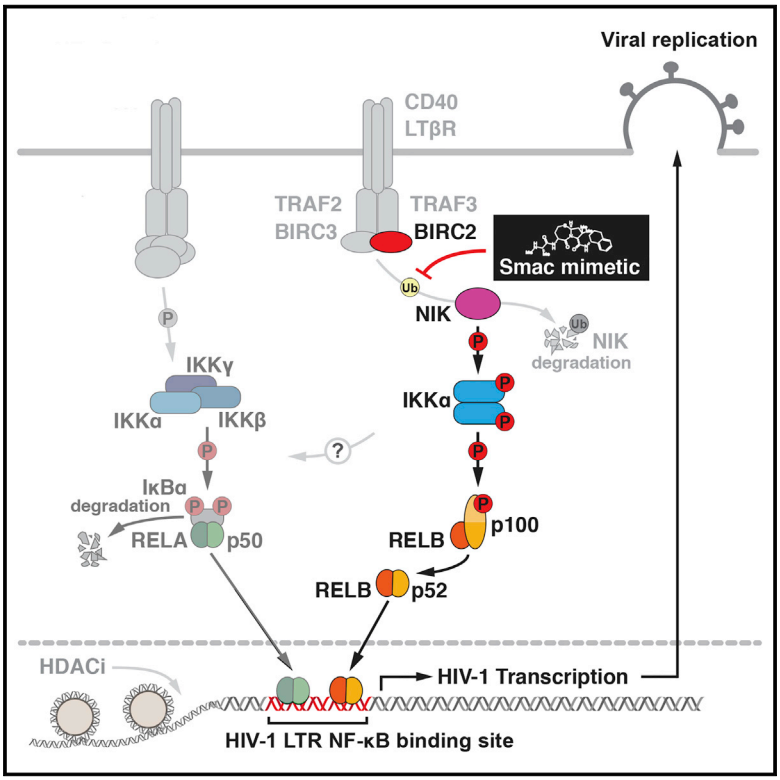
Short Article

Cell Host & Microbe

# BIRC2/cIAP1 Is a Negative Regulator of HIV-1 Transcription and Can Be Targeted by Smac Mimetics to Promote Reversal of Viral Latency

## Graphical Abstract

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## In Brief

Eradicating the latent HIV-1 reservoir represents a challenge. Pache et al. identify BIRC2/cIAP1 as a negative regulator of early HIV-1 transcription. Antagonism of BIRC2 by Smac mimetics, a class of molecules currently under evaluation for cancer, enhances HIV-1 transcription, reactivates latent virus, and synergizes with HDAC inhibitors to reverse latency.

## Highlights

- Targeted RNAi screen identifies host proteins that impede early-stage HIV-1 replication
- BIRC2/cIAP1 is a negative regulator of LTR-dependent HIV-1 transcription
- BIRC2 depletion by Smac mimetic activates NF-κB signaling and reverses HIV-1 latency
- Smac mimetic and HDAC inhibitor synergize to reverse HIV-1 latency in vitro and ex vivo

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# BIRC2/cIAP1 Is a Negative Regulator of HIV-1 Transcription and Can Be Targeted by Smac Mimetics to Promote Reversal of Viral Latency

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## SUMMARY

**Combination antiretroviral therapy (ART) is able to suppress HIV-1 replication to undetectable levels. However, the persistence of latent viral reservoirs allows for a rebound of viral load upon cessation of therapy. Thus, therapeutic strategies to eradicate the viral latent reservoir are critically needed. Employing a targeted RNAi screen, we identified the ubiquitin ligase BIRC2 (cIAP1), a repressor of the noncanonical NF-κB pathway, as a potent negative regulator of LTR-dependent HIV-1 transcription. Depletion of BIRC2 through treatment with small molecule antagonists known as Smac mimetics enhanced HIV-1 transcription, leading to a reversal of latency in a JLat latency model system. Critically, treatment of resting CD4+ T cells isolated from ART-suppressed patients with the histone deacetylase inhibitor (HDACi) panobinostat together with Smac mimetics resulted in synergistic activation of the latent reservoir. These data implicate Smac mimetics as useful agents for shock-and-kill strategies to eliminate the latent HIV reservoir.**

