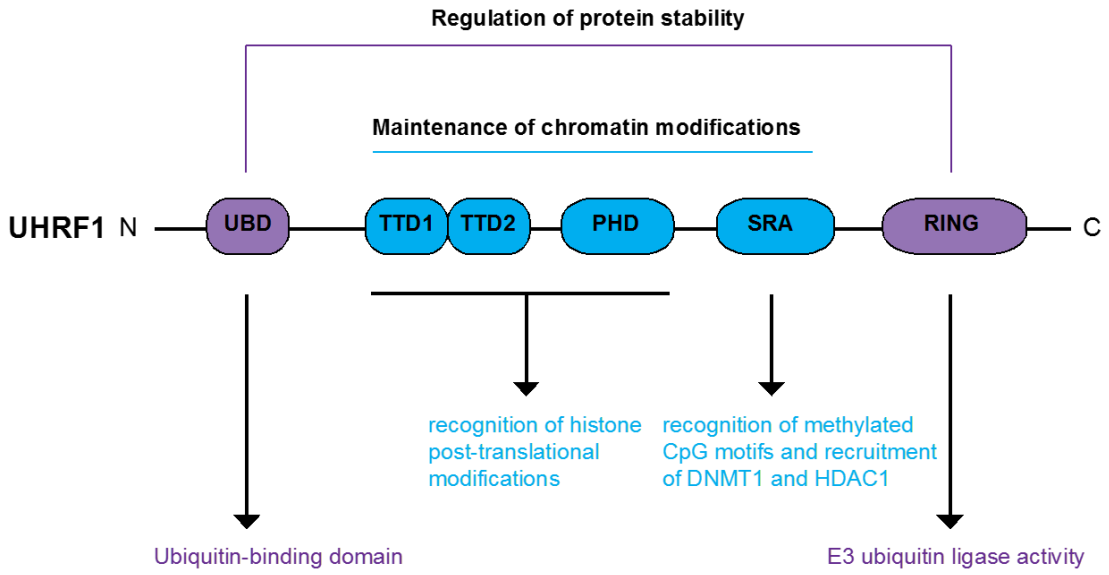

Figure 1-3 Structural domains of UHRF1.

UHRF1 has two major functions in cells. The Tudor-like (TTD1 and 2), PHD, and SRA domains recognize a variety of histone modifications and facilitate recruitment of chromatin modifying enzymes such as DNMT1 and HDAC1 to regulate gene expression and genomic methylation. The ubiquitin-binding (UBD) and RING domains confer E3 ligase activity, allowing UHRF1 to post-translationally modify histones and protein substrates. Together, these functions allow UHRF1 to affect gene expression on chromatin, genomic, and protein levels. Adapted from Alhosin *et al* (2016) (163).

histone deacetylase 1 (HDAC1) to promote DNA methyltransferase 1(DNMT1)-dependent DNA methylation (164-169). In contrast, the PHD domain of UHRF1 has also been shown to bind an unmethylated arginine residue on histone H3 that is associated with euchromatic regions of the genome (170), illustrating the context-dependent effects of UHRF1 in the nucleus.

Unlike the nuclear-localized, DNA and histone-binding function of UHRF1, its role as an E3 ubiquitin ligase is less well-characterized. Citterio *et al* (2004) was the first group to report on the ligase activity of the UHRF1 mouse homolog, NP95 (171). These results were further supported by Yasumichi Hitoshi's group, who demonstrated that the cysteine 724 and histidine 741 residues within the RING finger domain of UHRF1 were required for E3 ligase activity (172). Furthermore, these studies also demonstrated that the RING finger domain was also required for A549 lung cancer cells to resist chemotherapy agents including etoposide, suggesting that the E3 ligase function plays a role in drug response and cancer (172).

1.5 UHRF1 is as an oncogene that downregulates tumor suppressors

Multiple groups indicate UHRF1 as an oncogene in solid tumors and leukemia. For instance, *UHRF1* expression is higher in several cancer cell lines compared to non-cancerous cells (157), and it is suppressed in a p53 and p21-dependent manner in response to DNA damage (173). In contrast, UHRF1 interacts with the histone methyltransferase G9a to repress p21 expression (174). Similarly, UHRF1 promotes cell

proliferation and metastasis of colorectal cancer by downregulating the expression of the tumor suppressor $p16^{ink4a}$ (175). UHRF1 has also been implicated as a biomarker of bladder cancer and is associated with low-risk acute myeloid leukemia (176, 177). Collectively, these studies establish UHRF1 as an oncogene, yet its role in B cell ALL has yet to be defined.

The oncogenic role of UHRF1 has also been investigated based on interactions with protein target substrates. For example, UHRF1 ubiquitinates one of its binding partners, DNMT1, to signal for its degradation via the proteasome (178)—one example of how UHRF1 activity bridges DNA methylation with protein regulation. Additional UHRF1 protein substrates include p53, PML, and MEG3, all of which are known tumor suppressors (179-181). Together, these studies demonstrate UHRF1 negatively regulates multiple anti-tumorigenic proteins, but whether or not UHRF1 mediates other tumor-promoters—and its role in B cell ALL—has yet to be determined.

1.6 **Summary**

Despite dramatic improvement in survival rates of pediatric ALL with the advancement of chemotherapy and the birth of targeted therapy, there is still opportunity to better understand the biological mechanisms that drive therapy-resistant and relapsed disease. ROR1 is one example of how a disease is able to avoid complete elimination through a compensatory mechanism. t(1;19) pre-B-ALL patient mononuclear cells subjected to a siRNA screen revealed that *ROR1* was required for pre-BCR downstream signaling and cell survival (24). When pre-BCR effector kinases are

inhibited by dasatinib, *ROR1* is upregulated to promote leukemic cell survival (24). This rescue mechanism, therefore, is an intriguing point of therapeutic intervention. However, ROR1 cannot be directly targeted by traditional small molecule inhibitors because it lacks intrinsic kinase activity in t(1;19) pre-B-ALL. Therefore, it is important to identify regulators of ROR1 and its interacting partners that may function as surrogate drug targets to inhibit ROR1.

In this dissertation, I address two hypotheses. First, I hypothesize that the oncogene UHRF1 is required for the maintenance of ROR1 protein levels and t(1;19) pre-B-ALL survival. More importantly, this pathway can be targeted biochemically and pharmacologically in combination with dasatinib. My second hypothesis is that ROR1 interacts with members of mTORC2, a direct activator of AKT, to promote cell survival in t(1;19) pre-B-ALL. Collectively, this work establishes the preclinical rationale that ROR1 can be targeted, even indirectly, to improve the outcome for t(1;19) pre-B-ALL patients.

2 Materials and Methods

2.1 Apoptosis Assay

1.25e6 cells were treated with 25-150 nM naphthazarin for 8 hours. GuavaNexin reagent (Millipore) was added and apoptosis was measured according to the manufacturer's instructions. Annexin-V positive staining was normalized to unstained cells.

2.2 Cell Culture

2.2.1 *Cell lines and culturing conditions*

RCH-ACV and Kasumi-2 cells were purchased from the DSMZ German Collection of Microorganisms and Cell Cultures. REH and RCH-ACV+ROR1-V5 cells were a gift from Dr. Vincent T. Bicocca. U937 cells were from ATCC and provided by David K. Edwards V and SupB15 cells were a gift from Dr. Jessica Leonard. HEK293T17 cells were from ATCC and PC-3 cells were a gift from Dr. Joshi Alumkal. The following cell lines were gifts from Dr. Philip Koeffler: TC32, C666, HK1, SUNE1, S18, S26, SUNE2, HNE1.

RCH-ACV, Kasumi-2 REH, RCH-ACV+ROR1-V5, U937, TC32, C666, HK1, SUNE1, and PC-3 cells were cultured in R10 media: RPMI-1640 + 10% FBS + 1% Penicillin/Streptinomycin + 2% L-glutamine + 0.1% Fungizone. SupB15 cells were cultured in R20 media: RPMI-1640 + 20% FBS + 1% Penicillin/Streptinomycin + 2% L-

glutamine + 0.1% Fungizone. Kasumi-2 cells were thawed in R20 and transitioned into R10. HEK293T17, S18, S26, SUN3, and HNE1 cells were cultured in D10 media: DMEM + 10% FBS + 1% Penicillin/Streptinomycin + 2% L-glutamine + 0.1% Fungizone. All cell lines were maintained in 37°C with 5% CO_2. Refer to **Table 2-1** for cell culturing conditions.

2.2.2 *HEK293T17 transfections*

6-well plates were seeded with 3.5e5 cells per well (in 2 mL of D10) overnight to be at 40-60% confluency the next day. 10.9 μL Fugene6 (Promega) + 134.5 μL OPTI-MEM (Gibco/ThermoScientific) were mixed in a 1.5 mL tube and incubated at room temperature for 5 minutes. Up to 2 μg plasmid DNA was added and mixture was incubated at room temperature for 15 minutes. Reactions were added drop-wise to cells and incubated for 48 hours before harvesting cell lysates (section 2.8.1).

2.2.3 *MTS viability assay*

Total cell viability was measured using the Cell Titer96® AQueous One Solution Cell Proliferation Assay (MTS) according to the manufacturer's instructions (Promega). 20 μL per well was used for a 96-well plate and 5 μL per well was used for a 384-well plate.

Cell Line	Culture media	Cell density for routine passage	Approximate passage time (days)	Cell density for experiments (incubate overnight)
RCH-ACV	R10	5-6e5 cells per mL	24 hours	4e5 cells per mL
Kasumi-2	R20, transition to R10	6-7e5 cells per mL	24 hours	5e5 cells per mL
REH	R10	5e5 cells per mL	24 hours	3.5e5 cells per mL
RCH-ACV+ROR1V5	R10	5e5 cells per mL	24 hours	3.5e5 cells per mL
U937	R10	5e5 cells per mL	24 hours	4e5 cells per mL
SupB15	R20	7e5 cells per mL	24 hours	6e5 cells per mL
PC-3	R10	1:10 in 10mL	72 hours	3e5 cells per 2mL (6-well plate), 2500 cells/50uL (96-well plate)
HEK293T	D10	1:10 in 10mL	48 hours	3.5e5 cells per 2mL (6-well plate)

Table 2-1 List of cell lines and culturing conditions described in this dissertation.

2.2.4 *Small-interfering RNA (siRNA) gene silencing*

2.2.4.1 96-well plate format (pre-made tyrosine kinase siRNA screen, adherent cells described in the Appendix (Chapter 6))

52.2 μL oligofectamine (Invitrogen) was mixed with 11.55 mL OPTI-MEM and incubated at room temperature for 10 minutes. 110 μL for oligofectamine-OPTI-MEM mix was added per well in siRNA plate and incubated at room temperature for 20 minutes. 1.5-6e5 cells were pelleted, supernatant was aspirated, and cells were re-suspended in 21 mL media. 66 μL of cells were transferred to each well in a new 96-well plate (triplicate plates). 34uL oligofectamine-OPTI-MEM-siRNA mix was added to each well and incubated for 4 days before performing an MTS assay.

2.2.4.2 Design of custom 96-well plate format siRNA screens (as described in Section 3.3.1)

To identify potential regulators of ROR1 in t(1;19) pre-B-ALL, expression of transcription factors and epigenetic regulators were targeted with 40 μM siRNA SMARTpools (Dharmacon). Transcription factors were chosen based on prediction software using the *ROR1* promoter and locus (Softberry Inc., DECODE database from SA Biosciences). Epigenetic regulators were chosen based on Black *et al*. 2012 (182). *ROR1* and non-targeting (non-specific) siRNA were included on both screens as controls. All selected genes have been annotated according to the National Center for Biotechnology Information.

2.2.4.3 96-well plate format

For suspension cells, 1.5e7 cells were pelleted and re-suspended in 9.5 mL siPORT buffer (120 mM Trehalose, 20 mM HEPES pH8, 1 mM Myo-Inositol, 1 mM KCl, 1 mM $MgCl_2$, 1 mM K_2HPO_4, 0.4 mM KH_2PO_4, 2.14 mM KOH, 1 mM Glutathione). 95 μL cells were added to 5 μL siRNA (stocks reconstituted at 40 μM) in a 96-well electroporation plate on ice. Cells were electroporated twice (S=0.1), with current = 2000, resistance = 1000, and voltage and time at the following conditions: 300 volts, 2 ms (RCH-ACV and REH) or 250 volts, 3 ms (RCH-ACV+ROR1-V5). 20 μL cells were transferred in to 90 μL R10 media per well (7500 cells total per well) in triplicate and incubated for 4 days prior to performing an MTS assay.

For PC-3 cells, a 6-well plate was seeded with 3e5 cells per well (in 2 mL R10) and a 96-well plate with 2500 cells per well (in 50 μL R10 per well) overnight. For a 6-well plate, 100 μL 1 μM siRNA (diluted in OPTI-MEM) was mixed with 100 μL OPTI-MEM in one 1.5 mL tube. In a second 1.5 mL tube, 5 μL Lipofectamine 2000 (Invitrogen) was mixed with 195 μL OPTI-MEM. Both tubes were incubated at room temperature for 5 minutes. Contents of both tubes were combined, mixed by pipetting, and incubated at room temperature for 20 minutes. Mixture was added dropwise to cells and incubated for 5 days before harvesting lysate. For a 96-well plate used the same protocol with the following modifications (volumes written per well): 3 μL 1 μM siRNA (diluted in OPTI-MEM) was mixed with 3 μL OPTI-MEM in one 1.5 mL tube. In a second 1.5 mL tube,

0.15 μL Lipofectamine 2000 (Invitrogen) was mixed with 5.85 μL OPTI-MEM. The 96-well plate was incubated for 6 days prior to performing an MTS assay.

2.2.4.4 Single 4mm cuvette format (suspension cells only)

For every reaction, 180 μL siPORT buffer was mixed with 20 μL siRNA (stocks reconstituted at 40 μM, 1-2 μg siRNA per reaction). 8e5 cells were pelleted, re-suspended in 200 μL siPORT and mixed with siRNA. Cells were electroporated at the following conditions: 305 volts, 2 ms, 2 pulses. Cells were transferred to a 1.5 mL tube (on ice), mixed, and 7.5 μL were transferred to a 96-well plate in six replicates (15,000 cells total per well). 92.5 μL R10 was added to each well and incubated for 96 hours before performing an MTS assay. The remaining cells were pelleted (5000 rpm, 5 minutes), supernatant was aspirated, and pellet was re-suspended in 568 μL R10 (diluted to approximately 5e5 cells per mL). Cells were transferred to a 24-well plate and incubated for 72 hours before harvesting cell lysates. See section 2.5.3 for details regarding siRNA combinations with inhibitors.

2.3 **Cloning**

Full-length UHRF1 cDNA was purchased in a pDONR221 vector from the Harvard Plasmid Repository (plasmid ID HsCD00079664-1) and cloned into the pCS2-6Xmyc (a gift from Dr. Monika Davare) vector using InFusion cloning (Clonetech) and ClaI restriction sites. UHRF1 E3 ligase mutants, C724A and H741A, were generated

using the QuikChange site-directed mutagenesis XL kit (Agilent) using the following mutagenesis primers:

UHRF1 C724A: 5′ CTCCTGACACAGCAGATAGCCTGGAACGTCTCCTCC 3′, 5′ GGAGGAGACGTTCCAGGCTATCTGCTGTCAGGAG 3′,

UHRF1 H741A: 5′ CCTTGCACACGTTGGCCTGGCACACGGTCG 3′, 5′CGACCGTGTGCCAGGCCAACGTGTGCAAGG 3′.

All plasmids were validated by Sanger sequencing.

Full-length ROR1 cDNA in the pCDNA-pDEST3.2 vector (Invitrogen) was previously described (24). Plasmids (using the pCDNA-pDEST3.2 vector) encoding ROR1 mutants with deletions of individual structural domains were generated by 96 Proteins (South San Francisco, CA). Full-length Rictor cDNA was purchased in the Dev01.1 vector from Genecopoeia (plasmid ID GC-H3267B). It was cloned into the pCMV14-3XFLAG vector (a gift from Dr. Julia Maxson) using Gateway cloning technology (Invitrogen). All plasmids were validated by Sanger sequencing.

2.4 **Immunoprecipitation**

All experiments enriching for endogenous proteins used an NP-40-based lysis buffer (1% NP-40, 137 mM NaCl, 20 mM Tris-HCl, 20 mM HEPES pH 8, 2 mM EDTA pH 8, 10% Glycerol). All experiments using lysates with ectopically expressed proteins used a 10X lysis buffer from Cell Signaling (Section 2.8.1). Cell lysates were extracted as described in section 2.8.1. 0.5-1 mg of cell lysate was incubated with 4 μg antibody

overnight at 4°C with rotation. Protein G beads (Millipore) were blocked with 0.05% BSA/1XPBS for 1 hour at 4°C with rotation. Beads were added and incubated for 1 hour at 4°C with rotation. The unbound fraction was saved before beads were washed three times in NP-40 buffer (without NaCl) or 10X lysis buffer. Beads were boiled at 95°C in loading dye before performing SDS-PAGE.

Antibodies used for immunoprecipitation experiments are commercially available from: R&D Biosystems (ROR1), Invitrogen (V5), Sam Jackson Laboratories (Goat IgG), and Santa Cruz Biotechnology, Inc. (mouse IgG). See **Table 2-2** for more details.

2.5 **Inhibitor treatment – single and 96-well plate format**

2.5.1 *Inhibitors*

Commercially available inhibitors used in this work are from the following vendors: SelleckChem (Velcade (Bortezomib) and dasatinib), Cayman Chemical (anisomycin), Millipore (cycloheximide) and Sigma-Aldrich (actinomycin D, naphthazarin, thymoquinone). Cirmtuzimab and the ROR1 antibody-drug conjugate ADC-A were generously provided by Oncturnal Therapeutics (San Diego, CA).

Antibody	MW (kDa)	Species	Vendor	Cat#	Recommended Dilution	Notes
Actin	45	Mouse	Millipore	MAB1501	1:10,000	clone C4
AKT	60	Mouse	BD	610877	1:1000	good for WB
GAPDH	39	Mouse	Ambion	AM4300	1:10,000	good cytoplasmic fraction control
Goat IgG		Goat	Sam Jackson Laboratorie	005-000-003		Isotype control for IP, stock [ ] = 11.9mg/mL, use 4ug for IP
Iga (CD79a)	45-55	Rabbit	Cell Signaling	3351	1:1000	multibands but can distinguish
Igb (CD79B)	39	Sheep	R&D	AF6620	1:1000	better than mouse sc-166675
IKZF1 (IKAROS)	50-70	Mouse	BD	564475	1:1000	
KAT7 (HBO1)	83	Rabbit	SCBT	sc-25379	1:200	
LYN	53, 56	Rabbit	Cell Signaling	2732	1:1000	
Mouse IgG		Mouse	SCBT	sc-2025		Isotype control for IP, stock [ ] = 200ug/0.5mL, use 4ug for IP
mTOR	290	Rabbit	Cell Signaling	2983	1:1000	
p-AKT (S473)	60	Rabbit	Cell Signaling	4060	1:1000	
p-LYN (Y507)	53, 56	Rabbit	Cell Signaling	2731	1:1000	
p-mTOR (S2481)	290	Rabbit	Cell Signaling	2974	1:1000	
Raptor	150	Rabbit	Cell Signaling	2280	1:1000	
RhoH	21	Rabbit	SCBT	sc-366409	1:200	
Rictor	199	Rabbit	Cell Signaling	2114	1:1000	
ROR1	135	Rabbit	Cell Signaling	4102	1:1000	
ROR1	135	Goat	R&D	AF2000	1:1000	0.5mg/mL, good for IP, ok for WB
RUNX1 (AML1)	58	Rabbit	Calbiochem	PC285	1:1000	
SP1	95	Rabbit	Millipore	07-645	1:2500	nuclear protein control
Ubiquitin	7	Mouse	Boston Biochem	A-104	1:1000	block and primary in 5%Milk/1XTBST, secondary in 1XTBST only
UHRF1	95	Rabbit	Cell Signaling	12387	1:1000	
UHRF1 (ICBP90)	95	Mouse	BD	612264	1:1000	IP antibody
V5		Mouse	Invitrogen	46-0705	1:5000	for IP: ~1ug/rxn
β-tubulin	55	Mouse	Millipore	05-661	1:5000	
α-tubulin	50	Mouse	Sigma	T6074	1:10000	

Table 2-2 Antibodies used in this dissertation and recommended uses.

2.5.2 *384-well plate format*

5e5-1e6 cells were diluted in 26 mL R10 and 50 μL was pipetted into each well of a 384-well plate. Inhibitors were diluted in DMSO and added to the 384-well plate using the HP D300 Digital Dispenser. Plates were incubated for 3 days before performing an MTS assay.

For the experiments using Cirmtuzimab and the ROR1 antibody-drug conjugate, each reagent was diluted in media, and ten 1:2 serial dilutions were pipetted by hand in 384-well plate (maximum concentration 100 μg/mL). To test in combination with dasatinib, dasatinib was diluted in DMSO and added to the 384-well plate using the HP D300 Digital Dispenser. 6.5e5 cells were re-suspended in 13 mL media and added to the plate using the Multidrop Combi dispenser (ThermoScientific). Plates were incubated for 3 days before performing an MTS assay.

2.5.3 *Single agent drug treatment*

Dasatinib was used at 100 nM and protein cell lysates were collected over 24 hours. For combination treatments with siRNA, dasatinib was added 24 hours post-electroporation. 200 nM actinomycin D (up to 8 hours), 1 μg/mL cycloheximide (up to 24 hours), or 25 nM Velcade (up to 16 hours) was added 48 hours post-electroporation before collecting cell lysates.

