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Maintenance and Pharmacologic Targeting of ROR1 Protein Levels via UHRF1 in t(1;19) pre-B-ALL

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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), chronic lymphocytic leukemia, and many solid tumors. However, targeting ROR1 with small-molecules 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, we 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 axis. Finally, we show that ROR1-positive cells are twice as 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 naphthazarin with inhibitors of pre-B cell receptor signaling results in further reduction of cell survival compared with either inhibitor alone. Therefore, our 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.

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Conflict of Interest

Dr. Tyner's work has received 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 co-founder of Leap Oncology. All other authors declare no conflicts of interest.

Supplementary Information accompanies the paper on the *Oncogene* website (<http://www.nature.com/onc>).

Keywords

leukemia; ROR1; UHRF1; ALL

Introduction

Approximately 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) (1–4). The fusion protein resulting from this translocation, TCF3-PBX1, promotes aberrant expression and re-localization of WNT16B and β -catenin, respectively (5–7). 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 (pre-BCR) (8, 9). Recent studies have shown that signaling from the pre-BCR through tyrosine kinases such as BTK and SRC-family kinases (SFKs) is important to drive proliferation and survival of t(1;19) pre-B-ALL cells (10, 11). We and others have shown that the pseudokinase ROR1 is also critical for leukemic cell survival through cross-talk with the pre-BCR (10, 12, 13). Furthermore, t(1;19) pre-B-ALLs are sensitive to dasatinib, an inhibitor of pre-BCR effector kinases BTK and SFKs, while 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-BCR signaling, such as dasatinib (10).

ROR1 is highly expressed on the surface of both t(1;19) pre-B-ALL and chronic lymphocytic leukemia (CLL) cells in addition to multiple types of solid tumors including prostate, breast, and pancreatic cancer (14–19). ROR1 is thought to be rarely expressed in post-natal tissues, although a recent study revealed ROR1 may be present in several normal tissues (14, 20). Multiple groups are currently developing and testing methods of targeting ROR1-expressing tumor cells, some of which have seen initial success in clinical trials (21–24). 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 (10). Thus, we 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 (25) and NKX2-1 has been reported to induce *ROR1* expression in lung adenocarcinoma (26). In addition to transcriptional activation, ROR1 is thought to be post-translationally modified through glycosylation and ubiquitination (27), but the mediators of these modifications have yet to be elucidated.

The RING E3 ligase UHRF1 (also known as ICBP90) ubiquitinates several substrates, including p53 and histone H3, to mediate protein function and chromatin structure, respectively (28, 29). UHRF1 also has ubiquitin ligase-independent roles interacting with DNA and histones through its Tudor-like, PHD, and SRA/YDG domains (29–37). 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 (38, 39). Despite evidence that UHRF1 promotes solid tumor formation and progression and is associated with low-risk AML (31, 40–45), UHRF1 has not been thoroughly investigated in ALL.

Therefore, we sought to find new mechanisms that regulate ROR1 and, more importantly, may have therapeutic potential that can be targeted by small-molecule inhibitors. We 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-BCR targeting strategies, such as dasatinib, may prove to be a useful alternative regimen for ROR1-expressing cancers.

Results

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 we 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 1A, Supplementary Table 1). *RUNX1* has previously been shown to be a key regulator of pre-BCR expression (46), 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 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, we 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 (10). 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 1B). As a final control, we 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 1C). These data suggest UHRF1 specifically mediates t(1;19) cell viability through a mechanism associated with ROR1 expression.

UHRF1 is required for the maintenance of ROR1 protein, not mRNA, levels

To determine whether UHRF1 plays a direct role in regulating *ROR1* expression, we measured cell viability and levels of ROR1 mRNA and protein after *UHRF1* silencing. After confirming that UHRF1 is required for RCH-ACV cell viability (Supplementary Figure 1A), we observed that levels of ROR1 protein, but not mRNA, were significantly reduced after *UHRF1* silencing using pooled siRNA or two independent *UHRF1* siRNA duplexes (Figure

2A-B, Supplementary Figure 1B). Furthermore, we observed similar patterns with a second t(1;19) pre-B-ALL cell line, Kasumi-2 (Supplementary Figure 1C-D). Together, 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, we hypothesized that RCH+ROR1-V5 cells are rescued from UHRF1 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 levels of ectopic RCH+ROR1-V5 in these cells but would still be maintained above a threshold required to maintain cell viability. Consistent with this, we 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 2C).

Next, we asked whether the reduced levels of ROR1 protein that we 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, we suspected that regulation of ROR1 by UHRF1 is associated with the proteasome. We, therefore, measured ROR1 protein levels after *UHRF1* silencing in the absence or presence of the 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 2D). Collectively, these data suggest that loss of ROR1 protein following *UHRF1* silencing is mediated by the proteasome.

To complement the gene silencing experiments, we transiently expressed wild-type *UHRF1* in HEK293T17 cells, which express very low levels of *ROR1* and *UHRF1*. As shown in Figure 2E, 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.