## INTRODUCTION

HIV-1 latency is a state of nonproductive infection in which transcription of viral genes is repressed, likely through the concerted activities of multiple host pathways. While HIV-1 replication can be reduced to undetectable levels using combination antiretroviral therapy (ART), latently infected viral reservoirs can persist for decades (reviewed in Margolis, 2010). In well-suppressed patients, cessation of therapy typically leads to increased viremia within 3–4 weeks, and thus HIV-1-infected individuals must remain on ART throughout their lifetimes. Given the expense and toxicities associated with long-term therapies, pharmacological strategies designed to eradicate the viral latent reservoir represent a critical unmet need. Current "shock and kill" approaches seek to purge this reservoir by treating patients with therapeutics that activate latently infected cells, which are thought to be subsequently eliminated due to viral cytopathic effects or the immune response of the host (Xing and Siliciano, 2013). However, the optimal means for reactivating latent HIV-1 is at present unclear.

The establishment and maintenance of HIV-1 latency is controlled by a multitude of *cis*- and *trans*-acting mechanisms that include factors affecting the local chromatin environment or the levels of specific transcription factors, respectively (Donahue and Wainberg, 2013). Histone deacetylase inhibitors (HDACis) are known as general activators of transcription and have also been shown to reverse latency in multiple model systems (Margolis, 2011). Recent studies have indicated that most HDACi are unable to reactivate latent HIV-1 ex vivo with robust and consistent efficacies (Bullen et al., 2014; Wei et al., 2014). Therefore, it is presently not clear if treatment with an HDACi as single agent is sufficient to effectively reactivate latent HIV-1.

In addition to the local chromatin environment, specific transcription factors are also critical regulators of viral latency. A significant body of evidence indicates that NF-κB signaling plays an important role in the reactivation of latent HIV-1, implicating its regulation as an important therapeutic strategy for latency