2.6 Nuclear Fractionation

5e6-10e6 cells were pelleted at 500 x g for 5 minutes. Nuclear and cytoplasmic fractions were isolated using the NE-PER kit according to the manufacturer's instructions (ThermoScientific). Purified cellular fractions were quantified by BCA assay (ThermoScientific) prior to SDS-PAGE.

2.7 RNA extraction, cDNA synthesis, and qRT-PCR

2.7.1 *RNA extraction and cDNA synthesis*

Cell pellets were re-suspended in 350 μL RLT+β-mercaptoethanol buffer, and total RNA was extracted using the QiaShredder and RNAeasy extraction kits according to the manufacturer's instructions (Qiagen). RNA was quantified using the TAKE3 plate reader (BioTek Synergy2) and input RNA was normalized to the lowest concentrated sample. Equal amounts of RNA was used to generate cDNA using the SuperScript III/RNAseOUT cDNA synthesis kit (Invitrogen).

2.7.2 *qRT-PCR*

For the initial studies to validate the siRNA screen (discussed in Chapter 3), cDNA was used for qRT-PCR using primers designed against *ROR1* and *GUSB* as previously described (24) and fluorescence was detected by SYBR Green using the Opticon2 thermocycler (MJ Research). Additional qRT-PCR experiments were performed using primer sets with FAM probes (Taqman) and the QuantStudio7 (Life Technologies). All

qRT-PCR reactions were run using the following protocol: 50°C 2 min, 95°C 10 min, 40 cycles of 95°C 15 sec, 60°C 1 min. Technical replicate C(t) values were averaged and normalized to *GUSB* before further analysis.

2.8 Protein extraction and Immunoblots

2.8.1 *Whole cell protein lysate extraction*

1X cell lysis buffer was made using 10X cell lysis buffer with Triton X-100 (Cell Signaling), 1% phosphatase inhibitor cocktail 2 (Sigma), 1% PMSF (Sigma), and a complete mini protease inhibitor tablet (Roche, 1 tablet per 10 mL 1X cell lysis buffer). For cell pellets, cells were lysed with 1X cell lysis buffer, vortexed and rotated at 4°C for 15 minutes. Samples were vortexed three times before pelleting cellular debris (13,200 rpm, 10 minutes), and cell lysate (supernatant) was transferred to a new pre-chilled 1.5 mL tube. For adherent cells, media supernatant was aspirated. 100 μL 1X cell lysis buffer was added dropwise to cells and incubated for 5 minutes. Cell lifters were used to collect cells and pipet transferred to pre-chilled 1.5 mL tubes. Samples were centrifuged (13,200 rpm, 10 minutes), and the cell lysate (supernatant) was transferred to a new pre-chilled 1.5 mL tube. Lysates were quantified by BCA assay (ThermoScientific) and subjected to SDS-PAGE.

2.8.2 *SDS-PAGE, Western blot*

Quantified cell lysates were mixed with 3X GS + β-mercaptoethanol loading dye and boiled for 5 minutes at 95°C. 4-15% Tris-HCl gradient gels were used for SDS-PAGE

and membranes were blocked for 1-2 hours at room temperature and incubated with antibodies at 4°C overnight. See **Table 2-2** for details. The ROR1 antibody has been validated by the manufacturer and we have also tested this antibody in multiple cell lines to correlate immunoblot data with cell lines known to express or not express *ROR1* at the transcript level (**Figure 2-1**). Membranes were washed three times before HRP-conjugated secondary antibody was added, followed by incubation at room temperature for 1-1.5 hours. Afterwards, membranes were washed three times and images were obtained by chemiluminescence according to the manufacturer's instructions (BioRad ECL Clarity Substrate, ChemiDocMP). Densitometry was performed using ImageJ and bands were normalized to the loading control (Actin, GAPDH) before further analysis.

2.9 Statistical Analyses

All statistical analyses on MTS cell viability, qRT-PCR, immunoblot, and apoptosis assays were performed using Prism software package (GraphPad). Adjusted p-values were reported to reflect multiple comparisons used in statistical analyses when applicable.

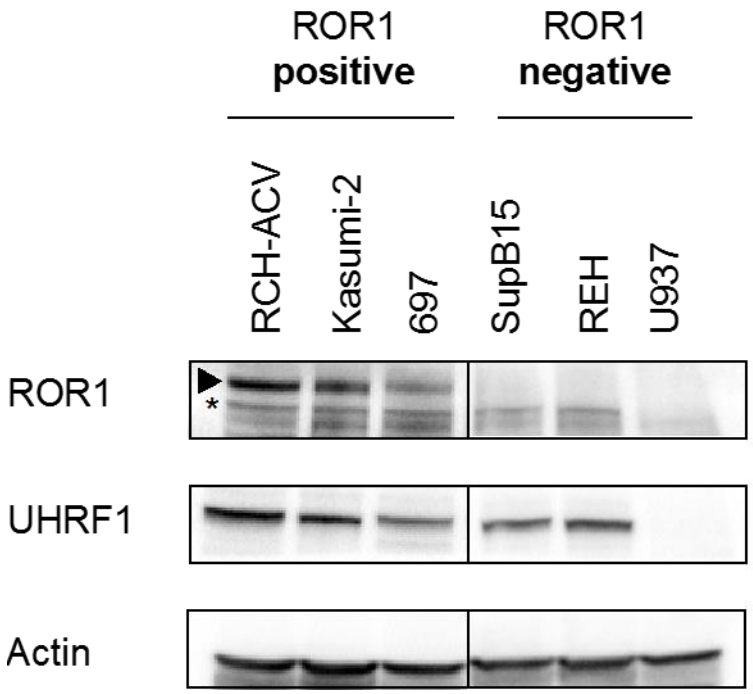

Figure 2-1 Detection of ROR1 protein is also associated with a non-specific band.

Full-length ROR1 (arrowhead) is detectable in *ROR1*-expressing cell lines (ROR1-positive). However, a lower band (asterisk) is also observed, but this is consistent in non-*ROR1*-expressing (ROR1-negative) cell lines, suggesting that this lower band is non-specific.

3 UHRF1 is necessary for the maintenance of levels of ROR1 protein in t(1;19) pre-B-ALL

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Part of this work was presented at the 57th annual American Society of Hematology meeting, December 5-8 2015, Orlando FL (Abstract #3648).

The data presented in this chapter is currently under preparation for submission.

3.1 **Abstract**

Expression of the transmembrane pseudokinase ROR1 is required for survival of t(1;19)-pre-B-cell acute lymphoblastic leukemia (t(1;19) pre-B-ALL), CLL, and many solid tumors. However, targeting ROR1 with small molecule inhibitors has been challenging due to the absence of ROR1 kinase activity. To identify genes that regulate ROR1 expression and may, therefore, serve as surrogate drug targets, I employed an siRNA screening approach and determined that the epigenetic regulator and E3 ubiquitin ligase, UHRF1, is required for t(1;19) pre-B-ALL cell viability in a ROR1-dependent manner. Upon UHRF1 silencing, ROR1 protein is reduced without altering ROR1 mRNA, and ectopically expressed UHRF1 is sufficient to increase ROR1 levels. Additionally, proteasome inhibition rescues loss of ROR1 protein after UHRF1 silencing, suggesting a role for the proteasome in the UHRF1-ROR1 mechanism. Finally, I show that ROR1-positive cells are more sensitive to the UHRF1-targeting drug, naphthazarin, and undergo increased apoptosis compared to ROR1-negative cells. Naphthazarin elicits reduced expression of UHRF1 and ROR1, and combination of napthazarin with inhibitors of pre-B-cell receptor signaling results in further reduction of cell survival compared with either inhibitor alone. Therefore, my work reveals a mechanism by which UHRF1 stabilizes ROR1, suggesting a potential targeting strategy to inhibit ROR1 in t(1;19) pre-B-ALL and other malignancies.

3.2 **Background**

Between 1-5% of patients with acute lymphoblastic leukemia carry a translocation between the long arm of chromosome 1 and the short arm of chromosome 19, leading to the arrest of normal B cell differentiation and development of pre B-cell acute lymphoblastic leukemia (t(1;19) pre-B-ALL). The fusion protein resulting from this translocation, TCF3-PBX1, promotes aberrant expression and re-localization of WNT16B and β-catenin, respectively (25, 28, 183). Consequently, t(1;19) pre-B-ALL cases uniformly exhibit differentiation arrest at an intermediate stage of B-lineage maturation where blasts express the pre B-cell receptor (23, 184). Recent studies have shown that signaling from the pre-B-cell receptor through tyrosine kinases such as BTK and SRC-family kinases is important to drive proliferation and survival of t(1;19) pre-B-ALL cells (24, 185). We and others have shown that the pseudokinase ROR1 is also critical for leukemic cell survival through cross-talk with the pre-BCR (24, 99, 186). Furthermore, t(1;19) pre-B-ALLs are sensitive to dasatinib, an inhibitor of pre-B cell receptor (pre-BCR) effector kinases BTK and SRC family kinases, but ROR1 is upregulated as a compensatory rescue response. Consequently, t(1;19) pre-B-ALL cell lines are maximally sensitive to silencing of ROR1 in combination with kinase inhibitors that block pre B-cell receptor signaling, such as dasatinib (24).

ROR1 is highly expressed on the surface of both t(1;19) pre-B-ALL and CLL cells in addition to multiple types of solid tumors including prostate, breast, and pancreatic cancer (109, 110, 118, 119, 125, 187). ROR1 is thought to be rarely expressed in post-natal

tissues, although a recent study revealed ROR1 may be present in several normal tissues (93, 119). Multiple groups are currently developing and testing methods of targeting ROR1-expressing tumor cells, some of which have seen initial success in clinical trials (94, 143, 147, 188). However, the targeting strategies to date have largely revolved around immunologic agents, since ROR1-specific small-molecule inhibitors have remained elusive due to the absence of ROR1 kinase activity (24). Thus, I sought to develop an improved understanding of the regulation of ROR1 expression in an effort to discover surrogate small-molecule targeting strategies. Previously, groups have shown that STAT3 directly binds the *ROR1* locus to promote its expression in CLL (153) and NKX2-1 has been reported to induce *ROR1* expression in lung adenocarcinoma (154). In addition to transcriptional activation, ROR1 is thought to be post-translationally modified through glycosylation and ubiquitination (189), but the mediators of these modifications have yet to be elucidated.

The RING E3 ligase UHRF1 ubiquitinates several substrates, including p53 and histone H3, to mediate protein function and chromatin structure, respectively (161, 180). UHRF1 also has ubiquitin ligase-independent roles interacting with DNA and histones through its Tudor-like, PHD, and SRA domains (161, 164, 166-168, 170, 174, 175, 190). Both UHRF1 functions can be inhibited through direct binding to or downregulation of *UHRF1* expression by a number of small molecule compounds, including NSC232003 and naphthazarin (163, 191). Despite evidence that UHRF1 promotes solid tumor formation and progression and is associated with low-risk acute myeloid leukemia (157, 175, 177, 192-195), UHRF1 has not been thoroughly investigated in B cell ALL.

Therefore, I sought to find new mechanisms that regulate ROR1 and, more importantly, may have therapeutic potential that can be targeted by small molecule inhibitors. I utilized an siRNA approach and identified UHRF1 as a regulator of levels of ROR1 protein in t(1;19) pre-B-ALL. Targeting the UHRF1-ROR1 axis in combination with readily available pre-B-cell receptor targeting strategies, such as dasatinib, may prove to be a useful alternative regimen for ROR1-expressing cancers.

In this chapter, I will present work generated from an siRNA screen to identify candidate regulators of ROR1 that are also required for t(1;19) pre-B-ALL viability. One of these candidates is UHRF1, which I hypothesize to be required for ROR1 expression in t(1;19) pre-B-ALL. Furthermore, I will describe work demonstrating that biological or pharmacological targeting of UHRF1 significantly reduces t(1;19) pre-B-ALL viability, especially in combination with dasatinib, a pre-BCR effector kinase inhibitor.

3.3 **Results**

3.3.1 *UHRF1 is required for t(1;19) pre-B-ALL in a ROR1-dependent manner*

To identify genes required for t(1;19) pre-B-ALL viability that also regulate *ROR1* expression I performed an siRNA screen targeting a broad range of transcription factors and epigenetic regulators using the t(1;19)-positive pre-B-ALL cell line, RCH-ACV. Gene targets were prioritized according to effects on overall cell viability after siRNA knockdown. Upon silencing, siRNA targets that reduced viability by at least one standard deviation were further investigated. UHRF1 and RUNX1 were among the gene

targets that, when silenced, significantly reduced RCH-ACV cell viability (**Figure 3-1 A, Table 3-1**). RUNX1 has previously been shown to be a key regulator of pre-BCR expression (196), consistent with the importance of the pre-BCR in t(1;19) pre-B-ALL cells. In contrast, UHRF1 has not been previously implicated in B cell ALL pathogenesis.

To determine whether these siRNA targets were important for t(1;19) pre-B-ALL cell survival in a ROR1-dependent or ROR1-independent manner, I repeated the screen with RCH-ACV cells that stably overexpress ROR1 with a V5 tag (RCH+ROR1-V5). These cells retained sensitivity to RUNX1 silencing, once again consistent with the role of RUNX1 in regulating the pre-BCR, which is a pathway orthogonal to ROR1 in t(1;19) cells (24). However, these cells did not exhibit sensitivity to UHRF1 silencing, suggesting that ectopically expressed ROR1 mitigates UHRF1-sensitivity in t(1;19) cells and, therefore, UHRF1 is important for maintaining t(1;19) cell viability in a ROR1-dependent manner (**Figure 3-1 B**). As a final control, I applied the same siRNA screen to REH cells, an ALL cell line that lacks the 1;19 translocation and do not express *ROR1*. REH cells generated a very different list of putative targets and, importantly, these cells were not sensitive to silencing of UHRF1 (**Figure 3-1 C**). These data suggest UHRF1 specifically mediates t(1;19) cell viability through a mechanism associated with *ROR1* expression.

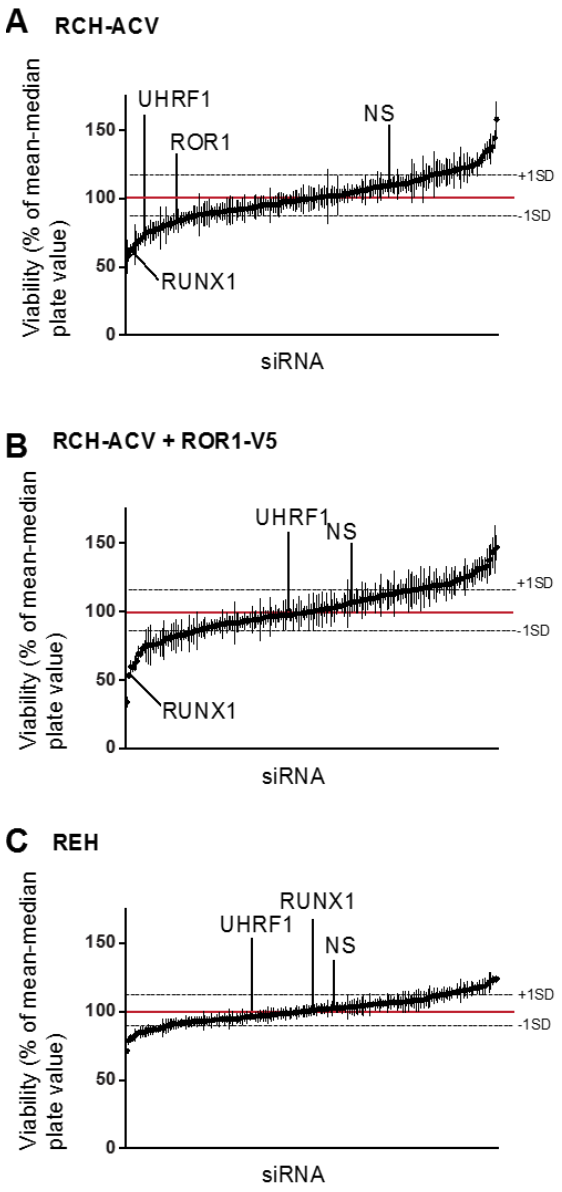


Figure 3-1 UHRF1 is a potential regulator of ROR1 in t(1;19) pre B-ALL.

(A) Parental RCH-ACV cells (N=4), **(B)** RCH-ACV stably expressing *ROR1*-V5 (N=3), or **(C)** REH (N=3) cells were electroporated with siRNAs targeting transcription factors and chromatin modifiers/epigenetic regulators. Cell viability was measured by MTS assay. Gene targets were ranked based on their effects on viability upon silencing. Targets that reduced viability by at least one standard deviation among all biological replicates were further investigated. Error bars represent S.D. NS = non-specific siRNA.

Plate 1 target	RCH-ACV Avg % viabiilty	Avg SD	RCH+ROR1-V5 Avg % viabiilty	Avg SD	REH Avg % viabiilty	Avg SD
	RCH-ACV		RCH+ROR1-V5		REH	
Plate 1 target	Avg % Viabillty	Avg SD	Avg % viabillty2	Avg SD3	Avg %viabillty	Avg SD4
IKZF1	58.61	5.35	75.30	13.79	82.47	4.62
RUNX1/AML1	62.27	4.34	33.79	4.07	100.30	4.03
RHOH	63.18	4.32	59.47	3.70	84.70	4.26
TCF3	68.60	6.52	102.37	11.37	84.96	3.69
ROR1	70.07	4.51	72.93	3.74	91.72	3.51
POU3F1	71.33	3.76	59.42	4.95	92.87	3.92
NFATC2	74.67	9.19	69.01	6.12	108.68	6.54
MYB	75.69	9.08	84.24	6.47	78.83	3.13
FOXL1	75.92	6.72	62.20	5.39	101.48	4.66
FOXF2	76.02	6.56	53.04	2.46	94.20	2.01
FOXO4	77.92	6.02	81.79	12.55	91.15	3.01
WT1	80.64	7.36	85.91	2.78	99.31	3.30
REST	81.38	5.47	93.08	6.31	90.95	4.47
NFE2L1	82.06	7.43	95.98	5.09	105.16	2.81
IRF4	82.93	5.63	80.21	5.61	116.52	5.87
USF1	83.97	3.88	74.44	4.61	102.79	2.92
ALX1	84.01	5.75	85.36	10.04	106.97	8.28
TGIF1	85.28	4.37	112.47	10.48	93.19	3.33
CEBPA	86.43	4.83	86.89	5.88	86.36	3.49
HSF1	87.46	7.64	100.94	12.32	103.00	8.81
USF2	87.84	6.61	119.50	6.43	86.96	2.50
CUX1	88.28	5.80	84.08	13.56	119.73	2.73
GATA6	88.50	8.60	93.51	3.10	90.99	3.62
FOXO3(a)	88.77	9.43	98.54	6.82	96.47	4.53
HOXA9	89.30	8.84	120.14	12.79	86.26	4.28
TAL1	89.68	4.84	88.70	6.08	83.86	1.84
LHX3	90.08	4.40	63.86	6.72	90.74	4.18
KLF12	90.25	5.99	91.70	15.95	106.82	6.63
FOXD1	90.49	5.92	106.13	17.68	94.42	3.14
SOX9	90.74	10.38	96.30	14.46	99.69	4.18
NME2	91.20	8.70	94.10	6.38	94.47	7.60
ATF2	91.48	2.51	107.54	9.32	71.04	1.68
GATA2	91.58	7.67	143.27	11.19	87.94	7.10
HNF1A	92.11	8.85	83.08	11.81	97.88	4.16
GTF2I/TFII-I	92.60	1.88	74.86	12.05	96.18	2.58
PPARG	92.94	4.18	100.88	11.22	107.99	8.05
FOXO1	94.07	7.14	86.00	14.07	98.58	5.28
NFYA	95.02	7.04	94.32	15.63	104.90	8.64
FOXOA2	95.16	7.13	113.07	17.15	80.89	3.16
STAT3	95.22	4.37	125.70	8.71	94.74	4.19
FOXI1	95.22	4.06	137.36	11.51	101.82	3.95
GATA1	95.42	4.67	87.98	7.73	111.17	5.86
SP1	95.49	6.24	75.52	15.27	107.91	4.69