UHRF1 indirectly regulates ROR1

Since silencing of *UHRF1* resulted in reduced levels of ROR1 protein, we 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 3A), which is consistent with previously published mass spectrometry studies (10). These observations were confirmed by co-immunoprecipitation from HEK293T cells with ectopically expressed wild-type *ROR1* (Figure 3B). Furthermore, these proteins do not co-localize in the same subcellular compartments 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 3C). Therefore, we conclude that UHRF1 regulates ROR1 indirectly.

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

Since targeting UHRF1 with siRNA led to reduced levels of ROR1 protein, we 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 (10). To address this, we 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. We also observed an enhanced loss of cell viability when *ROR1* expression was silenced in combination to dasatinib, which is consistent with previous studies (10). 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 4A). 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.

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

To further investigate the potential of targeting UHRF1, we screened t(1;19) positive and negative cell lines against several putative UHRF1 small-molecule compounds (38) 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 4B, Supplementary Figure 2A). In contrast, other putative UHRF1 inhibitors, including anisomycin, were observed to be toxic since their effects were similar in ROR1-expressing and ROR1-non-expressing cells. Since these other agents also did not result in discernible impact on UHRF1 expression levels, we could not rule out UHRF1-independent effects and did not pursue these other agents further (Supplementary Figure 2A-B).

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 (47–49). 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, the t(1;19)-positive pre-B-ALL RCH-ACV and Kasumi-2 cell lines showed reduced levels of UHRF1 and ROR1 protein (Figure 4C). To further confirm that the effects of naphthazarin mimicked the phenotype we previously observed with *UHRF1* silencing, we 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 t(1;19)-negative 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 staining (Figure 4D). In parallel to induced apoptosis, we also noticed that these cells underwent cell cycle arrest (Supplementary Figure 3). To determine whether naphthazarin can cooperate with inhibition of the pre-BCR pathway via dasatinib, we measured effects of cell viability in the presence of a naphthazarin-dasatinib combination compared to either agent alone. t(1;19)-positive RCH-ACV and Kasumi-2 cells were sensitive to naphthazarin and dasatinib as single agents, but this effect is enhanced when these small-molecule inhibitors are combined (Figure 4E). This data were in contrast to REH cells, which exhibited insensitivity to single agent or combination treatment. 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.

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, we 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. This mechanism may involve UHRF1-mediated maintenance of ROR1 through ubiquitination. Indeed, others have demonstrated ROR1 ubiquitination in CHO cells (27), and we have also confirmed this in HEK293 cells (Supplemental Figure 4). Although we show that UHRF1 is sufficient to increase levels of ROR1, we have presented evidence suggesting that UHRF1 indirectly regulates ROR1 and experiments to identify intermediaries of this mechanism will be essential for a complete understanding of the pathway. To preliminarily address this question, we performed mass spectrometry and identified several potential mediators of the UHRF1-ROR1 mechanism including nucleophosmin 1, which is implicated in AML and is an interesting candidate, given its ability to shuttle between the nucleus and cytoplasm (50) (Table 1). These putative UHRF1-ROR1 intermediaries merit further investigation.

While performing these siRNA studies, we also observed a significant siROR1-mediated loss of UHRF1 (Figure 2B). One potential explanation for these data is that ROR1 stability or function may be critical for UHRF1 expression in a positive feedback loop. This would not be surprising, given the roles of ROR1-associated AKT signaling and UHRF1 in cell proliferation (10, 13, 40, 51). However, this hypothesis will need to be further evaluated to fully elucidate the ROR1-mediated effects on UHRF1.

In addition to characterizing the UHRF1-ROR1 mechanism, we were 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 (10). First, we 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, we 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 5). 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.

Currently, few studies suggest a role for UHRF1 in leukemia (45, 52), and this is the first report for an oncogenic role of UHRF1 in t(1;19) pre-B-ALL. However, UHRF1 is an established oncogene in solid tumors, many of which overlap with those that overexpress ROR1 (18, 53). Indeed, it will be interesting to see if the UHRF1-ROR1 mechanism is conserved in other types of cancers.

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-cell ALL through ROR1-independent mechanisms. In addition to RUNX1, RhoH and KAT7 were required for t(1;19) pre-B-cell viability. RhoH is a hematopoietic-specific member of the Rho GTPase family and has roles in promoting CLL through cell intrinsic mechanisms and interactions with the microenvironment (54, 55). 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 (56, 57). 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 (38), yet here we show that UHRF1 regulates ROR1, a tumor-promoter. Thus, our 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-cell-ALL and other *ROR1*-expressing cancers.

Materials and Methods

Cell culture

RCH-ACV and Kasumi-2 cells were purchased from DSMZ (Germany). RCH-ACV +ROR1V5, SupB15, and REH cells were described previously (10). U937 cells were purchased from ATCC. All cell lines were cultured in RPMI1640 supplemented with 10% FBS, 2% L-Glutamine, 1% penicillin/streptomycin, and 0.1% Fungizone (R10). All cells were grown at 37°C with 5% CO₂.