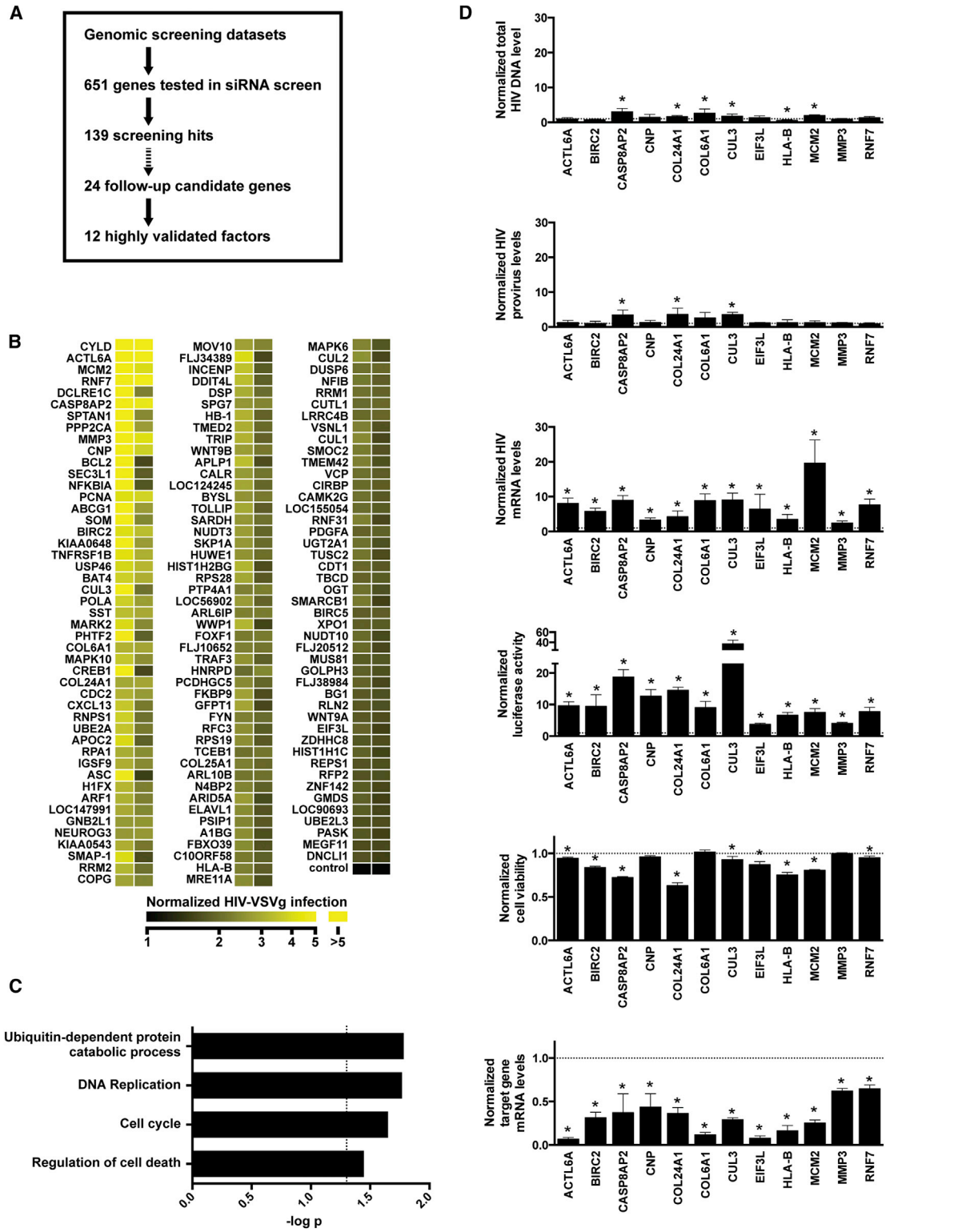


**Figure 1. Identification of Cellular Factors Impeding HIV-1 Replication through Targeted siRNA Screening**

(A) Candidate genes derived from genome-wide screening data sets were evaluated in a siRNA screen in HEK293T cells infected with HIV-1(VSVg). Twenty-four screening hits were selected for further validation, with 12 of these candidate genes being successfully validated using a set of more rigorous criteria (see text).

(B) Heatmap showing the activity of siRNAs targeting 139 genes identified in a siRNA screen described in (A). See also Table S1.

(C) Enrichment analysis of biological processes among screening hits. Shown are selected gene ontology terms enriched with $p < 0.05$ following Benjamini-Hochberg correction.

*(legend continued on next page)*

reversal (Nabel and Baltimore, 1987; Williams et al., 2006). In fact, among the most efficient proposed latency reversing agents (LRAs) are protein kinase C (PKC) activators, including bryostatin, ingenol, and phorbol esters such as PMA and prostratin. These compounds activate the canonical NF-κB pathway and have been found to reverse latency in cellular models and CD4+ T cells from HIV-1-infected patients (Bullen et al., 2014; Spivak et al., 2015; Xing and Siliciano, 2013). However, clinical uptake of these compounds has been complicated by concerns about tumorigenesis and other toxicities, particularly due to uncontrolled cytokine release (Xing and Siliciano, 2013).