	RCH-ACV		RCH+ROR1-V5		REH	
Plate 1 target	Avg % viabiilty	Avg SD	Avg % viabiilty	Avg SD	Avg % viabiilty	Avg SD
PBX1	95.63	8.03	76.78	4.11	105.86	2.67
MYCN	97.75	5.94	111.58	15.97	93.22	3.73
JUN	98.00	4.37	101.47	13.24	101.27	4.03
MYT1L	99.32	10.77	146.80	9.22	94.79	2.84
NR3C1	99.86	9.01	132.04	7.37	96.89	3.11
EN1	99.89	7.89	137.99	11.35	103.17	4.86
TFAP2A	100.53	9.60	100.75	5.91	94.11	2.83
MZF1	100.87	7.33	82.70	4.04	115.65	6.64
POU1F1	101.25	8.66	115.56	13.82	95.79	3.24
GFI1	101.59	5.47	93.62	5.79	113.28	5.30
EGR4	101.75	6.74	91.97	7.41	117.15	2.51
TEF	102.19	14.74	114.85	5.69	106.88	4.51
ELL3	103.26	6.31	93.75	10.20	112.63	3.28
CEBPZ	104.31	3.82	122.16	10.74	101.93	4.29
IRF7	104.92	5.37	126.98	8.50	121.24	7.78
GCFC2/GCF	105.38	3.80	112.14	5.98	92.93	7.25
ZNF238/RP58	105.65	8.75	91.47	7.68	101.81	2.40
BACH2	106.76	2.78	109.34	4.64	105.23	4.41
POU2F1	107.52	6.70	117.25	14.86	108.44	6.78
ETS1	108.25	12.08	91.64	4.92	90.50	4.25
POU2F2	109.26	4.52	101.25	4.78	97.74	4.64
ZBTB10	109.28	4.43	103.99	2.56	98.78	3.65
CRX	109.38	9.24	115.91	10.88	92.83	3.81
MSX1	110.25	11.27	97.68	11.55	99.98	4.71
SP3	110.92	5.63	108.37	13.47	111.18	3.37
KLF15	110.97	3.47	93.82	6.41	106.94	7.86
ESR1	111.11	7.04	116.86	16.74	85.25	3.84
TERF2IP	111.20	5.41	115.17	9.03	98.34	3.41
POU6F1	111.72	6.83	131.82	6.46	102.06	6.35
ZEB1	113.15	6.93	119.07	3.83	111.14	3.97
TFDP1	113.39	4.48	75.95	6.11	102.20	3.53
MYOD1	114.45	17.45	131.36	8.69	94.13	5.52
TBP	114.57	6.45	97.32	10.60	94.72	1.06
HSF2	115.50	9.95	126.51	5.43	98.31	4.87
GATA3	115.53	8.62	97.66	4.58	111.10	8.25
HIF1A	117.32	6.58	115.89	10.40	100.09	4.28
DLX4	118.11	9.37	131.41	9.34	106.82	4.28
NFKB2	118.17	10.36	112.25	3.92	115.43	6.48
ARID5B	118.21	9.68	124.24	5.03	86.79	2.04
MEIS1	119.23	5.86	105.01	9.34	93.42	5.81
AR	119.24	10.81	119.42	5.36	115.46	4.53
EP300	122.34	15.47	97.62	5.00	103.10	6.23
TP53	122.41	10.49	125.18	14.05	114.17	1.98
HES1	122.48	4.47	119.36	12.65	112.38	6.02
NFIL3	123.31	7.74	118.17	15.02	103.72	6.36
FOXJ2	125.02	3.08	116.39	7.65	122.28	6.29
POU3F2	125.27	8.90	99.75	14.26	122.88	3.97
RREB1	134.11	6.51	125.95	5.00	117.64	5.55
Non-Specific	134.97	7.09	112.29	11.36	113.51	4.33
SREBF1	136.09	7.35	116.96	4.77	114.10	2.25
MERTK	137.91	5.40	133.27	13.34	102.56	4.02
PAX4	144.28	5.59	145.45	17.48	106.62	5.06

Plate 2 target	RCH-ACV Avg % viabiilty	RCH-ACV Avg SD	RCH+ROR1-V5 Avg % viabiilty	RCH+ROR1-V5 Avg SD	REH Avg % viabiilty	REH Avg SD
	RCH-ACV		RCH+ROR1-V5		REH	
Plate 2 target	Avg % Viability	Avg SD	Avg % viability2	Avg SD3	Avg %viability	Avg SD4
KAT7	57.11	12.76	83.26	3.15	92.44	3.89
PRMT6	66.44	15.25	108.40	7.11	89.38	5.57
KAT8	67.62	9.13	92.11	4.11	111.77	5.79
UHRF1	74.74	5.22	97.11	5.39	96.11	2.21
CDY1B	75.65	4.93	83.51	5.18	86.98	2.48
KDM4E	77.56	6.34	76.93	9.69	89.43	5.48
KDM5D	78.19	15.72	88.97	3.49	106.33	7.49
RNF2	79.88	3.24	80.48	5.98	94.57	5.55
WHSC1L1	79.97	5.22	102.77	10.92	114.84	5.97
KDM5C	80.51	13.56	76.15	7.35	96.17	3.20
KAT5	81.71	3.91	90.33	5.96	95.55	6.10
EZH2	83.78	8.82	87.86	6.52	123.60	2.07
SUV420H2	84.64	5.39	95.23	4.67	108.47	1.99
SIRT2	86.33	4.31	91.50	5.42	93.48	2.29
DOT1L	86.79	4.33	89.04	5.64	115.00	3.02
PRDM9	87.57	7.50	77.09	10.45	86.37	3.70
GTF3C4	88.23	15.87	99.09	3.68	99.38	5.45
KDM4D	89.53	10.49	99.56	4.35	84.91	4.04
KDM2B	89.55	5.40	96.48	4.43	106.65	5.33
PRMT1	89.67	3.92	82.60	8.32	92.01	5.36
HDAC3	89.80	7.36	79.06	14.56	92.44	3.99
KDM3A	90.30	7.05	92.33	4.11	87.75	2.74
KDM1A	91.23	10.65	91.96	7.09	92.28	3.71
PRDM6	91.33	10.30	109.91	11.60	102.73	4.10
RNF40	92.04	6.59	81.05	3.86	98.98	2.40
NSD1	92.09	5.11	119.75	3.65	117.27	4.54
CDY1	92.42	10.29	69.04	3.03	87.96	3.96
UBR2	92.82	4.34	104.43	5.47	84.31	2.60
SUV420H1	92.93	5.15	75.82	4.15	92.51	2.39
EZH1	93.67	8.74	91.60	4.84	107.93	6.16
KAT6A	93.71	9.17	96.45	7.22	92.61	3.82
C14ORF169	94.90	5.50	96.67	4.96	80.79	2.35
SETDB1	95.34	12.82	115.00	4.94	96.32	2.17
MLL4	95.60	6.50	94.21	3.60	94.83	4.35
KDM6A	95.71	9.41	123.07	4.01	103.81	4.64
RING1	97.13	7.73	131.30	6.54	107.00	2.94
WHSC1	97.76	4.06	112.78	6.66	102.04	2.72
ROR1	97.85	5.13	81.10	10.35	94.38	2.76
PRMT7	97.91	5.45	97.61	4.15	95.95	5.01
KDM8	98.12	7.47	81.74	9.99	97.07	6.57
SETD8	98.13	8.29	103.96	6.22	98.66	2.48
RBX1	98.68	7.25	99.68	5.69	108.29	8.44
PHF2	98.69	5.30	98.62	12.51	104.27	8.09

	RCH-ACV		RCH+ROR1-V5		REH	
Plate 2 target	Avg % viabiilty	Avg SD	Avg % viabiilty	Avg SD	Avg % viabiilty	Avg SD
SIRT3	99.06	9.22	107.61	4.32	104.68	1.38
JHDM1D	99.37	3.57	97.32	3.80	99.80	2.54
SIRT6	99.73	3.81	97.49	11.40	100.64	3.86
KDM1B	99.83	10.01	101.79	5.68	109.36	7.13
MLL2	100.23	7.46	83.09	4.28	102.28	5.63
SMYD3	100.50	4.33	86.48	4.77	94.12	4.28
SIRT1	100.79	6.18	109.65	4.60	96.97	4.37
PRDM8	101.44	6.68	90.85	11.05	90.83	3.78
MYSM1	101.56	20.31	89.88	4.67	104.61	4.10
KDM4B	101.63	7.99	123.42	5.96	93.15	4.08
HAT1	102.03	4.96	114.37	6.50	97.10	2.67
DTX3L	102.30	4.82	110.24	3.94	105.66	3.36
CARM1	102.45	4.40	102.55	6.62	94.50	3.45
SETDB2	103.26	3.68	91.23	4.11	104.91	1.30
PRMT5	103.69	7.56	120.23	7.22	98.18	1.64
HDAC8	104.10	3.43	99.68	13.51	94.39	4.26
BRCC3	104.19	6.02	96.24	4.50	103.98	6.90
JMJD6	105.19	3.39	90.15	3.68	103.02	4.79
KDM6B	105.97	7.08	119.31	10.15	102.85	3.80
SETD2	105.98	11.27	88.15	3.78	96.34	3.53
CDYL	106.90	6.91	112.63	8.69	102.84	2.88
PRMT2	107.78	6.55	102.41	13.68	98.67	4.50
SETD7	108.23	5.75	95.53	7.50	117.83	1.38
RNF8	108.33	7.24	93.19	3.59	106.84	5.12
SETD1A	108.50	10.45	110.87	8.43	111.89	3.36
KDM4A	109.01	5.75	98.63	9.91	106.71	2.30
EHMT1	109.67	5.11	117.44	6.96	112.12	4.39
SETMAR	110.03	6.21	98.49	4.22	100.23	5.04
PRDM2	110.22	6.94	88.70	2.95	116.77	4.84
HDAC1	110.38	7.28	114.42	9.37	96.82	4.59
MLL3	110.41	5.47	105.24	7.00	91.96	2.46
PHF8	112.46	16.43	106.84	6.52	101.92	3.43
ASH1L	113.12	12.24	106.43	7.35	105.50	5.84
EHMT2	113.92	5.31	124.47	8.73	123.68	3.13
PRDM16	117.49	5.15	128.77	9.42	103.06	5.63
KDM5A	117.74	16.82	121.31	8.96	107.23	7.42
RNF20	118.34	7.53	108.72	12.30	105.37	3.52
NS	119.46	9.63	109.08	7.97	118.34	6.10
SETD1B	119.69	9.35	96.59	8.27	92.70	4.28
JMJD1C	119.94	5.66	115.23	16.12	105.90	2.43
KDM2A	120.30	13.50	115.43	12.43	115.72	1.81
SUV39H1	120.76	3.70	107.61	8.92	102.41	1.99
HDAC9	121.48	5.88	117.60	8.28	97.00	4.31
KDM3B	121.87	8.42	103.99	3.92	109.23	5.29
KDM5B	122.55	10.91	102.41	3.78	115.14	9.23
MLL	123.64	4.89	120.17	4.03	112.82	4.53
SUV39H2	123.79	6.11	99.13	5.28	102.80	4.23
SMYD1	126.70	3.69	113.71	5.81	107.72	4.12
MLL5	127.78	5.06	119.77	8.43	100.91	3.60
RNF168	130.73	5.42	106.22	10.14	118.18	6.22
KDM4C	136.17	11.29	120.78	11.55	97.43	5.16
SMYD2	157.67	13.43	109.61	5.75	97.79	2.38

Table 3-1 siRNA screen results.

siRNA SMARTpool targets are listed based on known functions as a transcription factor (Plate 1) or epigenetic regulator (Plate 2). Average percent viability and standard deviation (S.D.) are listed for all tested cell lines and targets are sorted based on their effects on RCH-ACV cell viability, where lowest values are ranked at the top of the lists. ROR1 and UHRF1 are highlighted in red.

3.3.2 *UHRF1 is required for the maintenance of ROR1 protein but not mRNA*

To determine whether UHRF1 plays a direct role in regulating *ROR1* expression, I measured cell viability and levels of ROR1 mRNA and protein after UHRF1 silencing. After confirming that UHRF1 is required for RCH-ACV cell viability (**Figure 3-2**), I observed that levels of ROR1 protein, but not mRNA, were significantly reduced after UHRF1 silencing (**Figure 3-3, Figure 3-4**). These results indicate that UHRF1 regulates ROR1 protein levels through a post-transcriptional mechanism. This was unexpected, since the RCH+ROR1-V5 cells were not sensitive to UHRF1 silencing, which initially suggested a transcriptional mechanism. To reconcile these results, I hypothesized that RCH+ROR1-V5 cells are rescued from UHRF1 siRNA sensitivity because ROR1 protein levels are present at highly elevated levels in these cells compared with parental RCH-ACV cells. In this case, UHRF1 silencing would also reduce the ectopic ROR1-V5 in these cells, but the levels would still be maintained above a threshold required to maintain cell viability. Consistent with this, I observed that upon UHRF1 silencing, ectopically expressed ROR1 was reduced in RCH+ROR1-V5 cells, but only to levels comparable to those levels seen at baseline in RCH-ACV parental cells (**Figure 3-5**).

Next, I asked whether the reduced levels of ROR1 protein that I observe after UHRF1 silencing could be attributed to one of the defined functions of UHRF1. Since UHRF1 can function as an E3 ubiquitin ligase to modulate protein turnover, I suspected that regulation of ROR1 by UHRF1 is associated with the proteasome. Therefore, I measured ROR1 protein levels after UHRF1 silencing in the absence or presence of the

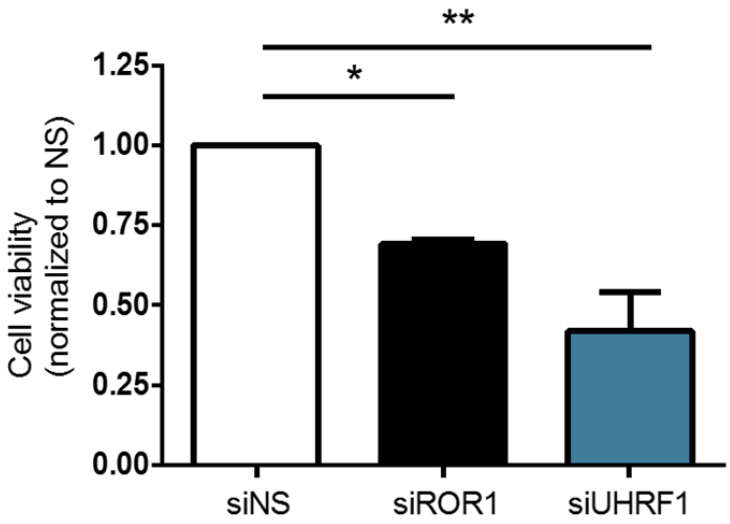


Figure 3-2 UHRF1 is required for t(1;19) pre-B-ALL cell viability.

RCH-ACV cells were electroporated with siRNA against ROR1 or UHRF1. Cell viability was measured by MTS assay (N=3 **$p<0.005$, *$p<0.05$). Data was normalized to a non-specific siRNA control (siNS). Error bars represent S.E.M.

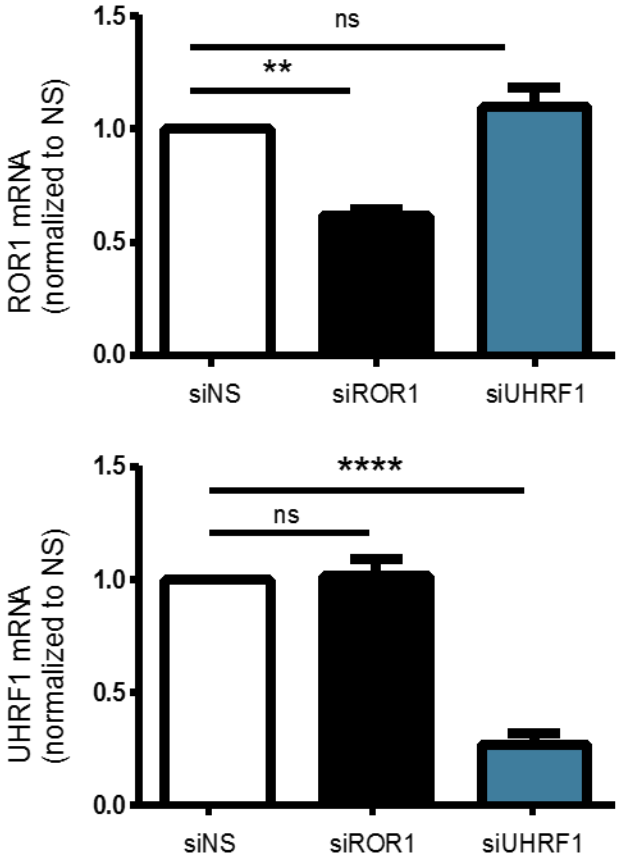


Figure 3-3 UHRF1 is not required for ROR1 mRNA expression in RCH-ACV cells.

RCH-ACV cells were electroporated with siRNA against ROR1 or UHRF1. 72 hours after electroporation, total RNA was harvested and tested for mRNA expression by qRT-PCR (N=3 **p=0.005, ****p<0.0001). mRNA expression was normalized to a non-specific siRNA control (siNS). Error bars represent S.E.M. "ns" = not significant.

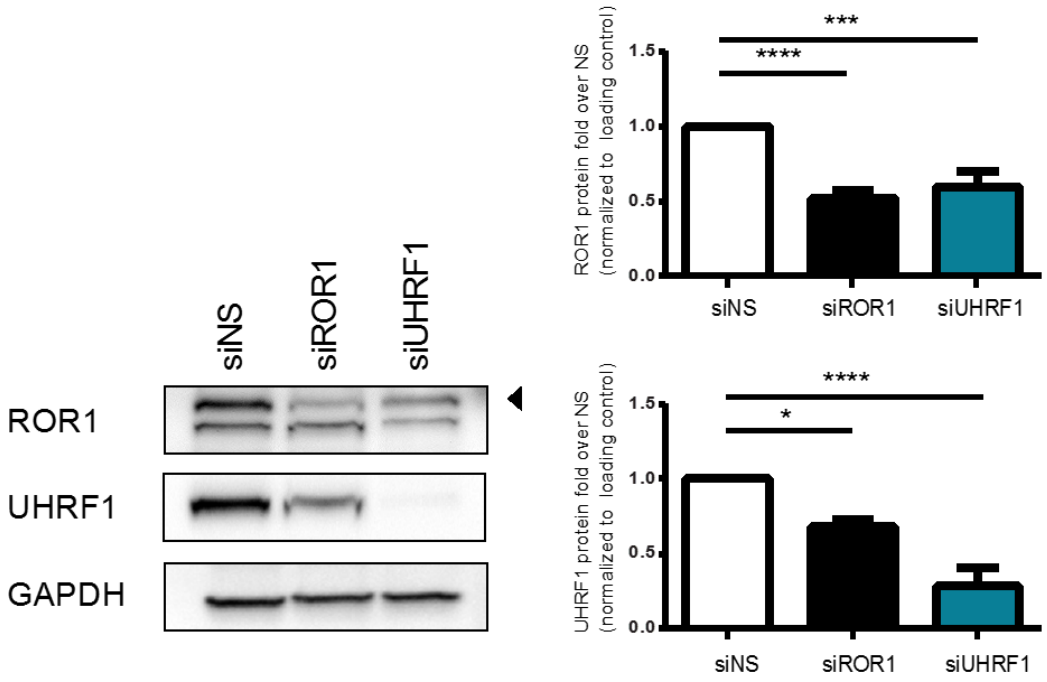


Figure 3-4 UHRF1 mediates ROR1 protein levels in RCH-ACV cells.

RCH-ACV cells were electroporated with siRNA against ROR1 or UHRF1. 72 hours after electroporation, protein lysates were harvested and tested by immunoblot (N=8 ****p<0.0001, ***p<0.0005, *p<0.05). Protein levels were normalized to a non-specific siRNA control (siNS). Error bars represent S.E.M. “ns” = not significant.

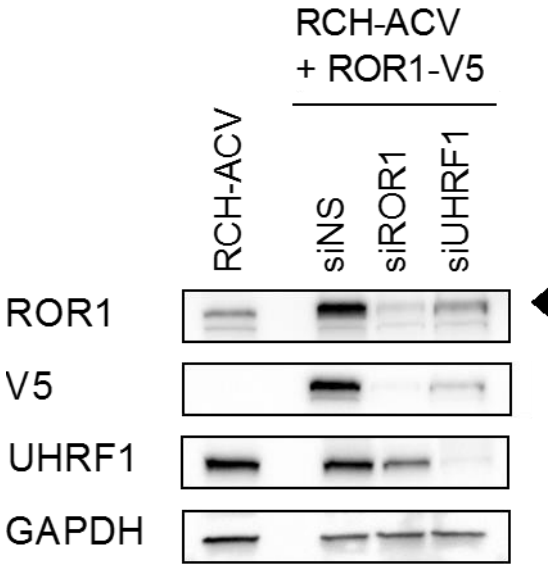


Figure 3-5 UHRF1 is required for ectopically expressed ROR1 protein.

RCH+ROR1-V5 cells were treated with siRNA against ROR1 or UHRF1. 72 hours after electroporation, whole cell lysates were harvested and tested by immunoblot (N=6).

proteasome inhibitor Velcade. Treatment with Velcade rescued ROR1 protein levels after UHRF1 silencing compared to vehicle-treated cells where UHRF1 silencing resulted in reduced levels of ROR1 protein (**Figure 3-6**). In contrast, loss of ROR1 after UHRF1 silencing was not rescued by the transcriptional and translational inhibitors, actinomycin D and cycloheximide, respectively (**Figure 3-7**). Collectively, these data further illustrate that UHRF1 regulates ROR1 through a post-transcriptional mechanism.

To complement the gene silencing experiments, I transiently expressed wild-type UHRF1 in HEK293T17 cells, which express very low levels of *ROR1* and *UHRF1*. As shown in **Figure 3-8**, ectopically expressed *UHRF1* correlates with an increase in ROR1 protein levels in a dose-dependent manner compared to the empty vector control. Therefore, these data suggest that overexpression of UHRF1 is sufficient to induce ROR1 protein levels, further support of UHRF1-mediated regulation of ROR1.