Reagents

Commercially available antibodies used in this study are: ROR1, UHRF1 (#4102, #12387, Cell Signaling, Danvers MA), ROR1 (#AF2000, R&D Systems, Minneapolis MN), UHRF1 (#612264, BD Biosciences, San Jose CA), goat IgG serum (#005-000-003, Jackson ImmunoResearch, West Grove PA), mouse IgG serum (#sc-2025, Santa Cruz Biotechnology, Santa Cruz CA), actin, GAPDH, SP1 (#AM4300, #07-645, Millipore, Burlington MA), V5 (#46-0705, Invitrogen, Carlsbad CA), and ubiquitin (#A-104, Boston Biochem, Cambridge

MA). 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 (Supplementary Figure 5). Commercially available inhibitors used in this study are: Velcade (Bortezomib), dasatinib (SelleckChem, Houston TX), anisomycin (Cayman Chemical, Ann Arbor MI), naphthazarin, thymoquinone (Sigma, St. Louis MO), and flavopiridol (LC Laboratories, Woburn MA).

Plasmids

UHRF1 wild-type was cloned into the pCS2-MT 6XMYC vector using In-Fusion cloning (Clontech) and ClaI restriction sites. The construct was confirmed by sequencing before use.

siRNA

siGENOME SMARTpools (and the equivalent individual siRNA duplexes) were purchased from Dharmacon (Lafayette CO) with the following sequences:

Non-specific pool #1: UAGCGACUAAACACAUCAA,
 UAAGGCUAUGAAGAGAUAC, AUGUAUUGGCCUGUAUUAG,
 AUGAACGUGAAUUGCUCAA

ROR1: GCGUGGCAACAAACGGCAA, CGGAUUAGAAACCUCGACA,
 UGACUUGUGUCGCGAUGAA, GAGAAUGUCCUGUGUCAAA

UHRF1: (siU#1) GCCAUACCCUCUUGCACUA, (siU#2)
 GGAACAGUCUUGUGAUCAG, UGGAGGAGGACCUCAUUUA,
 GAACGGCGUGGUCCAGAUG

Gene silencing studies were performed as previously described (10, 58, 59). In brief, 800,000 cells were electroporated with 1-2 μ g SMARTpool siRNA duplexes (Dharmacon) using the BioRad Gene Pulser XCell (305 V, 2 ms, 2 pulses). Cells were briefly centrifuged then resuspended in the appropriate growth medium. Cells were harvested after 72 hours and cell viability was measured by MTS assay 96 hours post-electroporation.

siRNA screen

RCH-ACV (305 V, 2 ms, 2 pulses), RCH-ACV+ROR1-V5 (250 V, 3 ms, 2 pulses), and REH (300 V, 2 ms, 2 pulses) cells were electroporated with 1-2 μ g siRNA in siPORT buffer (120 mM Trehalose, 20 mM HEPES pH8, 1 mM Myo-Inositol, 1 mM KCl, 1mM MgCl₂, 1 mM K₂HPO₄, 0.4 mM KH₂PO₄, 2.14 mM KOH, 1 mM Glutathione). Electroporation was performed using a 96-well BioRad Gene Pulser MXCell (BioRad, Hercules CA). Cells were re-plated in R10 media and incubated at 37°C for 96 hours. Total cell viability was measured using the Cell Titer96® Aqueous One Solution Cell Proliferation Assay (MTS) according to the manufacturer's instructions (Promega, Madison WI).

RNA extraction and qRT-PCR

Total cell RNA was extracted using RNeasy according to the manufacturer's instructions (QIAGEN), eluted in 30 μ L of water and quantified using the TAKE3 plate reader (BioTek Synergy2, Winooski VT). cDNA was synthesized according to the manufacturer's

instructions using equal amounts of RNA (Invitrogen Superstrand III/Superscript). qRT-PCR was performed using primer sets with Taqman FAM probes (ThermoFisher Scientific, Waltham MA). The Opticon2 thermocycler (MJ Research Inc., Waltham MA) and QuantStudio7 (Life Technologies, Carlsbad CA) were used to run 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.

Protein extraction and immunoblot

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, Basel Switzerland). Whole cell protein extract was quantified by BCA assay (ThermoScientific) and subjected to SDS-PAGE. Membranes were incubated with antibodies at 4°C overnight. Images were obtained by chemiluminescence according to the manufacturer's instructions (BioRad ECL Clarity Substrate, ChemiDocMP) and densitometry was performed using ImageJ (NIH, Bethesda MD). Bands were normalized to the loading control (Actin or GAPDH) before further analysis.