Toxicity associated with agonists of canonical NF-κB signaling, which is governed by TAK1/IKK signalosome activation, is in large part due to the acute, short-lived transcriptional activation that is initiated after pathway activation, resulting in a broad inflammatory response. Although abundant evidence implicates canonical NF-κB signaling in the control of HIV-1 transcription and latency, the influence of the noncanonical NF-κB pathway in regulating HIV-1 transcription and latency has not been established. In contrast to canonical NF-κB signaling, the noncanonical NF-κB pathway is characterized by a slower onset, long-lasting transcriptional response, and higher functional selectivity, restricting its impact to a limited number of cellular processes and cell types (reviewed in Sun, 2012). The activation of the noncanonical NF-κB pathway occurs only through a specific subset of tumor necrosis factor receptors (TNFRs), including lymphotoxin beta receptor (LTβR) and CD40. In the absence of stimulation, a complex of BIRC2 (cIAP1), BIRC3 (cIAP2), TRAF2, and TRAF3 constitutively degrades NF-κB-inducing kinase (NIK). Upon receptor activation, BIRC2 and BIRC3 promote the ubiquitination and subsequent degradation of TRAF3, thereby permitting an accumulation of NIK. In turn, NIK activation results in the phosphorylation IKKα, leading to the subsequent proteolytic processing of p100 to p52. p52 forms a heterodimer with the RELB transcription factor and translocates to the nucleus, inducing the expression of target genes. Crosstalk between various canonical and noncanonical NF-κB signaling components, resulting in both positive and negative pathway crossregulation, is considered to be a critical feature in shaping biological responses to a variety of extracellular stimuli (Basak et al., 2007; Shih et al., 2011; Zarnegar et al., 2008).

Here, using targeted RNAi screening, we have identified BIRC2, a repressor of the noncanonical NF-κB pathway, as a potent regulator of HIV-1 transcription and as a therapeutic target for the reversal of latency. Small molecule antagonists of BIRC2, in combination with HDAC inhibitors, activated latent proviruses in both cell line-based models of latency and in primary CD4+ T cells isolated from ART-suppressed HIV-1-infected patients. The results of this study indicate that the pharmacological activation of the noncanonical NF-κB pathway can be used as a component of a combinatorial regimen to reactivate latent HIV-1, while potentially limiting toxicity risks associated with systemic activation of NF-κB signaling by PKC agonists.

## RESULTS

### Identification of Host Cell Factors that Impede Early Stages of HIV-1 Replication

Using a genome-wide siRNA-based loss-of-function screen, we previously identified 295 cellular genes encoding proteins that support viral replication (König et al., 2008). To elucidate cellular factors that interfere with HIV-1 replication, we have reanalyzed this data set along with additional published and unpublished data sets from genome-wide gain- or loss-of-function analyses (Agarwal et al., 2006; Nguyen et al., 2007), identifying 651 genes that were predicted to have a likelihood of impeding HIV-1 replication. Using a triaging strategy based on results from an arrayed siRNA loss-of-function screen (Figure 1A), we evaluated the role of these factors in the early stages of HIV-1 replication, including LTR-mediated transcription. Four distinct siRNAs targeting each candidate gene were evaluated in HEK293T cells infected with a single-cycle, VSV-G-pseudotyped, HIV-1 reporter virus (HIV-1 [VSVg]) in the absence of interferon. Results from this analysis led to the identification of 139 host factors that, when depleted, enhanced viral infection by 50% or more compared to the negative control with at least two independent siRNAs (Figure 1B; see Table S1 available online). Proteins involved in cell cycle regulation, ubiquitination, apoptosis, and DNA replication were found to be enriched in the set of 139 genes (Figure 1C), including CYLD, a gene we recently reported to regulate LTR-dependent transcription (Manganaro et al., 2014).

### Cellular Factors that Limit Viral Transcription

We next performed a more rigorous validation study on 24 of the 139 factors that, when depleted, promoted HIV-1 infection. A gene was considered validated if at least two sequence-independent siRNAs enhanced expression of the luciferase reporter gene by 2.5-fold or more. We excluded genes where RNAi depletion altered cell viability >35% or the mRNA expression level of the targeted gene was not reduced by >35%. We found that 12 of the 24 selected genes met these more rigorous criteria (Table S2), including factors involved in apoptotic signaling (*BIRC2*, *CASP8AP2*), ubiquitin-mediated proteolysis (*CUL3*, *RNF7*), and antigen presentation (*HLA-B*). Only one factor induced G2/M cell cycle arrest upon depletion (Figure S1A) (Groschel and Bushman, 2005; Gummuluru and Emerman, 1999). We next used previously established assays to identify the specific steps of the early viral replication cycle that were influenced by these 12 host factors (Figures 1D and S1B). Knockdown of a subset of genes, including *CASP8AP2*, *CUL3*, and *COL6A1*, led to a modest increase in the levels of viral DNA and integrated provirus. Depletion of eight genes enhanced levels of HIV-1 transcription without significantly affecting levels of integrated provirus, among these the ubiquitin ligase *BIRC2*, a critical regulator of noncanonical NF-κB signaling.