3.3.3 *UHRF1 indirectly regulates ROR1*

Since silencing of UHRF1 resulted in reduced levels of ROR1 protein, I next asked whether UHRF1 and ROR1 are associated in a common complex that would allow UHRF1 to interact with or modify ROR1 directly. Co-immunoprecipitation experiments with either ROR1 or UHRF1 show no detectable endogenous interaction (**Figure 3-9**), which is consistent with previously published mass spectrometry studies (24). These observations were confirmed by co-immunoprecipitation from HEK293T cells with

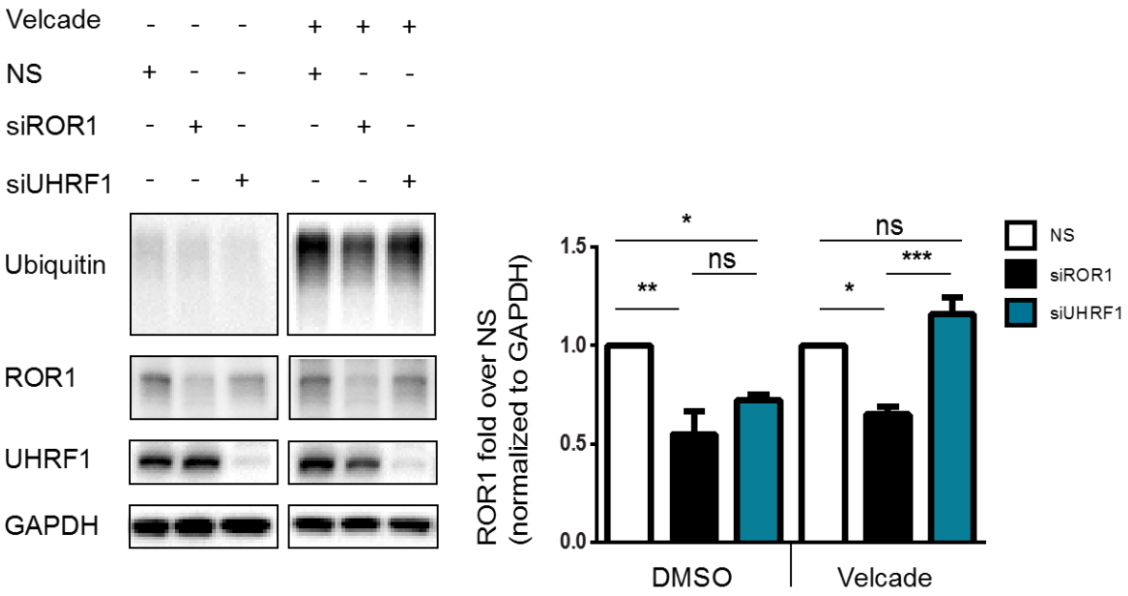


Figure 3-6 The proteasome is associated with UHRF1-mediated ROR1 protein levels in RCH-ACV cells.

RCH-ACV cells were treated with siRNA for 48 hours prior to treatment with DMSO or Velcade for 16 hours. Whole cell lysates were subjected to immunoblot. ROR1 protein was quantified and normalized to non-specific siRNA control treated with DMSO (*p<0.05, **p<0.005) or Velcade (*p<0.05, ***p<0.0005). UHRF1 protein was quantified and normalized to non-specific (NS) treated with DMSO (*p<0.05). Error bars represent S.E.M. “ns” = not significant.

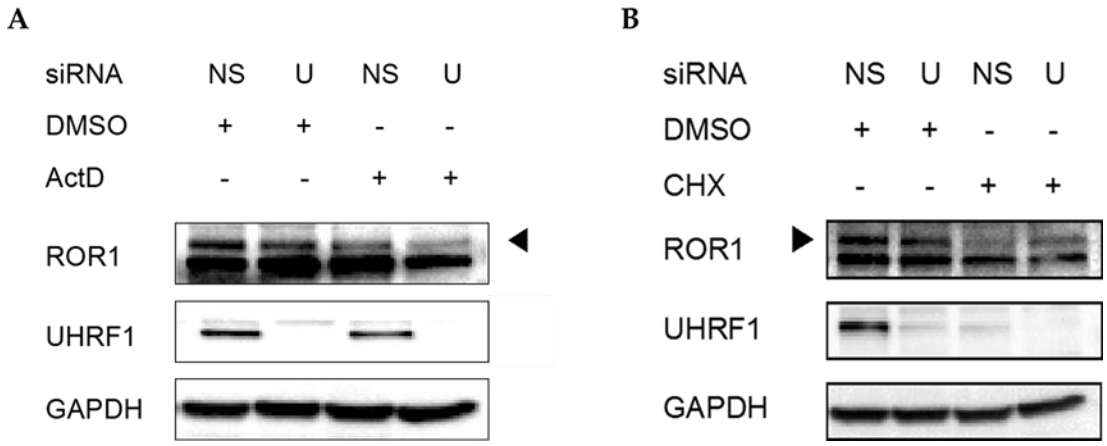


Figure 3-7 UHRF1-mediated ROR1 levels are not regulated at the transcriptional or translational level.

RCH-ACV cells were treated with siRNA for 48 hours before adding (**A**) 0.2 μM actinomycin D (ActD) for 8 hours or (**B**) 1 μg/mL cycloheximide (CHX) for 24 hours. Protein lysates were harvested for immunoblot. NS = non-specific siRNA. U = UHRF1 siRNA.

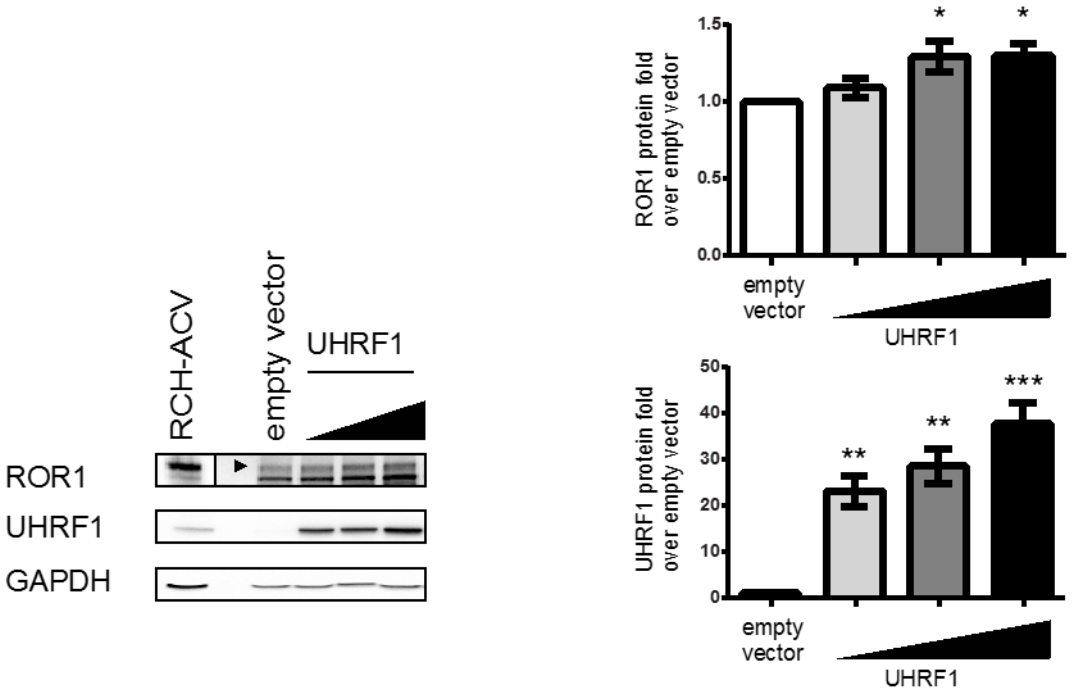


Figure 3-8 UHRF1 is sufficient to induce ROR1 expression.

HEK293T17 cells were transfected with either empty vector or WT *UHRF1*, and whole cell lysates were harvested 48 hours post-transfection. Levels of ROR1 (*$p<0.05$,) and UHRF1 (**$p<0.005$, ***$p<0.0005$) were detected by immunoblot (N=3) and normalized to empty vector. Error bars represent S.E.M.

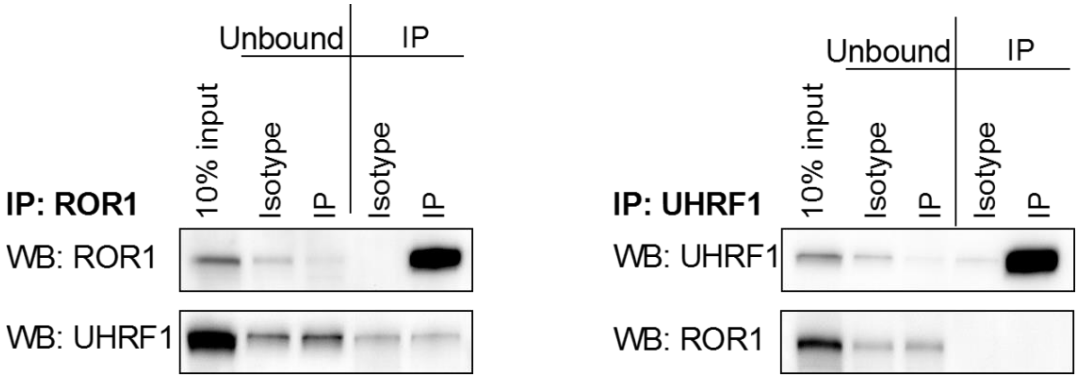


Figure 3-9 UHRF1 does not directly interact with ROR1.

RCH-ACV cell lysate was enriched for either ROR1 (left, IP: ROR1) or UHRF1 (right, IP: UHRF1) and tested for co-immunoprecipitation by immunoblot. Note that any UHRF1 signal from the ROR1 IP is also seen in the goat isotype control, suggesting that the signal is non-specific.

ectopically expressed wild-type ROR1 (**Figure 3-10**). Furthermore, these proteins localize to different subcellular spaces in RCH-ACV or RCH+ROR1-V5 cells as UHRF1 is abundant in the nucleus, while ROR1 is enriched in the cytoplasmic and membrane-bound fraction (**Figure 3-11**). Therefore, I conclude that UHRF1 regulates ROR1 indirectly.

3.3.4 *UHRF1 silencing sensitizes t(1;19) pre-B-ALL to dasatinib*

Since targeting of UHRF1 with siRNA led to reduced levels of ROR1 protein, I next investigated whether silencing of UHRF1 would cooperatively reduce t(1;19) cell viability when combined with inhibitors of pre-BCR signaling, as we had previously seen with direct ROR1 silencing (24). To address this, I silenced *UHRF1* expression before treating RCH-ACV cells with dasatinib, which inhibits SFKs and BTK that signal as part of the pre-BCR signaling complex. As expected, cells treated with non-specific siRNA responded to dasatinib in a dose-dependent manner. I also observed an enhanced loss of cell viability when *ROR1* expression was silenced in combination to dasatinib, which is consistent with previous studies (24). Similarly, silencing UHRF1 significantly reduced cell viability in the presence of dasatinib more than that observed with dasatinib in the presence of non-specific siRNA (**Figure 3-12**). These data demonstrate the potential of inhibiting UHRF1 in combination with dasatinib is comparable to targeting ROR1 directly in combination with the same kinase inhibitor to maximize loss of cell viability.

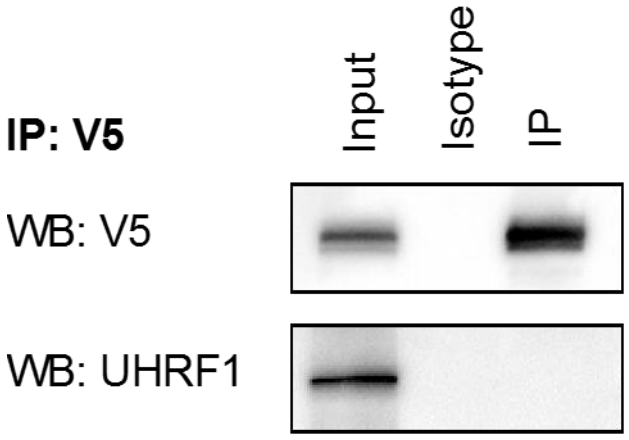


Figure 3-10 Ectopically expressed UHRF1 does not directly interact with ROR1.

HEK293T17 cells were transfected with plasmids encoding *ROR1* (V5-tagged) and *UHRF1*. Protein cell lysate was enriched using a V5 antibody and tested for co-immunoprecipitation by immunoblot.

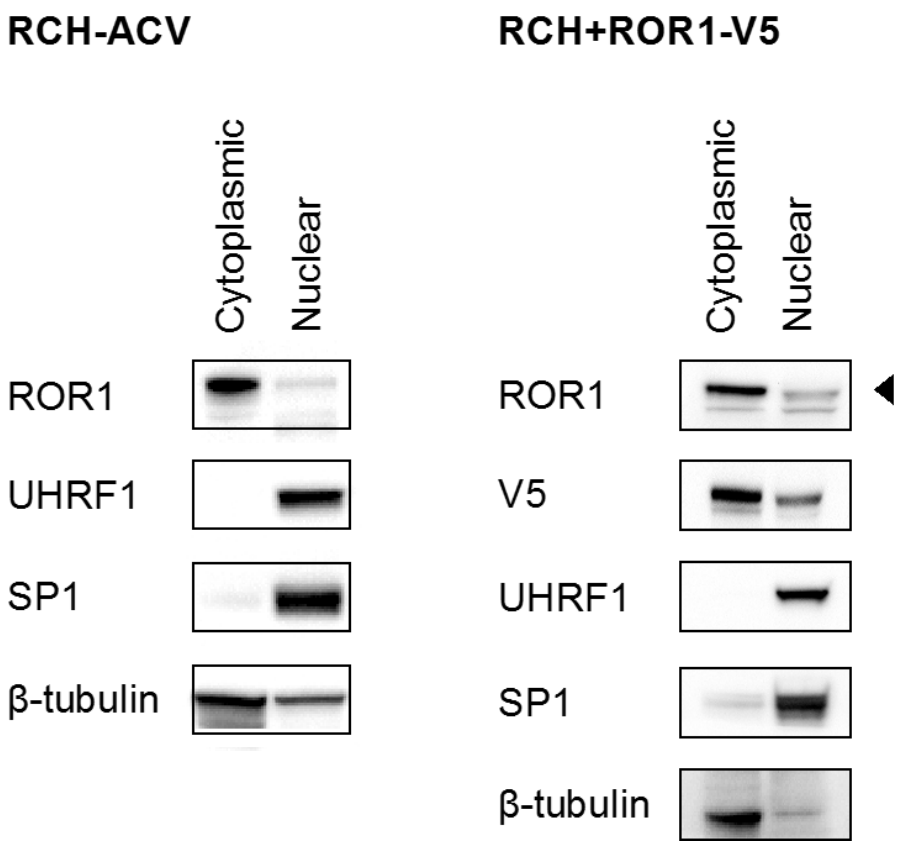


Figure 3-11 UHRF1 does not co-localize with ROR1.

RCH-ACV (left) and RCH+ROR1-V5 cells (right) were fractionated and localization of either ROR1 or UHRF1 was detected by immunoblot. "Cytoplasmic" fraction includes membrane-bound proteins. SP1 is a nuclear protein control and β-tubulin is a cytoplasmic control. Note that β-tubulin signal in the nuclear fraction is likely contamination between fractions.

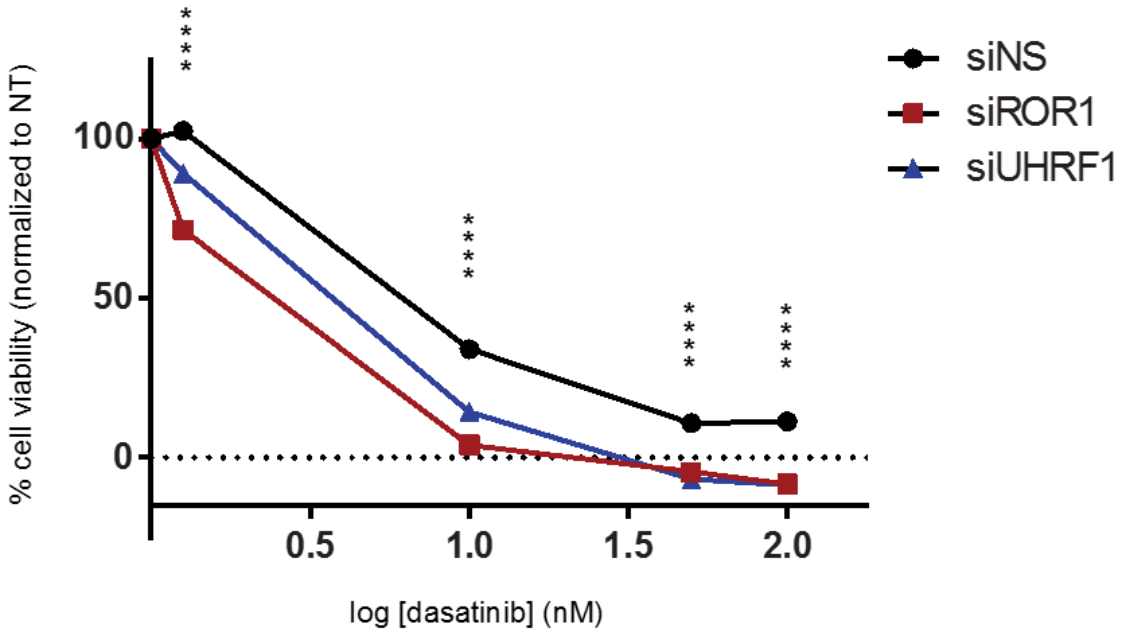


Figure 3-12 Silencing *UHRF1* expression sensitizes RCH-ACV cells to dasatinib.

RCH-ACV cells were treated with siRNA for 24 hours followed by exposure to dasatinib for 72 hours. Cell viability was measured by MTS assay and normalized to cells treated with siRNA only (NT = non-treated). Data shown is an average of three independent experiments (****p<0.0001). siNS = non-specific siRNA, Error bars represent S.E.M.

3.3.5 *Naphthazarin inhibits t(1;19) pre-B-ALL viability by downregulating the UHRF1-ROR1 axis*

To further investigate the potential of targeting UHRF1, I screened t(1;19) positive and negative cell lines against several putative UHRF1 small molecule compounds (163) and dasatinib. As expected, dasatinib was specifically effective against RCH-ACV cells. Of all other tested compounds, t(1;19) pre-B-ALL cells were twice as sensitive to the UHRF1 inhibitor naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) compared to REH cells, which lack the 1;19 translocation and do not express *ROR1* (**Figure 3-13**).

Naphthazarin has been reported to induce cell cycle arrest and apoptosis in lung and gastric cancers and reduce UHRF1 expression, sensitizing breast cancer to irradiation (197-199). To test the specificity of naphthazarin cytotoxicity on the UHRF1-ROR1 axis, cells were treated for up to 24 hours and levels of protein were measured. Within 24 hours of naphthazarin exposure, RCH-ACV cells showed reduced levels of UHRF1 and ROR1 protein, yet no significant change in mRNA (**Figure 3-14, Figure 3-15**). To further confirm that the effects of naphthazarin mimicked the phenotype I previously observed with UHRF1 silencing, I also treated RCH+ROR1-V5 cells with naphthazarin and observed a similar reduction in ectopically expressed ROR1. In contrast, this result was not observed in REH cells, which correlates with REH insensitivity to naphthazarin. Moreover, naphthazarin induced higher amounts of apoptosis in t(1;19)-positive but not negative cell lines, as demonstrated by Annexin-V

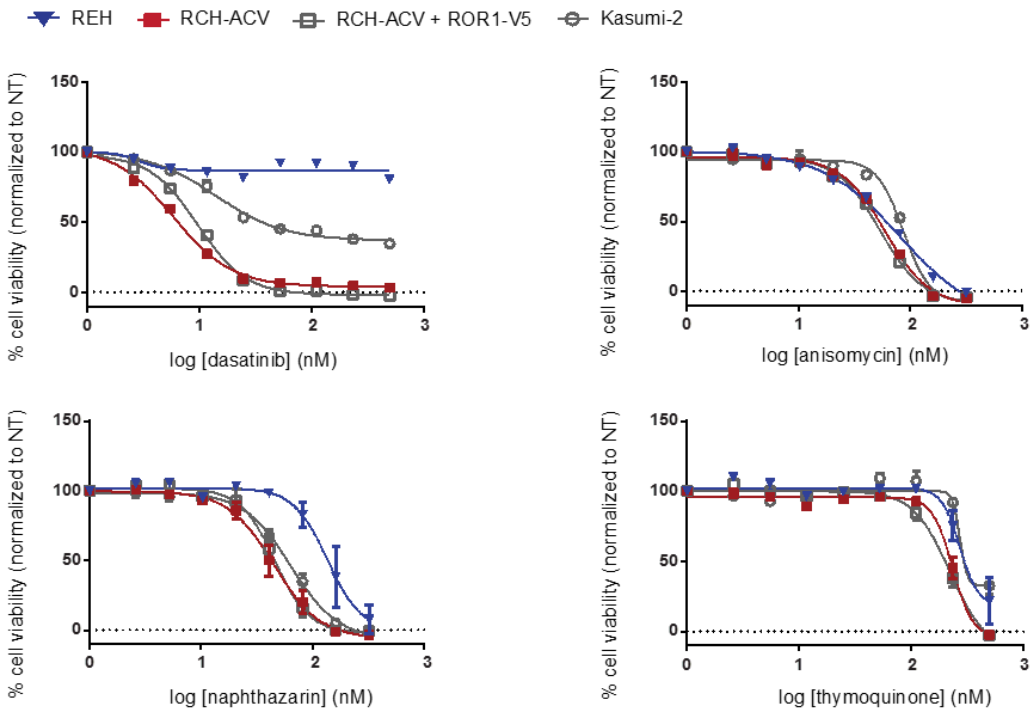


Figure 3-13 t(1;19) pre-B-ALL cells are selectively sensitive to naphthazarin.

Cell lines were treated with the indicated drugs (0-0.5 μM) for 72 hours and cell viability was measured by MTS assay. Cell viability was normalized to non-treated cells. Data shown is an average of three independent experiments. Error bars represent S.E.M.