Immunoprecipitation

Whole cell protein lysate was extracted in an NP-40-based buffer (1% NP-40, 137 mM NaCl, 20 mM Tris-HCl, 20 mM HEPES pH 8, 2 mM EDTA pH 8, 10% Glycerol). 0.5-1 mg of total whole protein 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. Beads were boiled at 95°C in loading dye before performing SDS-PAGE.

Cellular Fractionation

5e6-10e6 cells were pelleted at 500xg for 5 min. Nuclear and cytoplasmic fractions were isolated using the NE-PER kit according to the manufacturer's instructions (ThermoScientific).

HEK293 Transfections

6-well plates were seeded with 3e5 cells overnight. 1-2 µg DNA was transfected using Fugene6 (Promega) according to the manufacturer's protocol. Cells were harvested for protein lysate extraction 48-72 hours after transfection.

Inhibitor assays

5e5 –1e6 cells were treated with dasatinib, naphthazarin, thymoquinone, anisomycin, or a combination for 72 hours. Cell viability was measured by MTS assay and IC₅₀ values were calculated using Prism software package (GraphPad, La Jolla CA).

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.

Cell Cycle Analysis

7.5e5-1e6 RCH-ACV cells were treated with inhibitor for to 8 hours and subsequently fixed with pre-chilled ethanol for 2 hours at 4°C. Cells were washed with 1XPBS and stained with propidium iodide (Sigma-Aldrich, 0.5mg/mL) and RNase A (Sigma-Aldrich, 0.5ug/ml) for 30 minutes in the dark at 37°C. Cell cycle distribution was analyzed by flow cytometry and FlowJo.

Mass Spectrometry

1 mg of RCH-ACV or Kasumi-2 whole cell extracts were incubated with 4 µg ROR1, UHRF1, or a matched isotype control antibody overnight at 4°C. Samples were eluted in 1% SDS and submitted for mass spectrometry. Mass spectrometric analysis was performed by the OHSU Proteomics Shared Resource with partial support from the NIH instrument grant S10OD012246 (Orbitrap Fusion) and core grants P30EY010572 and P30CA069533. Only proteins identified with the ROR1-specific or UHRF1-specific antibody and not the isotype control were considered as candidate interacting proteins.

Statistical Analysis

All statistical analyses on MTS cell viability, qRT-PCR, immunoblot, and apoptosis assays were performed using Prism software package (GraphPad). Data shown in figure 2, 4A, and S1A were analyzed by ANOVA and adjusted p-values were reported to reflect multiple comparisons used in statistical analyses. ANOVA analysis includes statistical comparisons of variance between groups and were all found to be similar (residual ss values less than 1). T-tests were used to analyze data shown in figure 4D-E and S1C. T-tests assume equal variance between groups. Sample sizes listed in figure legends correspond to independent, biological replicates, and at least three independent experiments were performed for data to be analyzed for statistical significance. Error bars are noted in figure legends to represent as either S.D. = standard deviation or S.E.M. = standard error mean.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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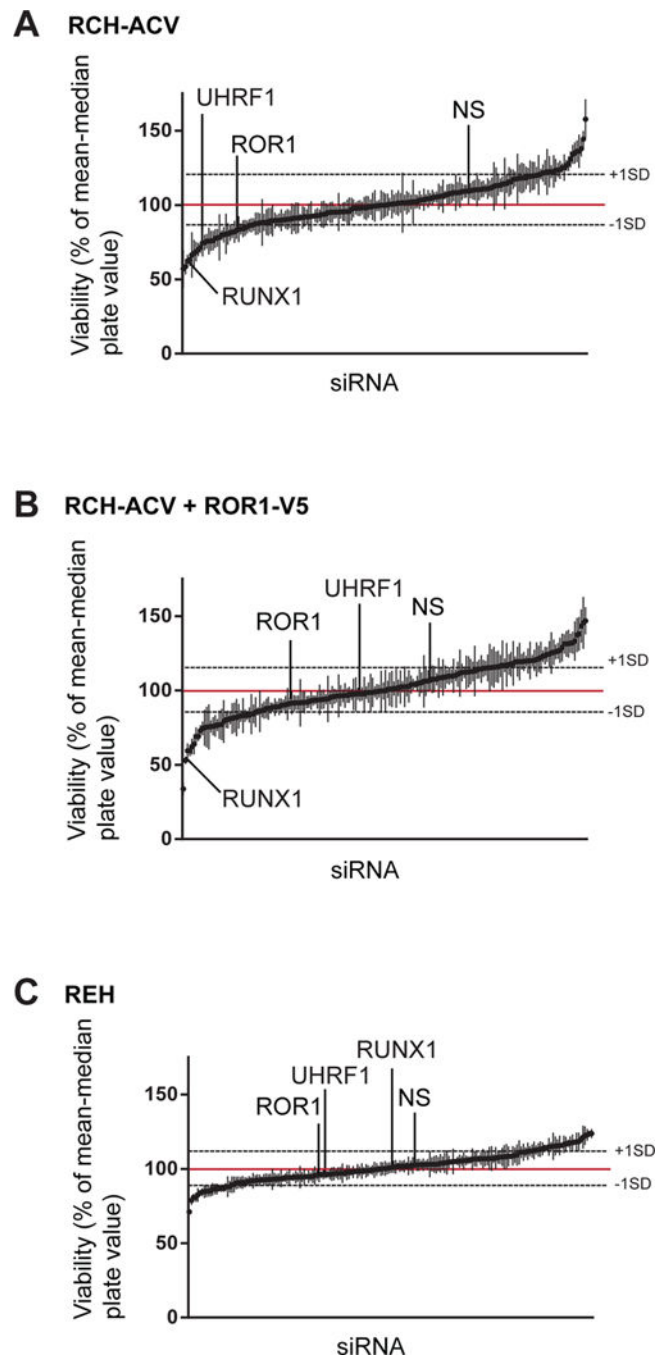