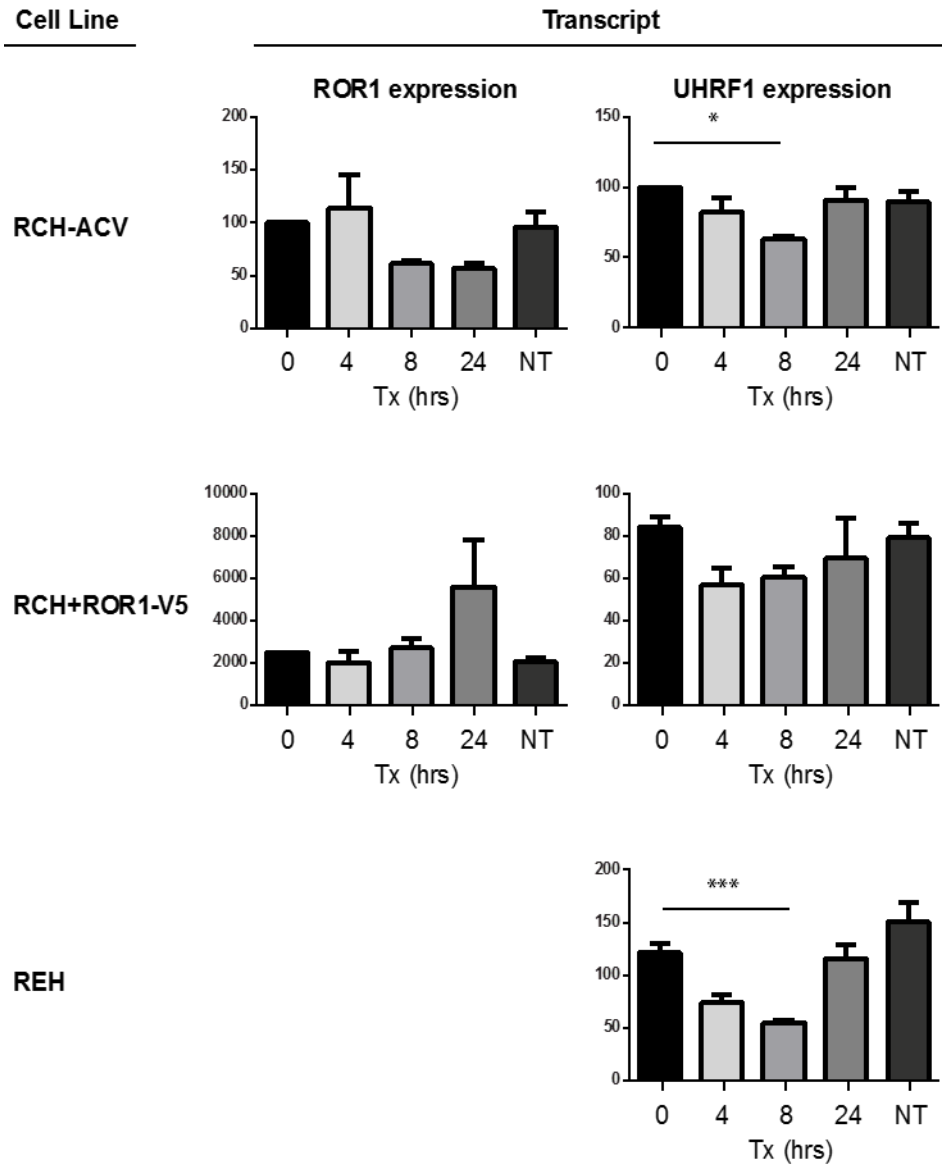


Figure 3-14 ROR1 and UHRF1 mRNA expression is not significantly affected by naphthazarin.

RCH-ACV, RCH+ROR1-V5, and REH cells were treated with naphthazarin for the indicated times. mRNA expression was measured by qRT-PCR and normalized to t=0 hours (*p<0.05, *** p<0.005). Data shown is an average of three independent experiments. NT = non-treated. Error bars represent S.E.M.

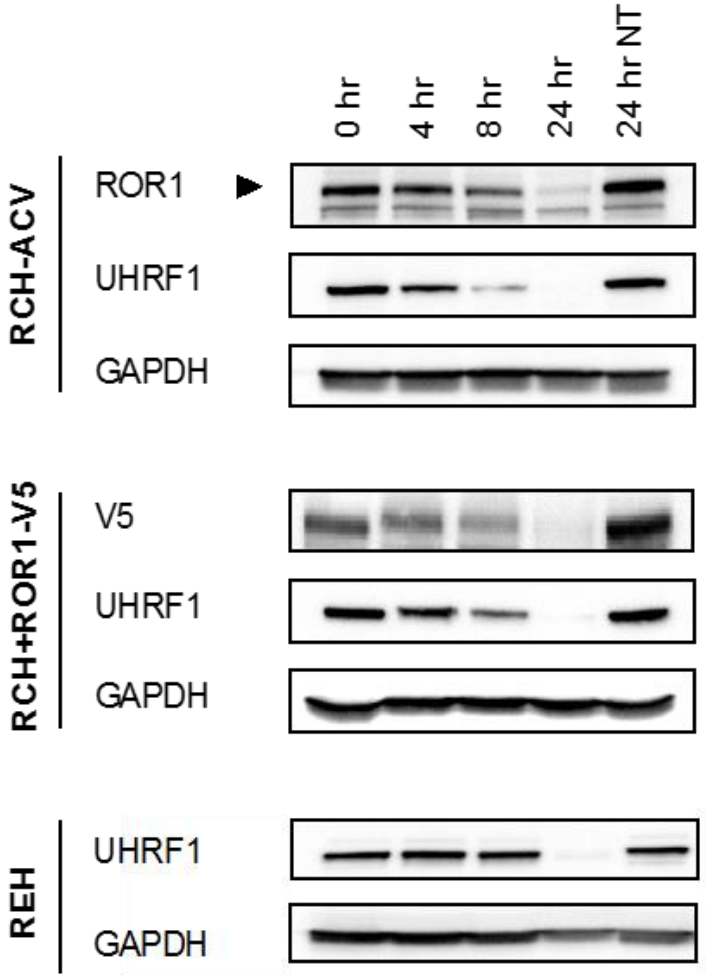


Figure 3-15 Naphthazarin reduces ROR1 and UHRF1 protein levels in t(1;19) pre-B-ALL.

RCH-ACV, RCH+ROR1-V5, and REH cells were treated with naphthazarin for the indicated times. Whole cell lysates were tested for ROR1 or UHRF1 by immunoblot. Data shown is an average of three independent experiments. NT = non-treated.

staining (**Figure 3-16**). To determine whether naphthazarin can cooperate with inhibition of the pre-B-cell receptor pathway via dasatinib, I measured effects of cell viability in the presence of a naphthazarin-dasatinib combination compared to either agent alone. RCH-ACV cells were sensitive to naphthazarin and dasatinib as single agents, but this effect is enhanced when these small molecule inhibitors are combined (**Figure 3-17**). Together, these data suggest that naphthazarin inhibits UHRF1-dependent protein levels of ROR1 and can be used in combination with dasatinib to maximize loss of t(1;19) pre-B-ALL cell viability.

3.4 Discussion

ROR1 is a pseudokinase that is required for the viability of t(1;19) pre-B-ALL and other hematologic and solid tumor malignancies. Despite ongoing efforts to utilize ROR1 for targeted therapies, there are no FDA-approved ROR1 small molecule inhibitors. To identify regulators of ROR1 expression that could be exploited to target ROR1, I screened 190 genes including transcription and chromatin modifying factors. Several candidates were generated including UHRF1. Sensitivity to UHRF1 siRNA was abrogated in RCH+ROR1-V5 cells, suggesting that UHRF1-mediated cell viability is associated with ROR1 expression and function. Follow-up siRNA experiments demonstrate that UHRF1 is critical for maintenance of levels of ROR1 protein, but not mRNA, indicating that UHRF1 regulates ROR1 through a post-transcriptional regulatory mechanism. Although I show that UHRF1 is sufficient to increase levels of ROR1, I have presented evidence suggesting that UHRF1 indirectly regulates ROR1 and

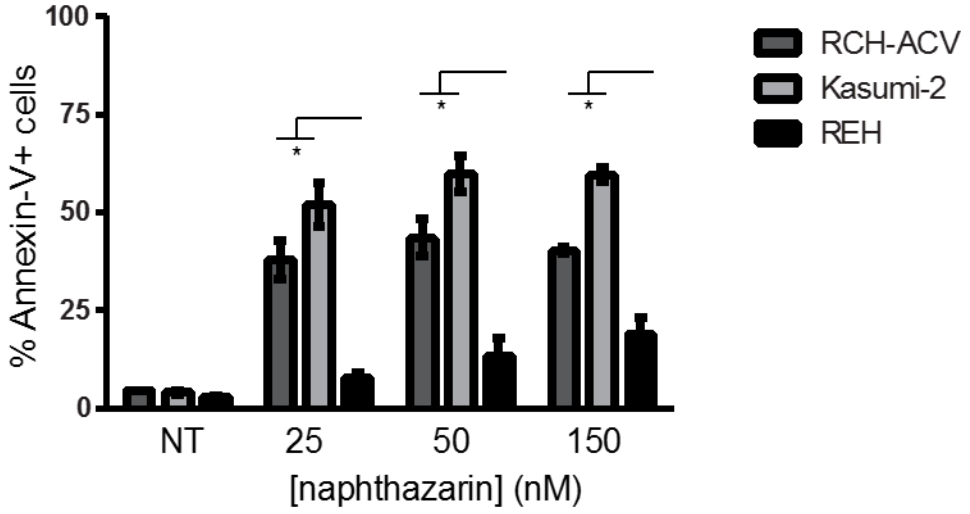


Figure 3-16 Naphthazarin induces apoptosis in t(1;19) pre-B-ALL.

Cells were treated with naphthazarin for 8 hours and Annexin-V was measured by GuavaNexin Assay. Annexin-V staining of RCH-ACV and Kasumi-2 cells was compared to REH cells for each treatment (*$p<0.05$). Data shown is an average of three independent experiments. NT = non-treated Error bars represent S.E.M.

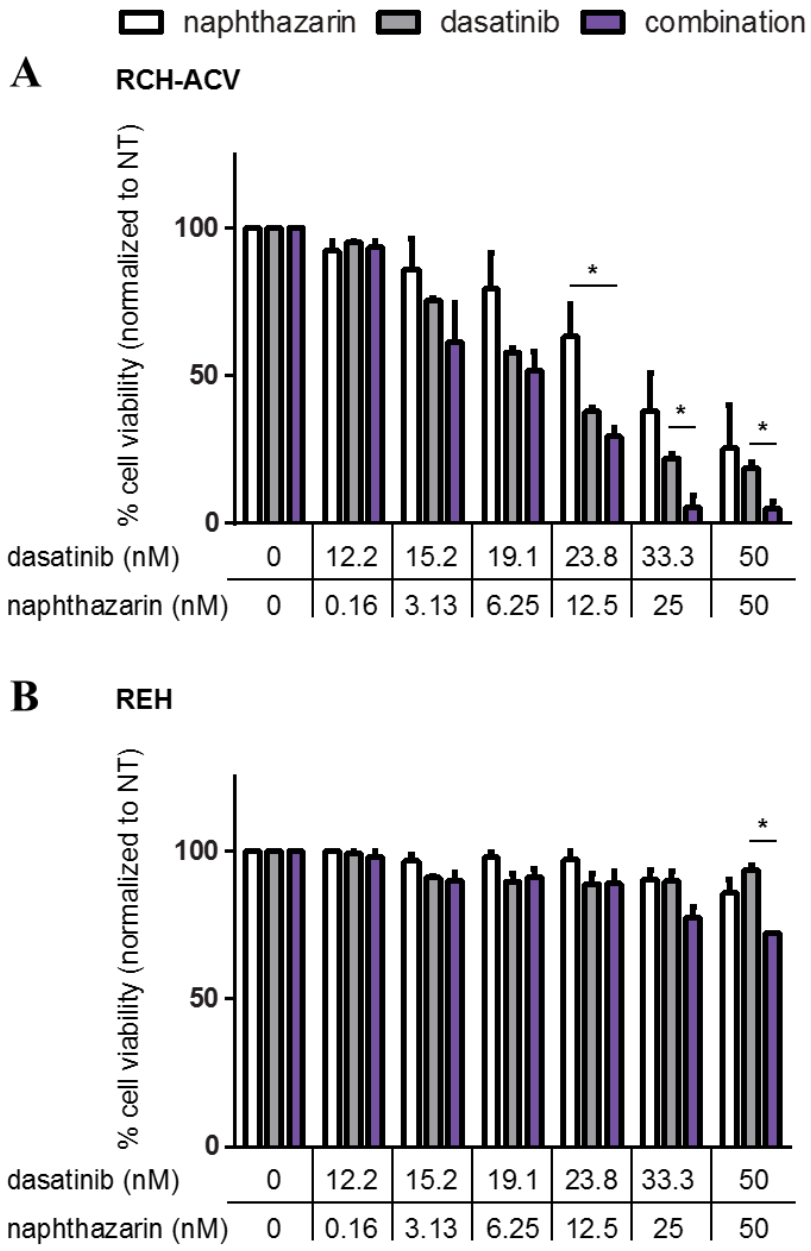


Figure 3-17 Naphthazarin can be combined with dasatinib to further reduce RCH-ACV cell survival.

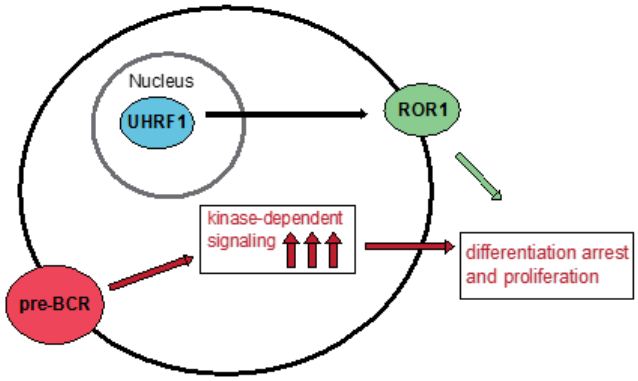
(A) RCH-ACV and **(B)** REH cells were treated with naphthazarin, dasatinib, or a combination of both for 72 hours. Cell viability was measured and normalized to non-treated cells (*$p<0.05$). Data shown is an average of three independent experiments. NT = non-treated Error bars represent S.E.M.

experiments to identify intermediaries of this mechanism will be essential for a complete understanding of the pathway.

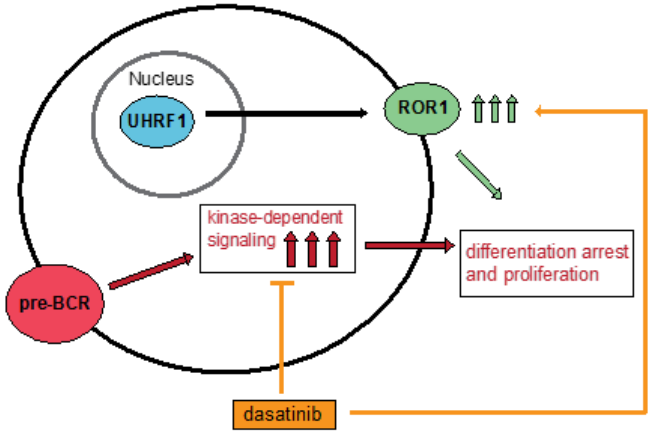
In addition to characterizing the UHRF1-ROR1 mechanism, I was interested in identifying agents that could target this pathway, since previous studies established the importance of ROR1 in t(1;19) pre-B-ALL and its ability to cross-talk with the pre-BCR (24). First, I have shown that silencing UHRF1 sensitizes t(1;19) pre-B-ALL to dasatinib to levels similar to that of ROR1 silencing, indicative of a potential therapeutic strategy through targeting of UHRF1. Next, I investigated candidate inhibitors of UHRF1 activity and demonstrated that naphthazarin leads to reduced levels of UHRF1 and ROR1 protein without significantly affecting levels of the *ROR1* transcript, phenocopying the effect of UHRF1 silencing on ROR1. Furthermore, naphthazarin induces significant apoptosis in t(1;19)-positive pre-B-ALL and can work in parallel with dasatinib to further reduce cell survival, suggesting that UHRF1-ROR1-pre-BCR combination therapy may be effective in treating t(1;19) pre-B-ALL (**Figure 3-18**). Together, these preclinical results provide opportunities to further investigate the mechanism by which the combination of dasatinib and naphthazarin suppress t(1;19) pre-B-ALL viability in future *in vitro* and *in vivo* studies.

Although UHRF1 was prioritized after the results of the screen with RCH+ROR1-V5 cells, other candidates are being investigated for their roles in maintaining t(1;19) pre-B-ALL through ROR1-independent mechanisms. In addition to RUNX1, RhoH and KAT7 were required for t(1;19) pre-B-ALL viability. RhoH is a hematopoietic

t(1;19) pre-B-ALL



t(1;19) pre-B-ALL + pre-BCR inhibition



t(1;19) pre-B-ALL + pre-BCR-UHRF1-ROR1 combinatorial inhibition

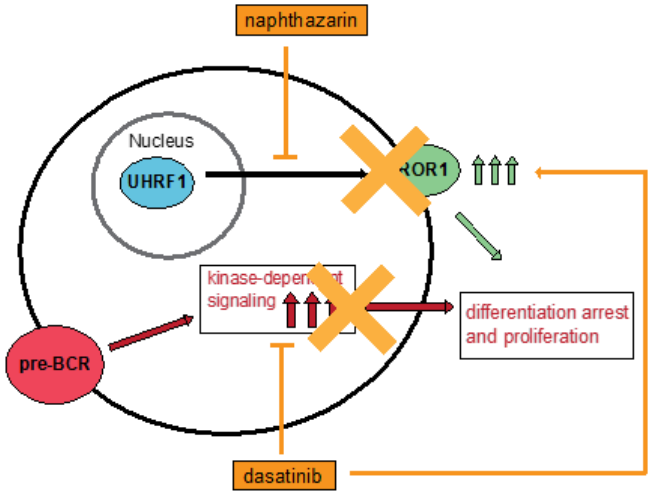


Figure 3-18 Model of UHRF1-ROR1 regulatory mechanism.

The translocation between chromosomes 1 and 19 leads to cell arrest and constitutively active pre-BCR signaling (top). This signaling can be suppressed by small molecule inhibitors such as dasatinib, but kinase inhibition is rescued by increased ROR1 expression (middle). Targeting the UHRF1-ROR1 mechanism with compounds such as naphthazarin can suppress baseline ROR1 levels. In combination with pre-BCR inhibition (via dasatinib), naphthazarin helps to prevent upregulation of ROR1 to maximize t(1;19) pre B-ALL apoptotic cell death (bottom).

specific member of the RhoH GTP-ase family and has roles in promoting CLL through cell intrinsic mechanisms and interactions with the microenvironment (200, 201). KAT7, also known as HBO1 and MYST2, is a lysine acetyltransferase that is critical for H3K14 and histone H4 acetylation during normal embryonic development and plays a role in DNA replication and cell cycle progression (202, 203). However, the roles of RhoH and KAT7 in t(1;19) pre-B-ALL have yet to be elucidated.

Collectively, this work suggests a new regulatory mechanism in which UHRF1 is necessary for maintaining the levels of ROR1 protein. When this mechanism is inhibited by siRNA or naphthazarin, the UHRF1-dependent loss of ROR1 leads to reduced t(1;19) pre-B-ALL viability. UHRF1 has been well-established as an oncogene that impairs the expression of various tumor suppressor genes (163), yet here I show that UHRF1 regulates ROR1, a tumor-promoter. Thus, my findings propose that targeting ROR1 through UHRF1 inhibition, potentially in combination with dasatinib, is a promising novel therapeutic strategy for t(1;19) pre B-ALL and other ROR1-expressing cancers.

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Authorship Contributions

M.C. designed and performed research, analyzed data, and wrote the manuscript. L.G., B.H.C., J.J.A., and J.W.T. designed research, analyzed data, and edited the manuscript. V.T.B. analyzed data and edited the manuscript. J.D.M. performed experiments.

Conflicts of Interest

J.W.T. receives research support from Agios Pharmaceuticals, Array Biopharma, Aptose Biosciences, AstraZeneca, Constellation Pharmaceuticals, Genentech, Gilead, Incyte Corporation, Janssen Pharmaceutica, Seattle Genetics, Syros, Takeda Pharmaceutical Company and is a consultant for Leap Oncology. All other authors declare no conflicts of interest.

4 ROR1 activates AKT signaling through interactions with mTORC1/2

4.1 Abstract

ROR1 is required for AKT activation and t(1;19) pre-B-ALL viability, yet the mechanistic details behind ROR1-dependent signaling are unclear. This is especially confounding since ROR1 lacks intrinsic kinase activity in this subtype of leukemia. Interestingly, ROR1 is required for phosphorylation of AKT on serine 473, which is directly added by the multi-subunit protein complex mTORC2. Therefore, I hypothesized that ROR1 interacts with members of mTORC2, including an essential component, Rictor. Surprisingly, the data presented here indicates an interaction between ROR1 and members of both mTOR complexes, but ROR1 may preferentially associate with Rictor and mTORC2. I also demonstrate that ROR1 is required for the Rictor protein levels and is upregulated upon siRNA-mediated knockdown of Rictor. Finally, I show that in response to dasatinib, partial rescue of AKT activation corresponds to an increase of ROR1 and Rictor protein levels, while Raptor protein levels are reduced. Taken together, these data suggest a role for ROR1 in AKT activation through dynamic interactions with mTORC1/2.

4.2 Background

4.2.1 *mTOR activity forms two different multi-subunit complexes that are required for cell growth, proliferation, and survival*

Mammalian target of rapamycin (mTOR), a serine/threonine kinase, is essential for normal development; knockout mice are embryonic lethal (204, 205). As the name

suggests, the macrolide drug rapamycin is allosteric inhibitor of mTOR and also an immunosuppressant (206-208). mTOR is most sensitive to rapamycin when mTOR is associated in the protein complex, mTORC1 (209, 210). The assembly of this complex requires the non-enzymatic protein, Raptor for mTORC1 activity (209, 210). mTORC1 facilitates cell growth and protein translation through activation of its downstream targets, including ribosomal protein S6 kinase and eukaryotic translation initiation factor 4E binding protein 1 (211). Because mTORC1 mediates metabolic and growth pathways, it is not surprising that it is affected by cellular stress, including hypoxia and DNA damage, many of which suppress mTORC1 activity through the TSC1/2 complex and p53 (212, 213). Downregulation of mTORC1 activity includes upregulation of kinase-dependent cell survival pathways, such as AKT and MAPK (214-218) (**Figure 4-1**).

In response to cellular stress mTORC1 activity is reduced in exchange for increased signaling through kinases such as AKT, which is mediated by a second mTOR complex, mTORC2. mTORC2 activates AKT by phosphorylating serine 473—a key residue required for optimal AKT activity (219). Unlike mTORC1, mTORC2 activity is more resistant to rapamycin but can also be inhibited with long-term treatment (220). mTORC2 assembly and function also requires a protein subunit Rictor (221, 222) (**Figure 4-1**). Interestingly, Rictor mouse knockout studies demonstrate that it is required for the maintenance of normal, mature B cells but not immature B cells in the bone marrow (223). Moreover, when *Rictor* expression is lost, several pre-BCR and mTOR-mediated pathways are suppressed, including AKT and NFκB (223).

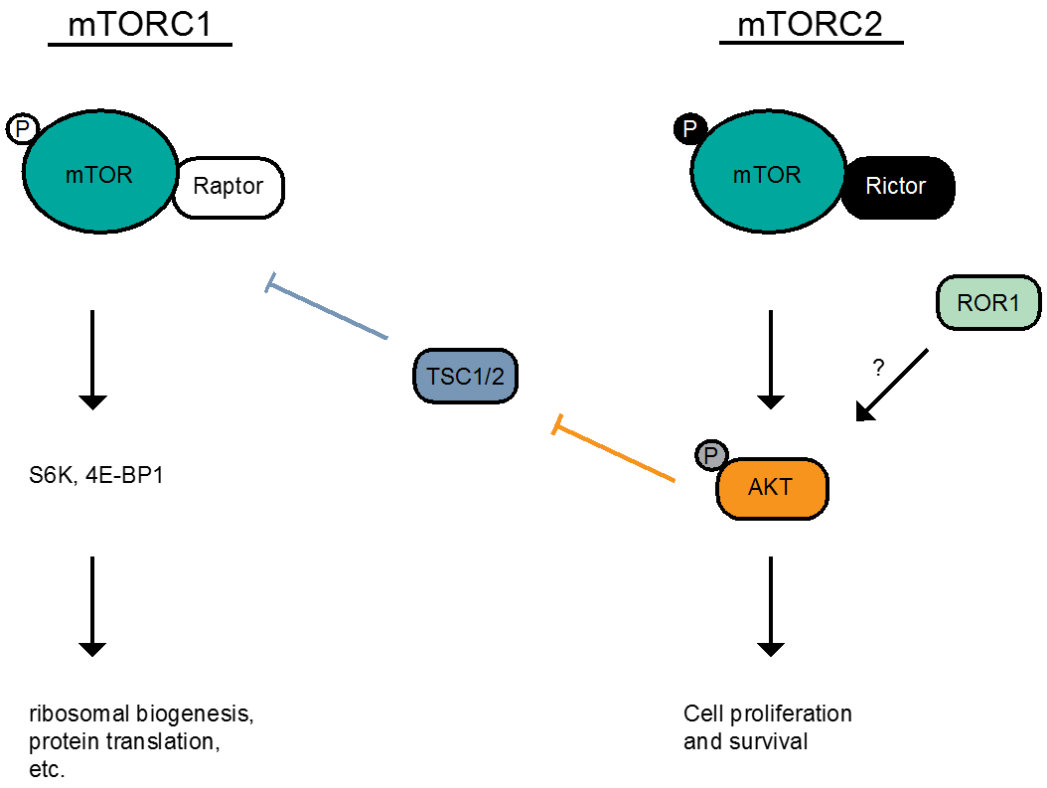

Figure 4-1 Schematic of mTOR complex signaling and the role of ROR1 in AKT addressed in this dissertation.

mTOR is a serine/threonine kinase that is regulated by phosphorylation and formation of different multi-subunit complexes. For simplicity, only one additional subunit is featured here. mTORC1 is assembled with proteins such as Raptor to promote cell growth. mTOR bound to Rictor forms mTORC2, which promotes cell proliferation and survival through downstream kinase signaling, including phosphorylation of serine 473 on AKT. As a result, activated AKT can induce mTORC1 activity by inhibiting TSC1/2. ROR1 is also required for AKT activation at serine 473 in t(1;19) pre-B-ALL (24) but through an unknown mechanism that is investigated in this dissertation. Adapted from Liu *et al* (2009) (224).

4.2.2 *Targeting mTORC1/2 activity is a promising therapeutic strategy*

mTORC1/2 activity mediates key cellular processes, including cellular metabolism, proliferation, and survival—all of which are dysregulated in cancer (67). Interestingly, there are no reports of mutations in AKT or mTOR in childhood cancers, but this is likely due to mutations in the mTOR-AKT mediators PI3K and PTEN (225). In addition to targeting these mediators, mTOR inhibitors are currently in development, especially since mTOR was originally studied in the context of inhibition by rapamycin (225). Indeed, rapamycin analogs (rapalogs) are under intense investigation for their efficacy in cancer, including Temsirolimus, which was approved in 2007 by the FDA to treat renal cell cancer (226). Thus, targeting mTOR signaling is an attractive therapeutic treatment, but the role of mTORC1/2 has not been fully elucidated in t(1;19) pre-B-ALL.

4.2.3 *ROR1 mediates AKT activation through an unknown mechanism*

In t(1;19) pre-B-ALL, siRNA studies suggest that ROR1 is required for AKT activation, specifically phosphorylation on the serine 473 residue (24). Furthermore, dasatinib-dependent ROR1 upregulation correlates with a partial rescue of phosphorylated AKT (24). These data indicate that ROR1 plays a role in rescuing kinase signaling downstream of the pre-B cell receptor in response to dasatinib (24). However, the mechanism behind ROR1-dependent AKT activation remains unclear. Since ROR1 lacks intrinsic kinase activity, it is possible that ROR1 may interact with functional kinases to promote AKT signaling. Therefore, I hypothesized that ROR1 interacts with mTORC2, which is required for AKT phosphorylation and activation. In this Chapter, I

present and discuss data suggesting that ROR1 interacts with Rictor to activate AKT through mTORC2.

4.3 **Results**

4.3.1 *Endogenous ROR1 is associated with mTOR complexes*

We and others have previously demonstrated the critical role that ROR1 plays in activating AKT in both solid tumors and hematologic malignancies (24, 114, 186). Furthermore, we have shown that serine 473, but not threonine 308, on AKT is important for t(1;19) pre-B-ALL survival (24). However, the details of this mechanism remain unclear.

I hypothesized that ROR1 interacts with mTORC2, which directly phosphorylates serine 473 on AKT. To test this, I performed a co-immunoprecipitation of endogenous ROR1 from RCH-ACV cells to detect any associations with the mTORC2-specific member, Rictor. Indeed, Rictor successfully co-immunoprecipitated with ROR1 (**Figure 4-2**). Interestingly, the mTORC1-specific protein Raptor was also detected with ROR1, albeit at lower levels (**Figure 4-2**). These results suggest that ROR1 is associated with members of the mTOR complexes, but there may be preferential interactions with mTORC2 through Rictor.

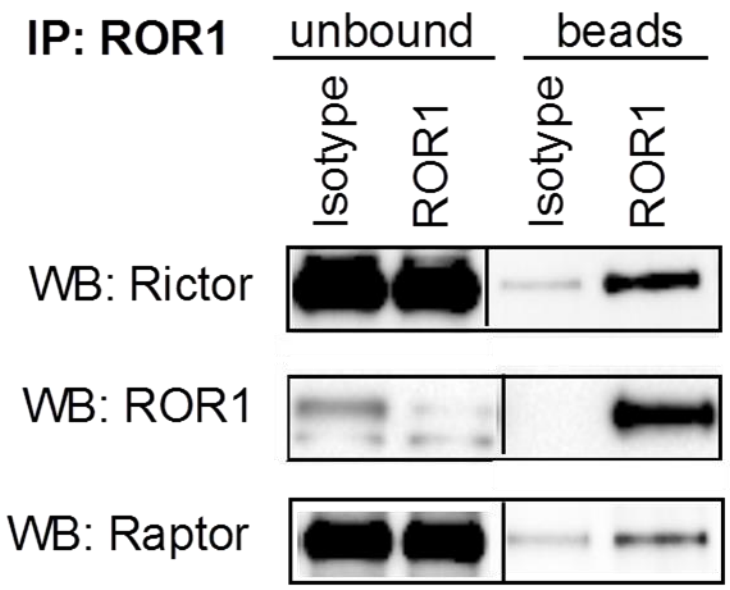

Figure 4-2 Endogenous ROR1 preferentially associates with the mTORC2 complex.

RCH-ACV cells were enriched for ROR1 and lysates were tested for co-immunoprecipitation with Rictor or Raptor. Note that signals from ROR1 IP ("beads") are higher than the signal from the goat isotype control. (N=2)

4.3.2 *ROR1 is required for Rictor expression*

After observing an association between ROR1 and Rictor, it became apparent that ROR1 may be functionally important for Rictor protein levels, similarly to AKT. To test this, I silenced the expression of either *ROR1* or *Rictor* in the t(1;19) pre-B-ALL cell line, RCH-ACV, and measured changes in cell viability and protein levels. As expected, silencing ROR1 reduced RCH-ACV cell viability and more importantly, I observed a reduction of Rictor protein (**Figure 4-3**). In contrast when Rictor expression is silenced by siRNA, cell viability is also reduced but ROR1 levels increase (**Figure 4-4**). I also observed a loss of phosphorylated mTOR on the serine 2481, which indicates mTORC2-specific activity (227). Together, these data indicate that ROR1 is required for Rictor expression and mTORC2 levels in t(1;19) pre-B-ALL.

4.3.3 *mTORC2 expression correlates with upregulation of ROR1 in response to dasatinib*

ROR1 expression increases in response to pre-B cell receptor inhibition via dasatinib, correlating with a partial rescue of phosphorylated AKT over 24 hours (24). Therefore, I evaluated the effects of dasatinib on mTORC1/2 subunit protein levels. To do this, I treated RCH-ACV cells with 100 nM dasatinib for up to 24 hours and measured the levels of Rictor and Raptor. As ROR1 proteins levels increase, Rictor is also upregulated and correlate with the partial rescue of phosphorylated AKT 24 hours after the addition of dasatinib. In contrast, Raptor levels slightly decrease in response to dasatinib (**Figure 4-5**). Collectively, these data suggest that in response to pre-BCR

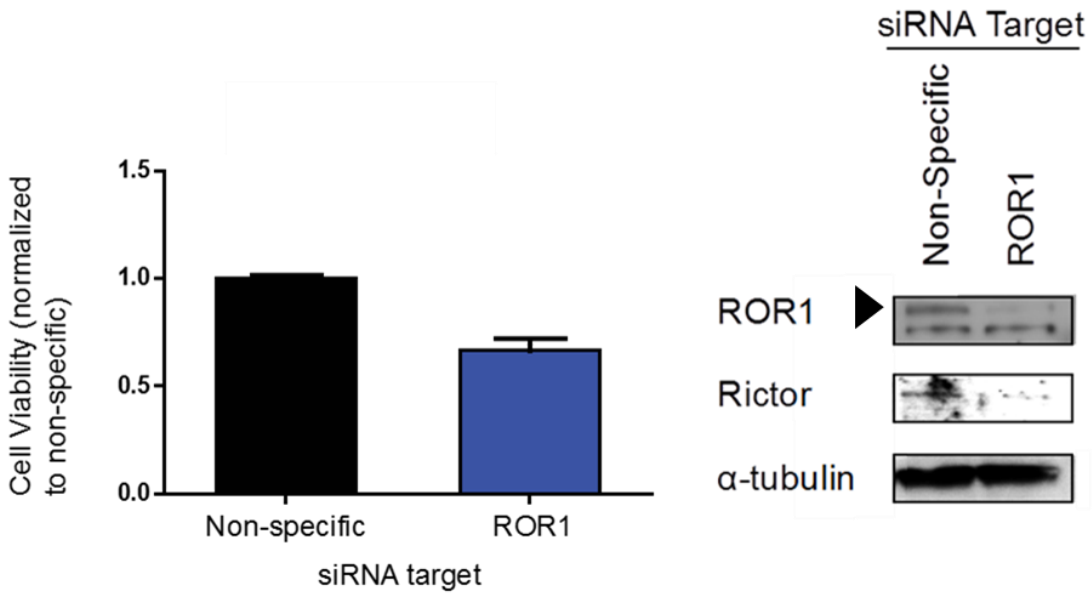

Figure 4-3 ROR1 is required for Rictor protein levels.

RCH-ACV cells were electroporated with a siRNA targeting *ROR1*. 96 hours later, cell viability was measured by MTS assay and values were normalized to non-specific siRNA (left). 72 hours post-electroporation, cell lysates were harvested and protein expression was measured by immunoblot (right). (N=2)

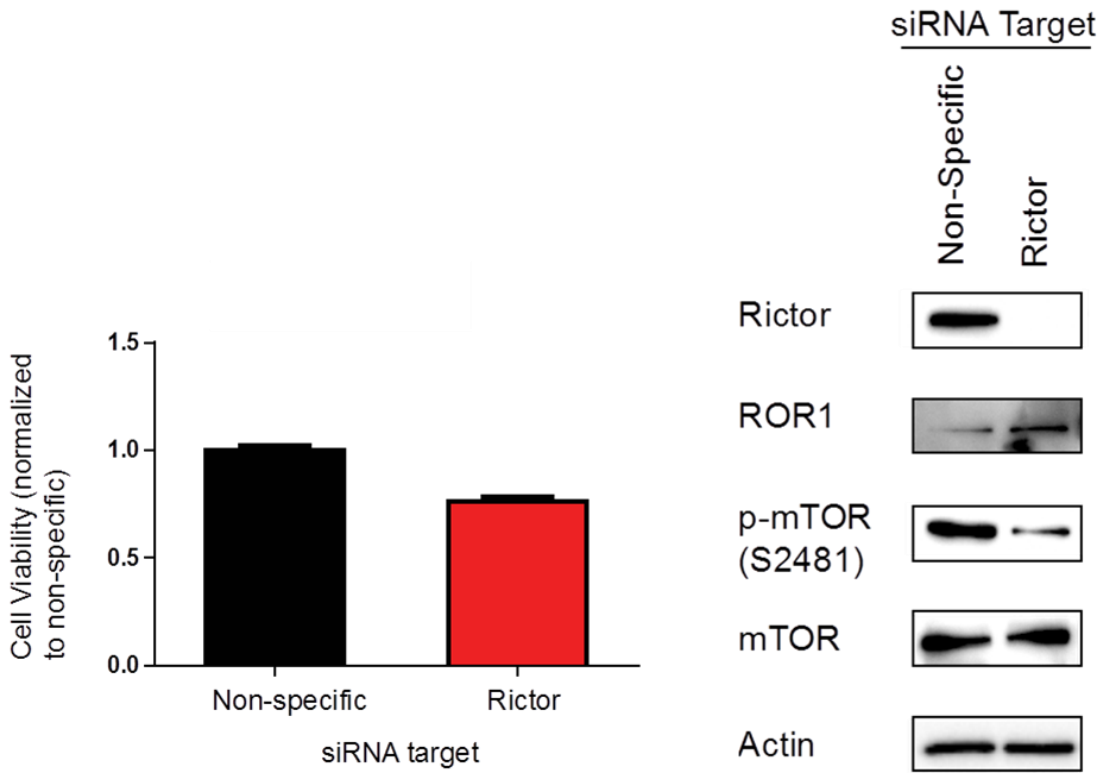

Figure 4-4 ROR1 is upregulated in response to Rictor silencing.

RCH-ACV cells were electroporated with siRNA targeting *Rictor*. 96 hours later, cell viability was measured by MTS assay and values were normalized to non-specific siRNA (left). 72 hours post-electroporation, cell lysates were harvested and protein expression was measured by immunoblot (right). (N=2)

inhibition, upregulation of ROR1 correlates with an increase of Rictor and downregulation of Raptor.

1.1 Discussion

In this Chapter, I presented data that addresses the role of ROR1 in AKT activation through interactions with mTORC2. My findings suggest that endogenous ROR1 is associated with mTORC1 and mTORC2, but there may be a preferential interaction with mTORC2 through Rictor (**Figure 4-6 A**). This observation is further corroborated by siRNA experiments that indicate that ROR1 is required for Rictor—and likely mTORC2—expression. Of note, ROR1 was upregulated in response to siRNA-mediated knockdown of *Rictor*, further illustrating the importance of ROR1 in mTORC2 signaling. However, the effects on Raptor and mTORC1 after siRNA-mediated knockdown of *ROR1* need to be determined.

Pre-BCR inhibition with dasatinib leads to upregulation of ROR1 and partial re-activation of AKT (23). Here, I show that Rictor levels negatively correlate with those of Raptor in response to dasatinib. This suggests that ROR1 mediates mTORC2-dependent activation of AKT while suppressing mTORC1 activity in response to dasatinib (**Figure 4-6 B**). This may be more conclusive if known mTORC1 signaling targets, such as ribosomal protein S6 kinase and eukaryotic translation initiation factor 4E binding protein 1, are tested in response to dasatinib. Furthermore, it would be interesting to

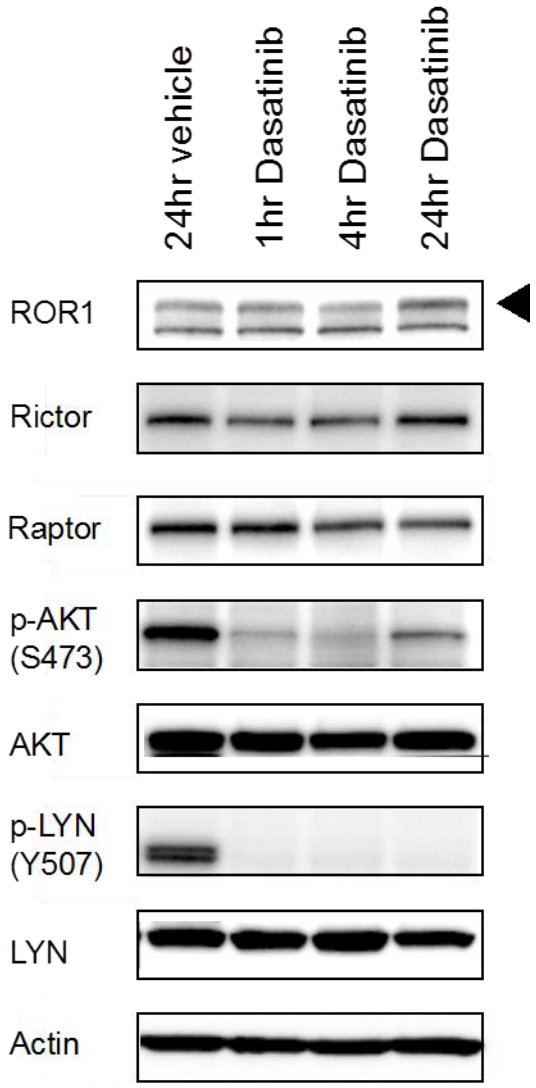

Figure 4-5 Rictor expression is upregulated with dasatinib.