Figure 1. UHRF1 is a potential regulator of ROR1 in t(1;19) pre B-ALL
 (A) Parental RCH-ACV cells (N=4), (B) RCH-ACV stably expressing ROR1-V5 (N=3), or (C) REH (N=3) cells were electroporated with siRNAs targeting transcription factors or chromatin modifiers/epigenetic regulators. Cell viability was measured by MTS assay. siRNA 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 = S.D. NS = non-specific siRNA.

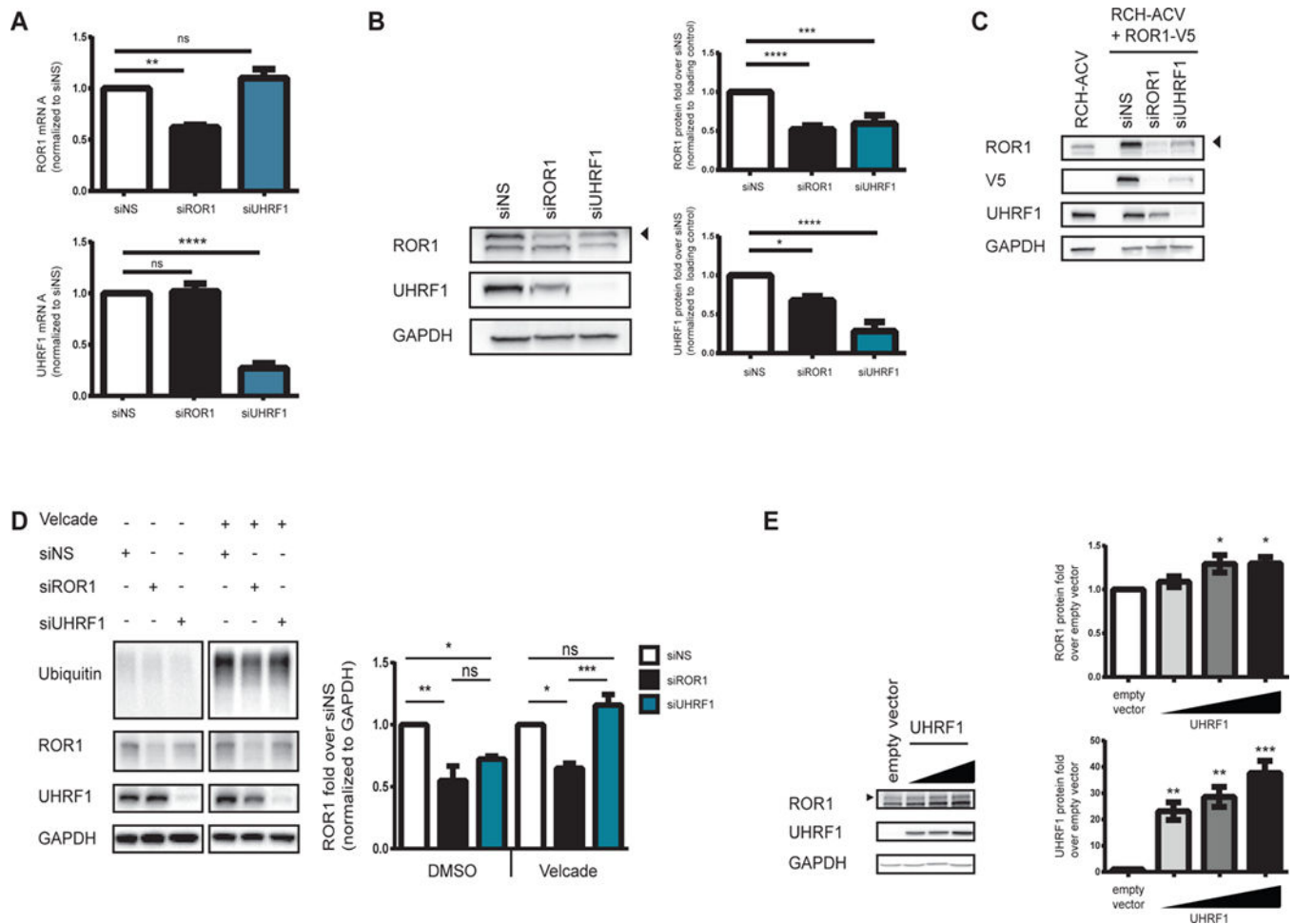


Figure 2. UHRF1 mediates ROR1 protein but not mRNA expression in RCH-ACV cells
 Cells were electroporated with siRNA and (A) 72 hours after electroporation, total RNA and whole protein lysates were harvested and tested for mRNA expression by qRT-PCR (N=3 **p=0.005, ***p<0.0001) and protein levels by (B) immunoblot (N=6 ****p<0.0001, ***p<0.0005, *p<0.05). mRNA and protein levels were normalized to a non-specific siRNA control (siNS). (C) RCH+ROR1-V5 cells were treated with siRNA and whole cell lysates were subjected to immunoblot (N=4). (D) 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 siNS samples treated with DMSO (*p<0.05, **p<0.005) or Velcade (*p<0.05, ***p<0.0005). UHRF1 protein was quantified and normalized to siNS samples treated with DMSO (*p<0.05). Note that these data are from a contiguous blot (N=3). (E) HEK293T17 cells were transfected with either empty vector or a plasmid encoding 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. Error bars represent S.E.M. “ns” = not significant.

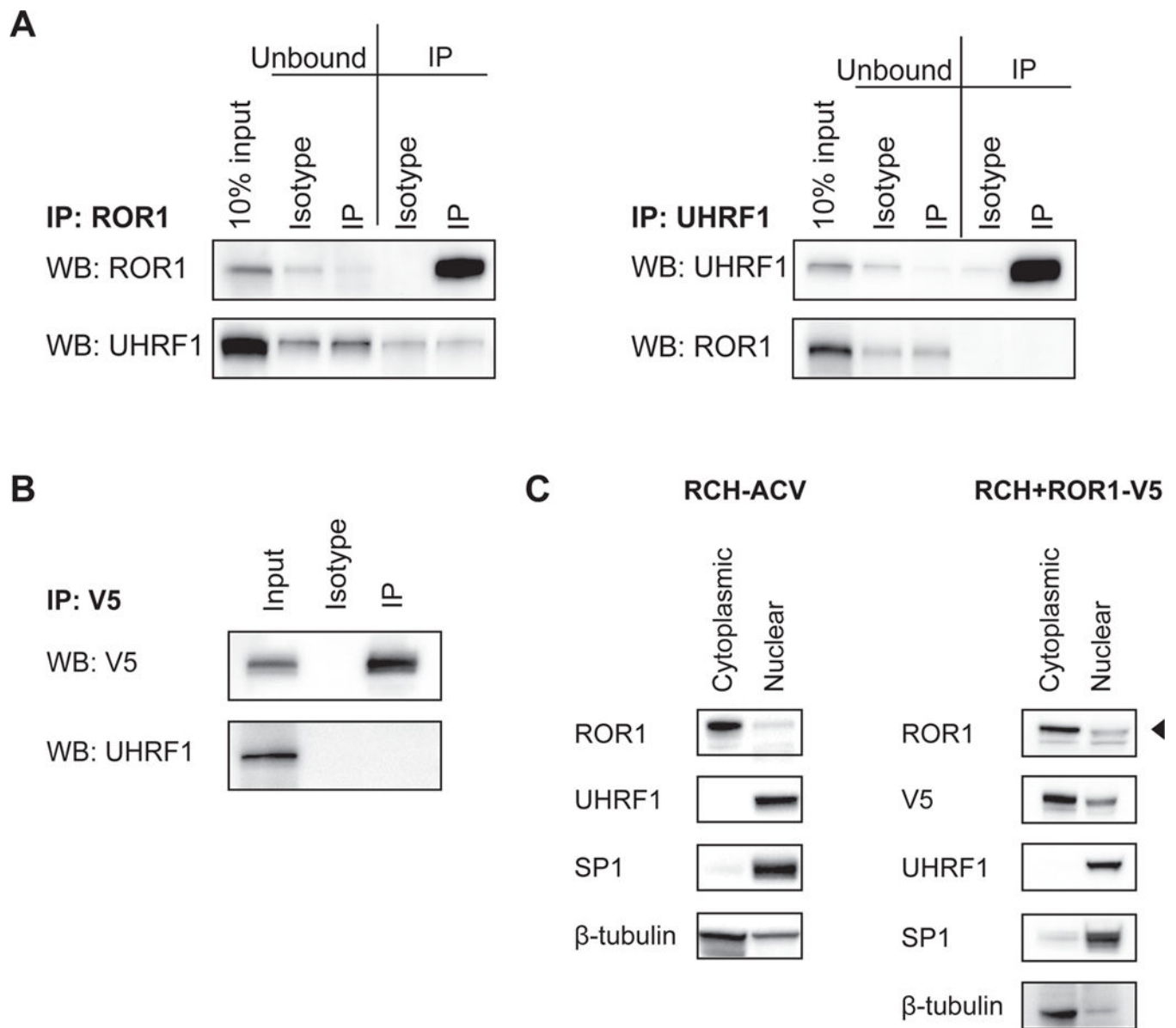


Figure 3. UHRF1 indirectly regulates ROR1

(A) RCH-ACV cell lysate was enriched for either ROR1 (left, IP: ROR1) or UHRF1 (right, IP: UHRF1) and tested for co-immunoprecipitation by immunoblot. (B) HEK293T17 cells were transfected with plasmids encoding V5-tagged ROR1 and UHRF1. Whole cell lysate was enriched using a V5 antibody and tested for co-immunoprecipitation by