RCH-ACV cells were treated with 100 nM dasatinib for up to 24 hours and cell lysates were harvested for immunoblot. DMSO was used as the vehicle control. (N=2)

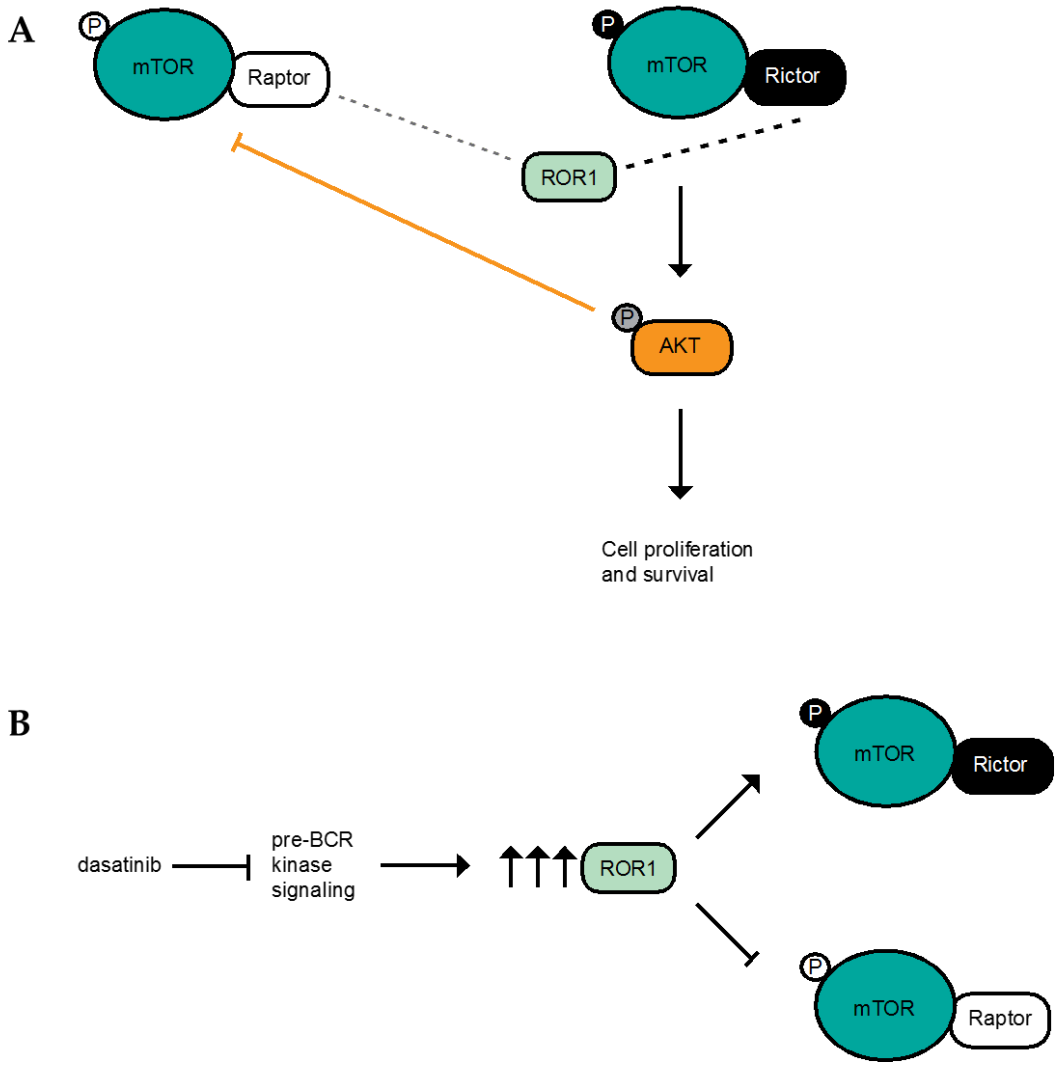

Figure 4-6 Model of ROR1-dependent AKT activation in t(1;19) pre-B-ALL.

(A) ROR1 interacts with both mTOR complexes through Rictor and Raptor, with preference for Rictor. **(B)** However, in response to dasatinib, *ROR1* expression is upregulated (24) and leads to an increase in Rictor levels to rescue AKT activity through mTORC2 signaling. This rescue correlates with a reduction of Raptor levels and may also result in lower mTORC1 activity. Therefore, ROR1-dependent AKT signaling allows t(1;19) pre-B-ALL cells to survive inhibition of pre-BCR kinase signaling.

investigate the mechanism driving the transition between mTORC1 and 2. This may be tested by measuring changes in interactions with endogenous or ectopically expressed *ROR1*, *Rictor*, and *Raptor* in response to dasatinib treatment. Given that ROR1 is a pseudokinase, it is also important to identify the specific regions on ROR1 required for interactions with Rictor or Raptor. These experiments will not only address what domains of ROR1 are required for t(1;19) pre-B-ALL cell viability, but whether or not ROR1 acts as a scaffold to interact with kinase complexes, including mTORC1/2. Plasmids encoding *ROR1* domain mutants and *Rictor* have been generated and are ready to utilize for these studies.

Finally, it is important to note that endogenous interactions do not show all Rictor or Raptor associated with immunoprecipitated ROR1 (**Figure 4-2**)[8]. This suggests that these interactions may not be necessary to be at high levels to have a biological effect (and may change in response to a perturbation like dasatinib) or these proteins have additional roles in the cell. Rictor, for example, is phosphorylated on the Threonine 1135 residue, which appears to not have an effect mTORC2 activity but is mediated by the mTORC1 effector ribosomal protein S6 kinase 1 (228, 229). Therefore, it will be important to further elucidate the roles of ROR1 and mTOR complexes to better inform the potential effects of mTOR-AKT therapeutic strategies in t(1;19) pre-B-ALL.

[8] Assuming no technical issues that prevented full depletion of Rictor or Raptor during the immunoprecipitation.

5 Conclusions and Future Directions

5.1 UHRF1 regulates ROR1 in t(1;19) pre-B-ALL

Previously, our lab reported that ROR1 is required for t(1;19) pre-B-ALL survival (24), but the lack of ROR1 kinase activity presented a challenge to target it directly with inhibitors. Therefore, I sought to target ROR1 by identifying and inhibiting proteins required for *ROR1* expression. Using an siRNA screen, I found that UHRF1 is important for t(1;19) pre-B-ALL cell viability and a candidate regulator of ROR1 (**Figure 3-1**, **Figure 3-2**). In this dissertation, I hypothesized that UHRF1 was required for *ROR1* expression in t(1;19) pre-B-ALL.

To characterize the role of UHRF1 in *ROR1* expression, I used biochemical and pharmacological experimental approaches. I found that UHRF1 was required for ROR1 protein, but not mRNA, expression (**Figure 3-3**, **Figure 3-4**). This observation was confirmed by my subsequent work using inhibitors to block transcription, translation, and proteasome activity, demonstrating that UHRF1 post-transcriptionally regulates ROR1 (**Figure 3-6, Figure 3-7**). To complement the siRNA studies, I determined that ectopic expression of wild-type *UHRF1* is sufficient to increase ROR1 protein levels (**Figure 3-8**). This data further illustrated that UHRF1 is functionally important to maintain levels of ROR1. Current studies by other groups have merely focused on the regulation of the *ROR1* transcript (Section 1.3.8). Yet here, I have presented data that

describes the first report of a post-transcriptional mechanism that maintains ROR1 protein levels.

While the mechanism behind UHRF1 regulation of ROR1 is not fully elucidated, I have demonstrated that inhibiting UHRF1 significantly reduces t(1;19) pre-B-ALL cell viability and can increase sensitivity to dasatinib, which inhibits pre-BCR effector kinases (**Figure 3-12**). Moreover, UHRF1 and ROR1 expression is reduced after naphthazarin treatment (**Figure 3-14**, **Figure 3-15**), although changes in mRNA expression are not statistically significant. Consistent with the idea that UHRF1 regulates ROR1 protein levels, naphthazarin dramatically reduced levels of UHRF1 and ROR1 (**Figure 3-15**). These results demonstrate that biochemical and pharmacological inhibition of UHRF1 downregulates ROR1 protein levels, inhibiting t(1;19) pre-B-ALL survival.

Although naphthazarin inhibits t(1;19) pre-B-ALL, these data do not consider the effects of this small molecule in combination with pre-BCR inhibition. Dasatinib, like naphthazarin, is very effective against t(1;19) pre-B-ALL, but some cells survive kinase inhibition through upregulation of *ROR1* expression (24). The role of UHRF1 in dasatinib-dependent effects on ROR1 have yet to be fully elucidated. In Chapter 3, I show that naphthazarin reduces t(1;19) pre-B-ALL viability, and this effect is maximized in the presence of dasatinib (**Figure 3-17**). However, the specific effects on UHRF1 and ROR1 in response to combination treatment need to be investigated further. Our group has previously shown that dasatinib upregulates ROR1 mRNA and protein levels (24).

This result suggests that UHRF1 either regulates only mature ROR1 protein or can also regulate newly synthesized ROR1 protein. Testing naphthazarin and dasatinib sequentially may clarify the mechanism by which UHRF1 maintains ROR1 in addition to further elucidating the mechanism of action of this inhibitor combination on t(1;19) pre-B-ALL.

5.2 Characterization of the mechanism by which UHRF1 maintains levels of ROR1 protein in t(1;19) pre-B-ALL

Although I show that UHRF1 is required to maintain levels of ROR1 protein in t(1;19) pre-B-ALL, this phenomenon does not involve a physical association between these proteins (**Figure 3-9**, **Figure 3-10**, **Figure 3-11**). These data suggest that UHRF1 indirectly regulates ROR1. Identifying the UHRF1 target that affects ROR1 would, in turn, be a new opportunity to target ROR1 more directly than through UHRF1 in t(1;19) pre-B-ALL. Since I observed that UHRF1 and ROR1 do not co-localize (**Figure 3-11**), I hypothesize that this intermediate may translocate from the nucleus towards the plasma membrane to affect mature ROR1. This intermediary could either be a transcript that is expressed in the nucleus or a protein that is directly modified by UHRF1.

Since UHRF1 has two major functions in the cell—gene expression and protein stability—the intermediate substrate, which is directly regulated by UHRF1, may be a transcript or a protein that subsequently maintains ROR1 protein levels. mRNA targets could be identified through RNA-seq analysis of cells treated with UHRF1 siRNA compared to non-targeting siRNA. Similarly, candidate UHRF1 protein substrates may

be identified using mass spectrometry of siRNA-treated cells. To date, no prior investigations have identified UHRF1 targets in ALL.

Since UHRF1 and ROR1 do not physically associate with each other, it is also important to consider the possibility that changes in ROR1 protein is an off-target effect of overexpression or siRNA gene silencing. To confirm that UHRF1 specifically regulates ROR1, wild-type *UHRF1* can be ectopically expressed in the presence of silencing endogenous *UHRF1* expression. This experiment would address whether or not loss of ROR1 protein levels after UHRF1 silencing is compensated by simultaneous overexpression of wild-type UHRF1. Therefore, I hypothesize that ectopic expression of wild-type *UHRF1* can rescue t(1;19) pre-B-ALL cell viability and levels of ROR1 protein. If ROR1 protein is not rescued by ectopic expression of UHRF1, then this would indicate that UHRF1 does not specifically regulate ROR1. Additional studies using UHRF1 mutants—either lacking a domain or carrying point mutations—may determine the functions and regions of UHRF1 that are required for ROR1 protein levels. Plasmids encoding wild-type and mutant *UHRF1* and an siRNA that recognizes the 3′ UTR of *UHRF1* to silence endogenous expression are available for these experiments.

5.3 **Mechanism of naphthazarin in t(1;19) pre-B-ALL**

Although I have demonstrated that naphthazarin reduces t(1;19) pre-B-ALL cell viability as well as levels of ROR1 and UHRF1 (**Figure 3-13**, **Figure 3-14**, **Figure 3-15**), I have not fully investigated the mechanism of action of naphthazarin in this subtype of leukemia. To confirm the specificity of naphthazarin's effects on UHRF1 and ROR1, I

could ectopically express wild-type *UHRF1* before treating cells with naphthazarin. I hypothesize that naphthazarin would not significantly reduce ROR1 protein levels with ectopic expression of *UHRF1*.

Despite naphthazarin inhibition of UHRF1 and ROR1, it is also important to determine any potential off-target effects. Naphthazarin is a quinone; it generates reactive oxygen species, inducing DNA damage and apoptosis, in lung and gastric cancers (197, 199). This mechanism of action would be consistent with my work so far: I have demonstrated that t(1;19) pre-B-ALL positive cell lines have significantly higher Annexin-V staining, a marker of early apoptosis, than a t(1;19) pre-B-ALL negative cell line (**Figure 3-16**). Also, UHRF1 is an antagonist of p53 (173, 174), and naphthazarin downregulates UHRF1 to promote p53-dependent signaling in breast and lung cancer cell lines (197, 198). Therefore, t(1;19) pre-B-ALL cells treated with naphthazarin may also upregulate p53 and its effector p21, but this potential mechanism has yet to be confirmed.

5.4 **ROR1 effects on UHRF1**

While much attention throughout this dissertation project focused on the effects of UHRF1 on ROR1, little attention has been given to the role that ROR1 may have on UHRF1. In section 0, I demonstrated that UHRF1 is required for maintenance of ROR1 protein levels. Interestingly, silencing *ROR1* expression slightly reduced UHRF1 protein levels (**Figure 3-4**). This observation is counter-intuitive: if UHRF1 is required for ROR1, silencing ROR1 expression with siRNA would likely increase UHRF1 in an attempt to

rescue ROR1 levels. One explanation for these results is that ROR1 may be important for UHRF1 stability or function. ROR1 has been previously shown to promote cell survival and proliferation (Section 1.3.3), and perhaps some of ROR1's downstream effects also suppress negative cell cycle regulators such as p53. Yamaguchi *et al* (2016) previously showed that ROR1 acts a scaffold for cavin-1 and caveolin-1, which are structural components of the lipid transport vesicles, caveolae (130). Caveolin-1, in particular, inhibits p53 in lung cancer (230). UHRF1, which also opposes p53, may play a role in this anti-apoptotic ROR1-dependent response. However, additional studies are needed to further investigate potential effects of ROR1 on UHRF1.

5.5 Conservation of UHRF1-dependent maintenance of ROR1 in other cancers

ROR1 and UHRF1 are expressed in many tumor types, making both candidates for therapeutic targets (Section 1.3.3, Section 1.5). Therefore, assessing the potential of the UHRF1-ROR1 mechanism in other hematologic malignancies and solid tumors may provide rationale for developing additional targeting strategies to inhibit UHRF1 and ROR1. Since ROR1 is a tumor-promoter that is important for the progression of CLL (99, 118-121, 147, 186, 188), this disease may benefit directly from preliminary studies investigating the role of UHRF1. Similarly, UHRF1 is important for prostate cancer progression (231) and ROR1 expression is also high in this cancer type (126). Interestingly, I found that the prostate cancer cell line PC-3 exhibits UHRF1-dependent levels of ROR1, similarly to t(1;19) pre-B-ALL (**Figure 5-1**). However, the effects of

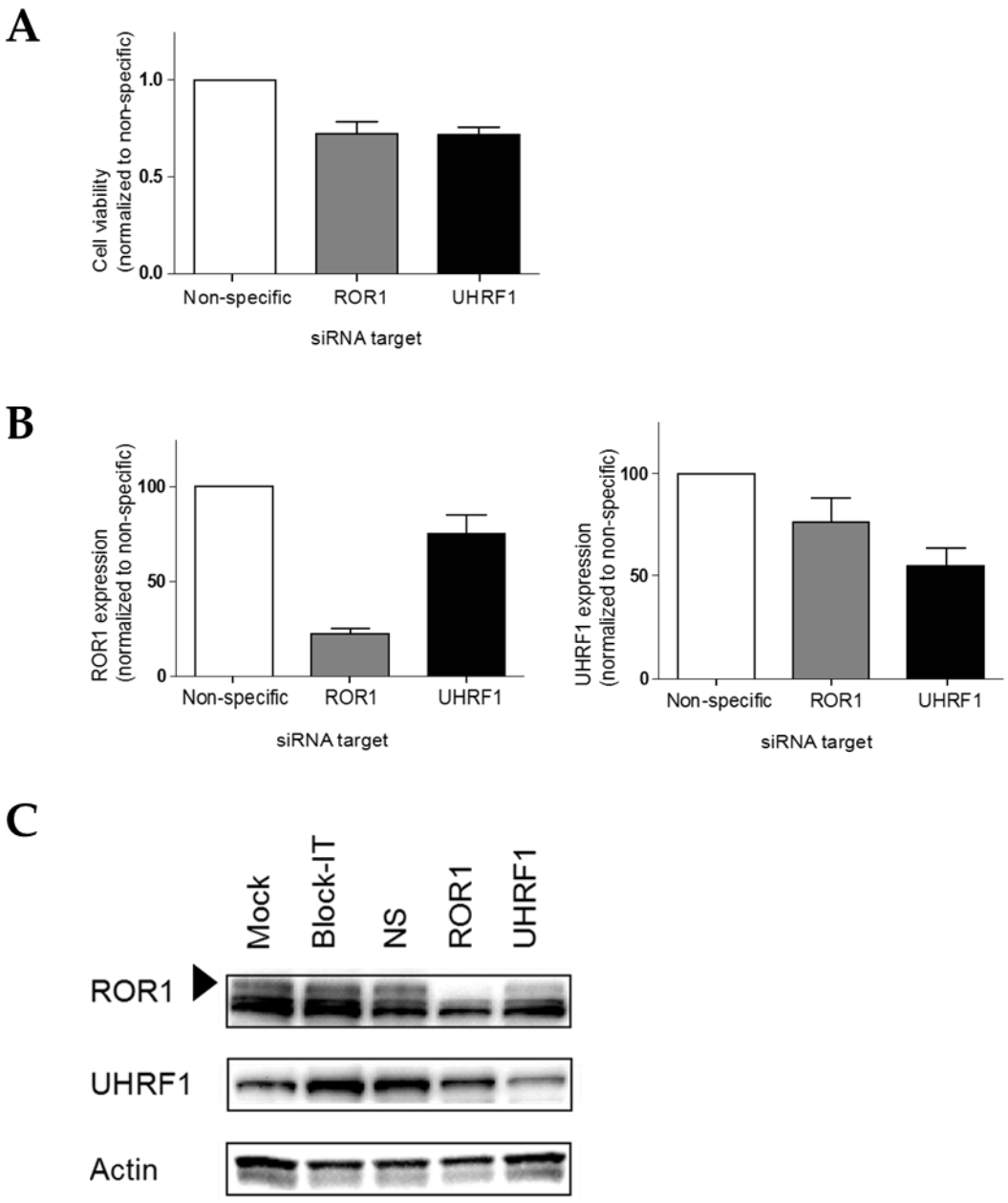

Figure 5-1 UHRF1 is required for ROR1 protein levels in PC-3 cells.

PC-3 cells were transfected with siRNA. **(A)** 6 days later, cell viability was assessed by MTS assay and normalized to mock treated cells. 5 days post-transfection RNA and protein cell lysates were extracted. RNA and protein were tested for **(B)** mRNA expression by qRT-PCR and **(C)** protein levels by immunoblot. Block-IT is a fluorescent oligo used as a visual marker of transfection efficiency. (N=2)

naphthazarin on these cells have yet to be determined. Furthermore, it is important to note that additional work presented in the Appendix indicate that ROR1 is also important for Ewing sarcoma cell survival, making this tumor type another candidate for UHRF1-ROR1 studies.

5.6 Mechanism of ROR1-dependent activation of AKT in t(1;19) pre-B-ALL

ROR1 is required for AKT activation through phosphorylation of serine 473 in t(1;19) pre-B-ALL (24) but the mechanism of this ROR1 function is unclear. This conundrum is especially puzzling since ROR1 lacks intrinsic kinase activity (Section 1.3.4). However, phosphorylation of AKT on serine 473 is added by a multi-subunit kinase complex, mTORC2 (219). Therefore, I hypothesized that ROR1 mediates AKT activation through interactions with members of mTORC2. Data presented in Chapter 4 suggests that ROR1 may preferentially interact with Rictor, an essential component of mTORC2 (**Figure 4-2**) and this interaction is essential for the maintenance of Rictor protein levels and t(1;19) pre-B-ALL viability (**Figure 4-3**, **Figure 4-4**).

Dasatinib inhibits pre-BCR kinase signaling, including AKT, but leukemic cells are not completely eliminated (24). AKT signaling is partially rescued through an upregulation of ROR1 expression, demonstrating one mechanism of resistance to small molecule inhibitors (24). In the presence of dasatinib, Rictor protein levels increase and correlates with upregulated ROR1 and re-activation of AKT (**Figure 4-5**). Furthermore, levels of Raptor—a required subunit of another mTOR complex mTORC1—begin to decrease in response to dasatinib (**Figure 4-5**). Together, my data suggests that ROR1

activates AKT by mediating the expression of mTOR complex subunits Rictor and Raptor (**Figure 4-6**). The mechanistic details, such as the spatial and temporal dynamics of ROR1-mTORC1/2 interactions, still need to be determined. Nevertheless, these data identify new therapeutic targets to consider in future studies of t(1;19) pre-B-ALL, including mTOR and AKT. However, *ROR1* expression increases in response to AKT inhibitors in t(1;19) pre-B-ALL (24), supporting the need to also design new therapeutics to target ROR1.

5.7 Strategies to target ROR1 and UHRF1 in cancer

In light of the work presented in this dissertation, there is much to consider when designing new therapies to target UHRF1. Work from Chapter 3 suggests that naphthazarin significantly reduces t(1;19) pre-B-ALL survival (**Figure 3-13**). Until the mechanism of action is fully elucidated, however, alternative inhibitors may also need to be investigated. So far, one potential small molecule has been described to bind the SRA domain of UHRF1, potentially inhibiting it directly (191), but the development of this compound is still in its infancy.

Given that UHRF1 is also an E3 ubiquitin ligase, this function may be another promising avenue for drug development. Indeed, targeting E3 ligases has been successful with the development of MDM2 inhibitors that stabilize p53 (232, 233), but there are challenges with targeting E3 ligases as well. Similar to current targeted therapies, small molecules that inhibit E3 ligases have had problems with efficacy due to off-target effects and low bioavailability (234). However, similar to MDM2 inhibitors,

suppressing UHRF1 E3 ligase activity may also promote p53-dependent tumor suppressor function, which is normally downregulated by UHRF1 (173, 180).