immunoblot. (C) RCH-ACV (left) and RCH+ROR1-V5 (right) cells were fractionated and localization was detected by immunoblot. “Cytoplasmic” fraction includes membrane-bound proteins. Note that β -tubulin signal in the nuclear fraction is likely contamination between fractions.

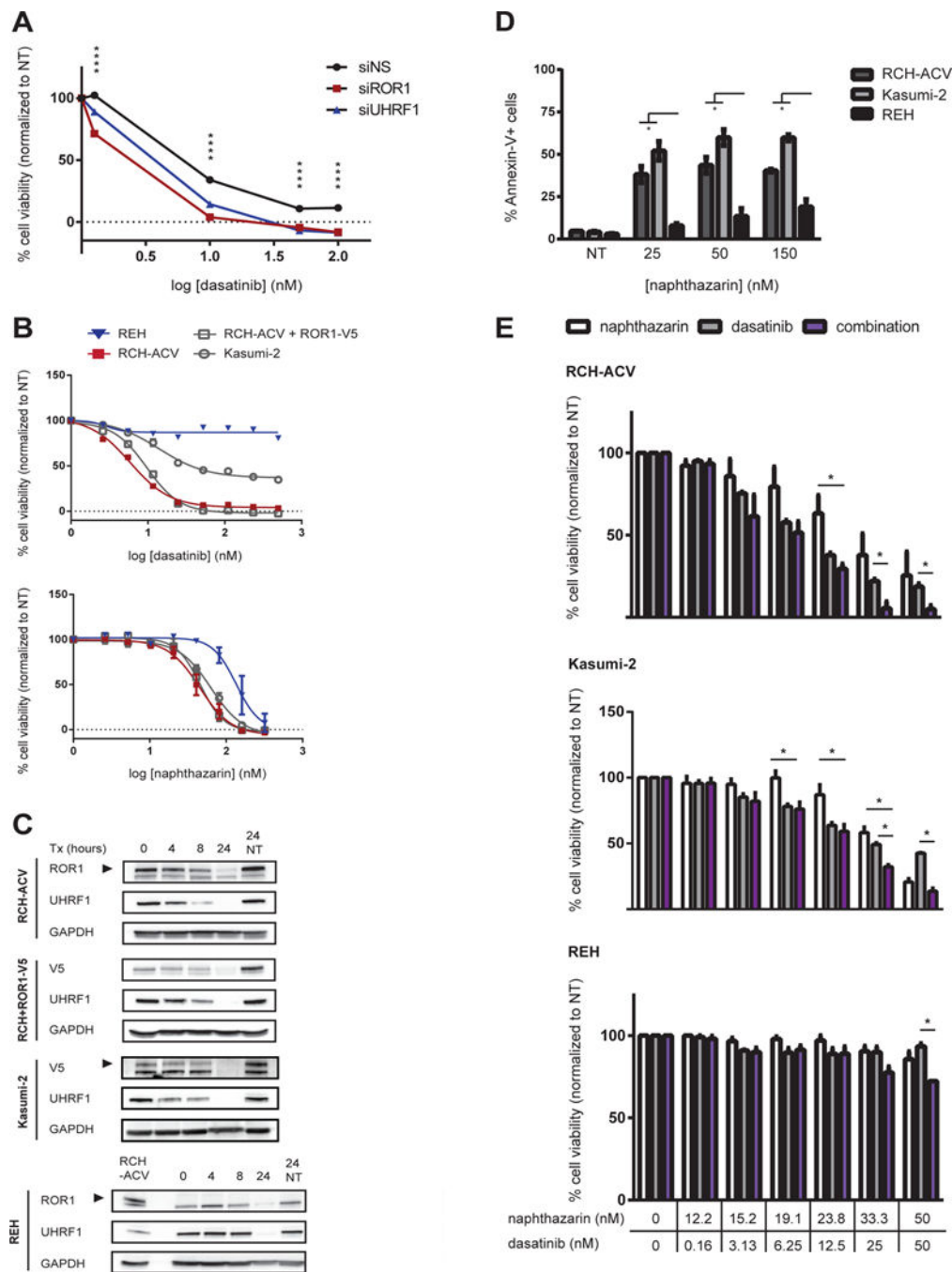


Figure 4. Targeting the UHRF1-ROR1 axis with naphthazarin and dasatinib inhibits t(1;19) pre B-ALL