Unlike UHRF1, there are relatively more therapeutic strategies currently in development to target ROR1 (Section 1.3.7). Recently, I have started to test the efficacy of a humanized ROR1 antibody (Cirmtuzimab) and an antibody-drug conjugate that links this ROR1 antibody to the toxic drug, monomethyl auristatin E, on t(1;19) pre-B-ALL. In collaboration with Oncturnal Therapeutics, I determined that the humanized monoclonal ROR1 antibody Cirmtuzimab did not affect cell viability as a single agent (**Figure 5-2**). Moreover, any effect in combination with dasatinib is likely due to dasatinib since t(1;19) pre-B-ALL is already sensitive to this kinase inhibitor (**Figure 5-2**). These results indicate that Cirmtuzimab is not effective against t(1;19) pre-B-ALL cell lines, even in combination with dasatinib.

In contrast, the antibody-drug conjugate with the ROR1 antibody greatly reduced the viability of t(1;19) pre-B-ALL positive and negative cells (**Figure 5-2**). Furthermore, this result was consistent when the agent was added alone or in combination with dasatinib (**Figure 5-2**). Taken together, these data suggest that the ROR1 antibody-drug conjugate does not selectively kill t(1;19)-positive cells and likely has off-target effects. However, cytotoxicity of the antibody drug conjugate may be due to degradation or unlinking of monomethyl auristatin E from the ROR1 antibody. Follow-up experiments to determine specificity are ongoing.

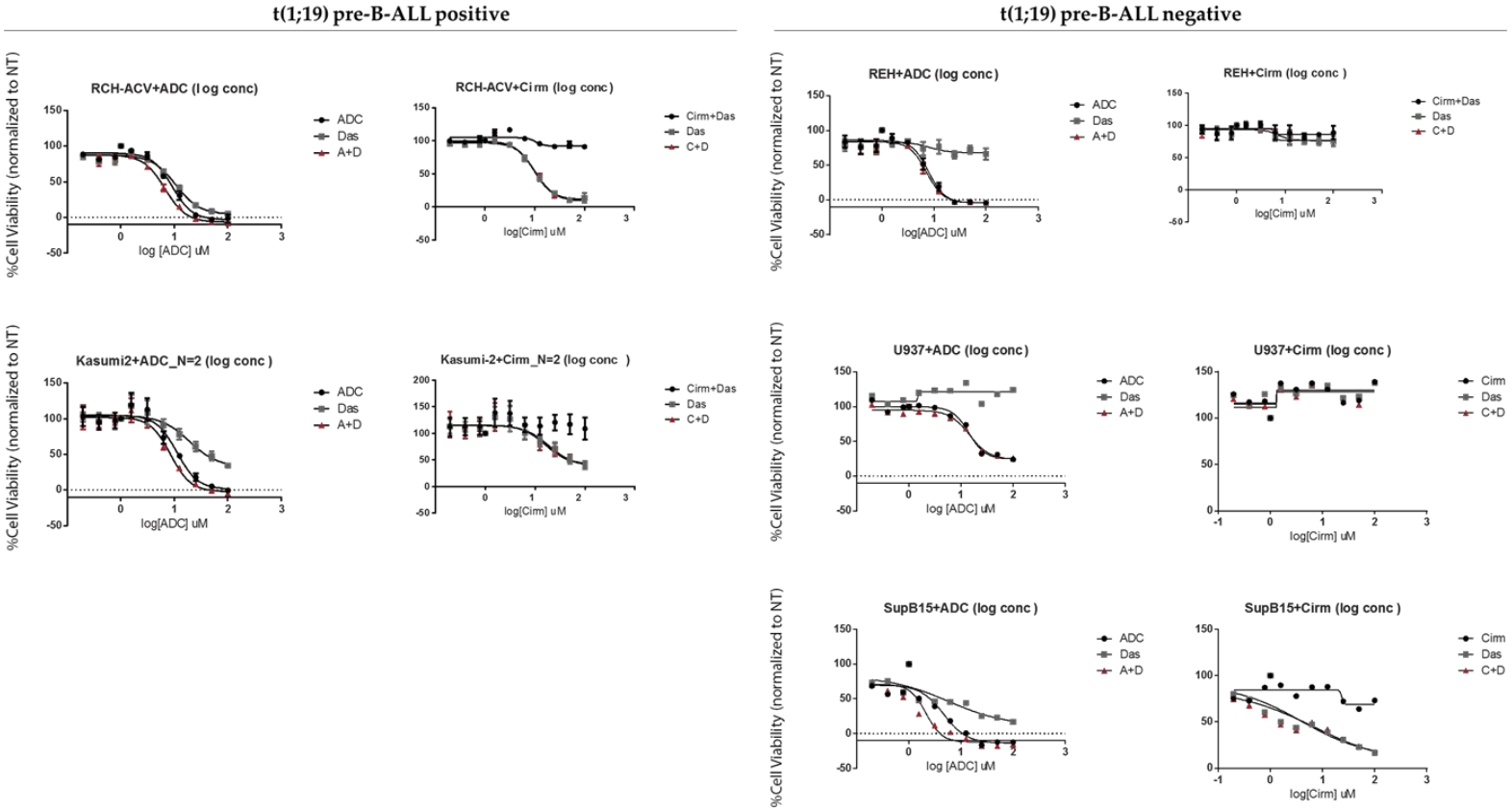

Figure 5-2 ROR1-ADC and Cirmtuzimab are cytotoxic to t(1;19) positive and negative cell lines.

RCH-ACV (N=3), Kasumi-2 (N=2), REH (N=3), U937 (N=1), SupB15 (N=1) were treated with up to 100 µM of ROR1-ADC (ADC, left) or Cirmtuzimab (Cirm, right) as single agents or in combination with dasatinib (Das) for 72 hours. Cell viability was measured by MTS assay and values were normalized to non-treated cells. Error bars represent S.E.M.

Finally, to overcome the off-target effects of the therapeutic agents discussed throughout this dissertation, there is an interesting strategy to target either UHRF1 or ROR1. Proteolysis targeting chimeras are bi-functional small molecules that are conjugated with a drug on one end and a ligand of an E3 ligase on the other. This technology was originally developed in 2001 by Sakamoto and colleagues, who were able to show that proteolysis targeting chimeras mark proteins for degradation by the proteasome (235). This may be a promising strategy as ROR1 protein turnover is associated with the proteasome (**Figure 3-6**). Subsequent studies illustrated the efficacy of proteolysis targeting chimeras in the context of other difficult-to-target proteins, including BRD4 and BCR-ABL (236, 237). However, this approach requires some additional studies before it can be applied to UHRF1. Specifically, the role of UHRF1 E3 ligase activity in the maintenance of ROR1 will need to be confirmed and the UHRF1 target that regulates ROR1 will also need to be identified. The future studies presented earlier in section 5.2 would help to address these issues and provide the rationale to develop proteolysis targeting chimeras to target one or more components of the UHRF1-ROR1 mechanism.

5.8 **Final Conclusions**

siRNA-mediated knockdown of UHRF1 sensitizes t(1;19) pre-B-ALL cells to dasatinib, which has previously been shown to upregulate ROR1 expression to promote leukemic cell survival (24). In cancer, UHRF1 is an oncogene that downregulates

expression and activity of tumor suppressor genes (163). Similarly, ROR1 is a tumor-promoter that is required for the progression of many solid tumors and leukemias (109-111, 115, 117, 238). The work from this dissertation not only demonstrates a relationship between UHRF1 and ROR1 in t(1;19) pre-B-ALL, but it is the first evidence of UHRF1 being required for the maintenance of another tumor-promoter in B cell leukemias.

In conclusion, my dissertation research presents two mechanisms: (1) ROR1 post-transcriptional regulation by UHRF1 and (2) ROR1-dependent AKT activation through interactions with mTORC1/2. This work is the first investigation of how levels of ROR1 protein are maintained and how it is able to promote downstream kinase signaling as a pseudokinase. More importantly, these findings generate new potential therapeutic targets and further evidence that targeting ROR1—through its regulation or its signaling—is a promising strategy to improve treatment of t(1;19) pre-B-ALL and other ROR1-expressing cancers.

6 Appendix: siRNA screens for solid tumors

Section 6.2.1: Sun, S., Cao, Q. Pang, B., Gae, D.D., Lee V.K.M., Lim, H.J., Doan, N., Said, J.W., Gery, S., **Chow, M.**, Mayakonda, A., Forscher, C., Tyner, J.W., Lin, D., Koeffler, H.P. *Identification of a Novel SYK/c-MYC/MALAT1 Signaling Pathway and Its Potential Therapeutic Value in Ewing Sarcoma*

This manuscript was published in Clinical Cancer Research on August 1 2017 Volume 23(15): 4376-4387.

Section 6.2.2: Jiang Y., Mayakonda A., Huang, M. Ding, L., Lin, H., Yu, F., Loh K.S., **Chow, M.**, Savage, S., Tyner J.W., Lin, D., Koeffler, *Super-Enhancers Promote Transcriptional Dysregulation in Nasopharyngeal Carcinoma*

This manuscript is in press at Cancer Research as of September 26 2017 (DOI: 10.1158/0008-5472.CAN-17-1143)

6.1 siRNA and small molecule inhibitor screens can identify critical tumor-promoters and potential small-molecules to treat cancer

siRNA screens are a useful, high-throughput method to identify candidate genes required for the viability of a cell population. This technology identifies major oncogenic drivers of a cell line or a patient sample, and our group developed this assay for a 96-well plate format to systematically and consistently test leukemic cells against the human kinome (239, 240). Indeed, the landscape of multiple cancer subtypes have been profiled using kinome-specific siRNA screens. These studies have generated many hypotheses to further investigate the biological mechanisms that drive hematologic malignancies and solid tumors (239, 241-243).

The concept of an siRNA screen has been adapted to directly interrogate potential small molecule inhibitors that may be effective in pharmacologically inhibiting leukemic cell survival (244-246). Many of these inhibitors share overlapping targets with the siRNA screen, increasing the power and consistency of these assays. Furthermore, testing inhibitors, rather than siRNA, provides an opportunity to quickly determine clinical therapeutic relevance while simultaneously identifying additional non-kinase targets critical for leukemic cell viability.

Investigations of solid tumors using functional screens developed in our lab, however, is limited. For example, the ephrin receptor B4 and platelet-derived growth factor receptor beta kinases were found through the siRNA screen to be required for the survival of alveolar rhabdomyosarcoma, an aggressive childhood malignancy (243).

Ewing sarcoma and nasopharyngeal cancer are both rare (247, 248), and may benefit from the use of high-throughput functional screens to identify potential oncogenic drivers and candidate small-molecules for treatment. Although patient samples may not be readily available for these cancer subtypes, cell line models allow investigations of these diseases.

In this Appendix, I will briefly describe two studies using functional screens to determine key drivers of tumorigenesis and candidate inhibitors in Ewing sarcoma and nasopharyngeal cancer cell lines. The roles of kinases in both of these cancers have yet to be fully investigated. Therefore, functional assays may provide insight into the mechanisms that drive tumor progression or response to small molecule inhibitors.

6.2 **Results**

6.2.1 *SYK is a targetable kinase in Ewing sarcoma*

Ewing sarcoma is an aggressive soft tissue tumor that is only about 30% curative once metastasis occurs (249). Interestingly, tyrosine kinases have been shown to be important for Ewing sarcoma progression and are potential targets for therapeutic strategies (250, 251). To identify candidate kinases and small molecules to inhibit them in Ewing sarcomas, I performed siRNA and inhibitor screens with the Ewing sarcoma cell line T32. I found that the kinase SYK was required for cell viability, and more importantly, these cell lines were also sensitive to the SYK inhibitor PRT062607 (**Figure 6-1, Figure 6-2**). Members of the Koeffler lab followed up on these results and found

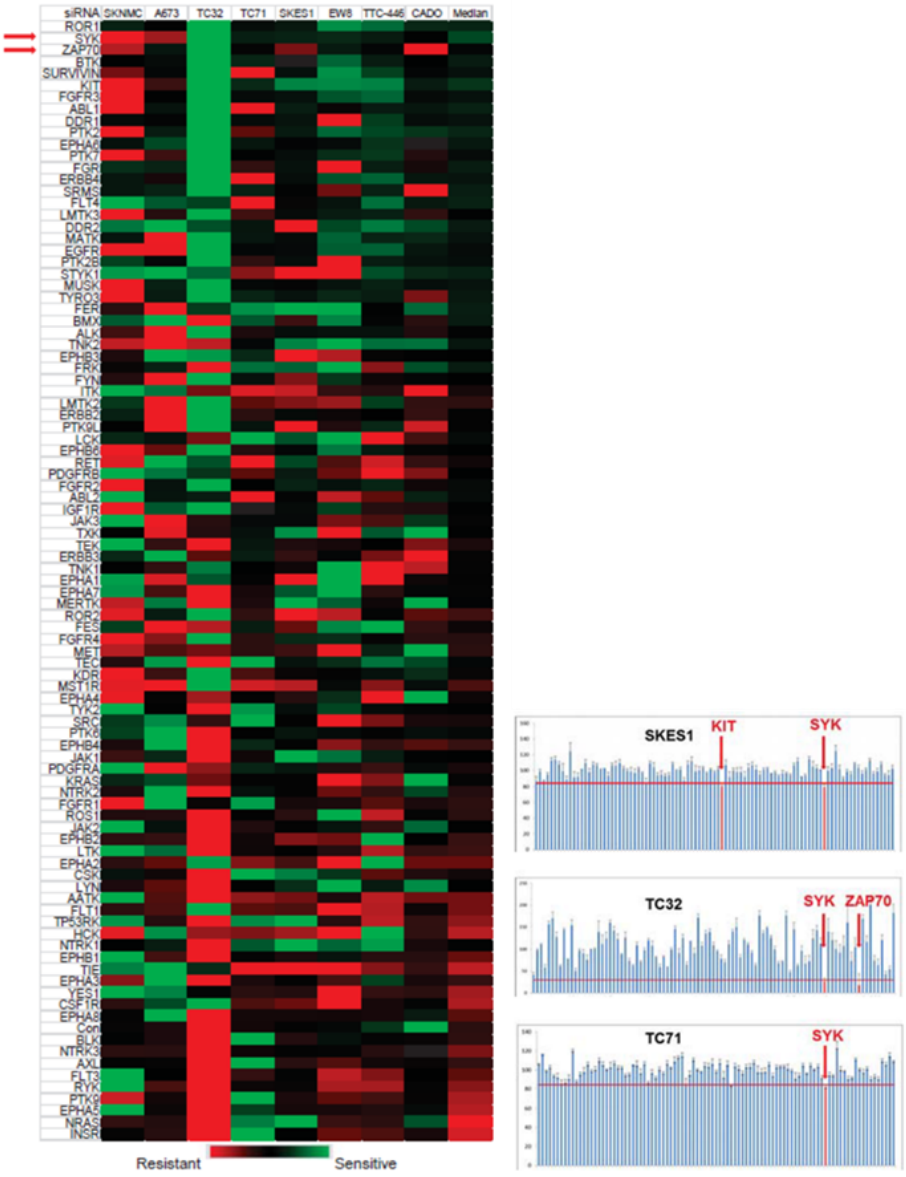

Figure 6-1 SYK is required for the survival of Ewing sarcoma cell lines.

Cell lines were cultured in the presence of siRNA for four days prior to measuring cell viability by MTS assay. Viability was normalized to a non-specific siRNA. (Left) Heat map of all tested siRNAs. (Right) Representative histograms of cells lines showing sensitivity to kinase inhibition. Red line represents two S.D. Data from 2017 Sun *et al. Clin Cancer Res*.

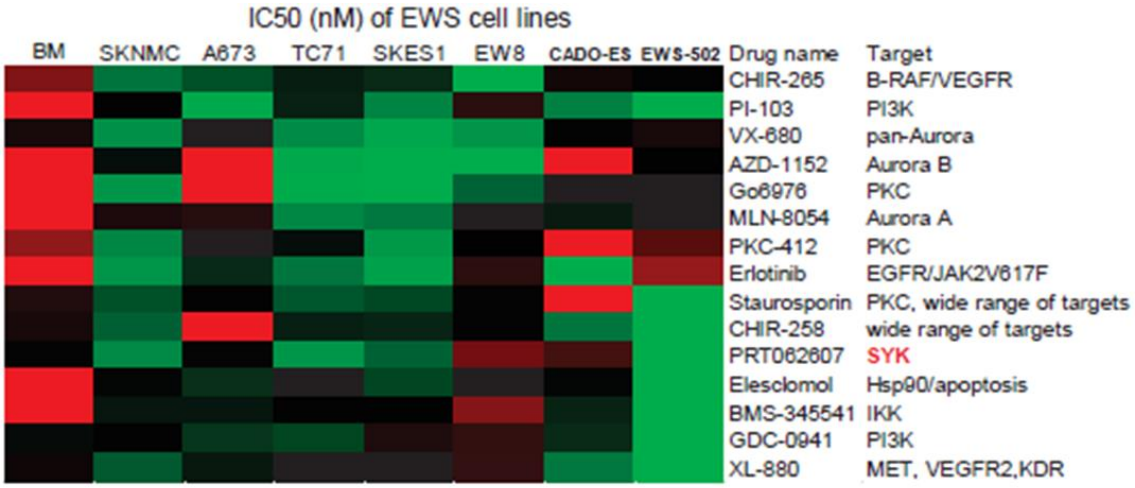

Figure 6-2 Ewing sarcoma cell lines are sensitive to SYK inhibition.

Cell lines were incubated with small-molecule inhibitors for three days prior to measuring cell viability by MTS assay. IC_{50} values were calculated based on effects on cell viability. Red = resistant Green = sensitive. Data from 2017 Sun *et al. Clin Cancer Res*.

that SYK was hyper-activated in Ewing sarcomas. Increased levels of phosphorylated SYK led to an upregulation of a c-myc-dependent expression of the long non-coding RNA, MALAT1, which was proposed to control the G1 transition in the cell cycle and promote tumorigenesis (252).

6.2.2 *Nasopharyngeal cancer cell lines are sensitive to cyclin dependent kinase inhibitors*

Genomic profiling of nasopharyngeal cancer revealed that genetic aberrations in tumor suppressors were more common than oncogenes, specifically in negative regulators of NFκB signaling (253, 254). This provides an opportunity to identify other pathways that may be readily targetable using a screening approach. Therefore, I performed an inhibitor screen on seven nasopharyngeal cell lines. I found that the top three inhibitors that were most effective against these cell lines targeted cyclin dependent kinases (**Figure 6-3**). Again, the Koeffler lab further investigated the biological mechanism underlying this sensitivity. They found that super enhancers and their target genes are upregulated in response to cyclin dependent kinase inhibition and may play a major role in disease response to inhibition.

6.3 **Discussion**

Here, I presented two vignettes utilizing siRNA and inhibitor functional assays to inform follow-up studies on tumor progression and drug response in solid tumors. The first story involved both siRNA and inhibitor screens that identified SYK as a kinase

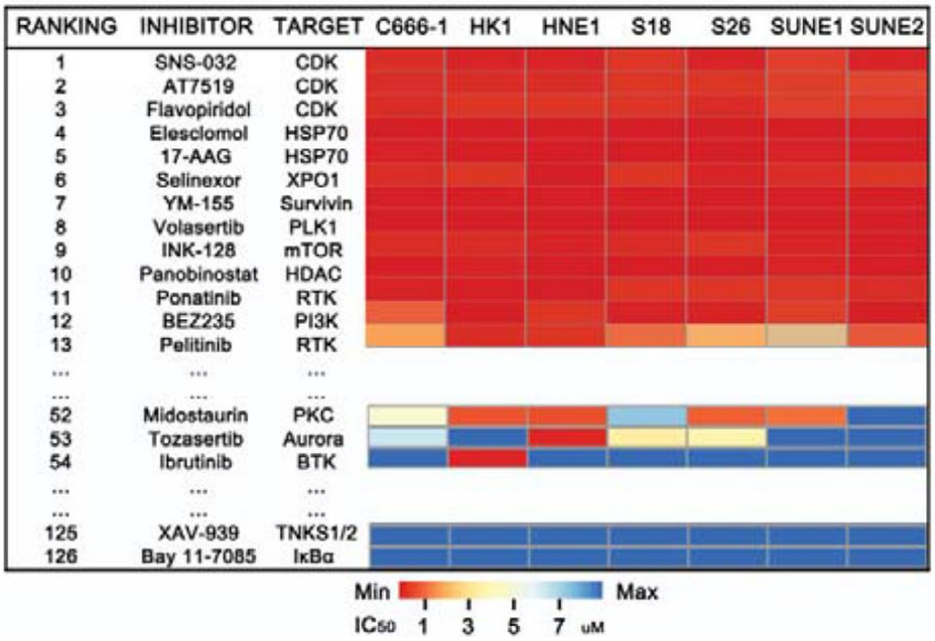

Figure 6-3 Nasopharyngeal carcinoma cell lines are sensitive to cyclin dependent kinase inhibitors.

Cell lines were incubated with small-molecule inhibitors for three days prior to measuring cell viability by MTS assay. IC_{50} values were calculated based on effects on cell viability. Data from 2017 Jiang *et al.*

required for the survival of Ewing sarcoma. The second example was based on the use of the inhibitor screen to show that nasopharyngeal cancer cell lines exhibited sensitivity to cyclin dependent kinase inhibitors and led to the discovery of super enhancers that upregulate the expression of target transcripts in response to drug treatment. While both applications of functional screens identified key regulators of cancer cell line survival, the biological mechanisms were quite different—investigating the oncogenic contributions of kinase signaling compared to studying the functional consequence of inhibitor treatment. These studies, along with work described in Chapter 3, demonstrate the versatility of high-throughput, unbiased functional screens as a hypothesis-generating tool. Overall, functional screens provide direction for subsequent studies investigating biological mechanism as well as potential therapeutic options, demonstrating the applicability of functional assays to translational biomedical research.

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