(A) 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 (****p<0.0001). (B) Cell lines were treated with the indicated drugs (0-0.5 μ M) for 72 hours and cell viability was measured by MTS assay. (C) Cells were treated with naphthazarin for the indicated times and whole cell lysates were tested by immunoblot. RCH-ACV cell lysate was used as a positive control for REH immunoblot. (D)

Cells were treated with naphthazarin for 8 hours and Annexin-V was measured by GuavaNexin Assay (* $p < 0.05$). (E) Cells were treated with naphthazarin, dasatinib, or a combination of both for 72 hours (* $p < 0.05$). Cell viability was measured and normalized to non-treated cells. Data shown is an average of three independent experiments. NT = non-treated Error bars = S.E.M.

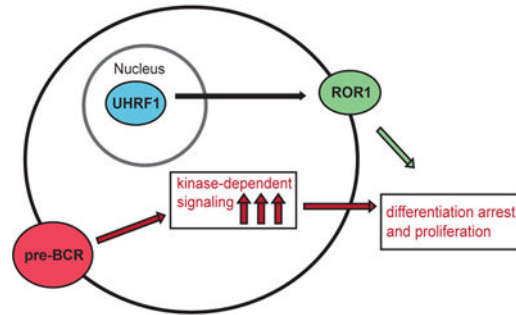
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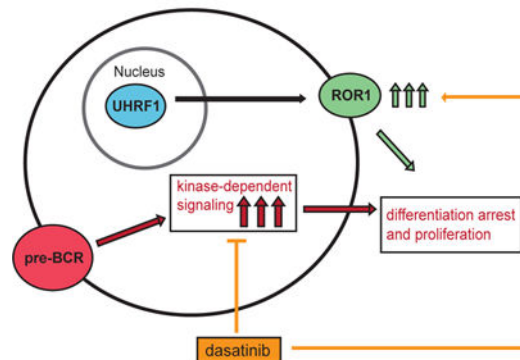
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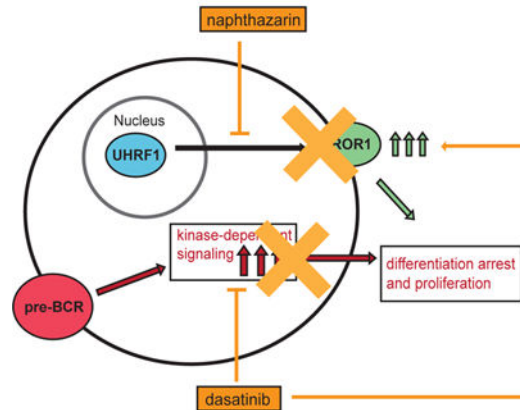
t(1;19) pre-B-ALL



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



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

**Figure 5. Model of UHRF1-ROR1 regulatory mechanism**

The 1;19 fusion product 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).

Table 1

Candidate UHRF1-ROR1 intermediaries

Whole cell lysates from t(1;19) pre-B-ALL cell lines (Kasumi-2 and RCH-ACV) were incubated with a ROR1-specific antibody or an isotype matched control. Protein names, UniProt accession number, and corrected MS/MS spectral counts are shown for each identified protein.

Protein	Description	Accession #	Isotype		ROR1 Antibody	
			Kasumi-2	RCH-ACV	Kasumi-2	RCH-ACV
ALDOA	Fructose-bisphosphate aldolase A	P04075	0	0	3	3
DSG1	Desmoglein-1	Q02413	0	0	6	1
ENPL	Endoplasmic reticulum protein	P14625	0	0	1.471	7.78
IMA1	Importin subunit alpha-1	P52292	0	0	1	3
MCM5	DNA replication licensing factor MCM5	P33992	0	0	3	5
NPM	Nucleophosmin	P06748	0	0	7	7
SRSF3	Serine/arginine-rich splicing factor 3	P84103	0	0	3	4
XRCC6	X-ray repair cross-complementing protein 6	P12956	0	0	5	6

		Accession #	Isotype		UHRF1 Antibody	
			Kasumi-2	RCH-ACV	Kasumi-2	RCH-ACV
ALDOA	Fructose-bisphosphate aldolase A	P04075	0	0	4	1
CEI170	Centrosomal protein of 170 kDa	Q55W79	0	0	10	3
DESP	Desmoplakin	P15924	0	0	6	4
DSG1	Desmoglein-1	Q02413	0	0	5	2
FILA2	Filaggrin-2	Q5D862	0	0	4	1
IGHA1	Ig alpha-1 chain C region	P01876	0	0	4	1
SFPQ	Splicing factor, proline- and glutamine-rich	P23246	0	0	3.027	2.533