### BIRC2 Antagonist Treatment Enhances HIV-1 Infection

Smac mimetics are synthetic molecules that mimic a critical tetrapeptide sequence from the second mitochondria-derived

(D) Mapping of 12 validated genes to the HIV-1 replication cycle. HEK293T cells were treated with siRNAs targeting the indicated genes. Following infection with HIV-1(VSVg), the levels of HIV-1 total DNA, integrated provirus, and HIV-1 mRNA were determined by qPCR. Also shown are luciferase expression levels, target gene knockdown levels, and cell viability upon target gene knockdown. All values are normalized to nontargeting control siRNAs and represent mean ± SD of at least three biological replicates. * indicates statistical significance as determined by Holm-Sidak t test (alpha = 0.05).

activator of caspase (Smac/Diablo). Smac binds to the baculoviral IAP repeat (BIR) domains that are common to the eight members of the inhibitor of apoptosis (IAP) family of proteins, which includes XIAP, BIRC2, and BIRC3 (Fulda and Vucic, 2012). The IAP proteins differ in function, and only BIRC2 and BIRC3 are known regulators of noncanonical NF-κB signaling. Most Smac mimetics directly compete with caspases for XIAP binding, but also can allosterically activate the E3 ubiquitin ligase activity of BIRC2 and BIRC3, leading to autoubiquitination and subsequent degradation of these proteins. Primarily through their ability to bind XIAP, Smac mimetics can elicit proapoptotic activities, and thus have been developed to treat both solid and hematological cancers (Bai et al., 2014). We have previously described the small molecule SBI-0637142 as a potent Smac mimetic that preferentially targets BIRC2 (Finlay et al., 2014; Vamos et al., 2013). Here, we find that treating HEK293T cells with SBI-0637142 resulted in enhanced HIV-1 replication, similar to the effects of siRNA-mediated *BIRC2* knockdown. This activity was concordant with the depletion of BIRC2 protein, while no change in BIRC3 protein levels was observed (Figure 2A). Furthermore, the loss of BIRC2 led to the accumulation of NIK, indicating that treatment with the Smac mimetic resulted in the activation of the noncanonical NF-κB pathway.

Next, CD4+ T cells isolated from six healthy donors were treated with SBI-0637142 or LCL161, a second Smac mimetic that has been evaluated in phase I/II clinical trials for patients with advanced solid tumors (Bai et al., 2014; Infante et al., 2014). Treatment with SBI-0637142 and LCL161 both enhanced expression of the viral luciferase reporter gene 2- to 10-fold relative to the DMSO control upon HIV-1(VSVg) infection, without inducing significant cytotoxicity (Figure 2B). As expected, both compounds decreased BIRC2 protein levels and resulted in the stabilization of NIK.

## BIRC2 Affects Viral Transcription via NF-κB-Dependent Signaling

The HIV LTR contains two copies of an NF-κB enhancer element known to bind the RELA:p50 heterodimer in response to the activation of canonical NF-κB signaling (Nabel and Baltimore, 1987). Observations using in vitro biochemical systems indicate that the noncanonical RELB:p52 heterodimers also can bind these sequences (Britanova et al., 2008; Fusco et al., 2009). Since knockdown of *BIRC2*, a negative regulator of noncanonical NF-κB signaling, increased expression of HIV-1 mRNA (Figure 2C), we hypothesized that the effects of BIRC2 depletion were mediated through NIK-dependent activation of NF-κB signaling and subsequent interaction of transcription factors with the NF-κB binding sites in the HIV-1 LTR. To test this hypothesis, siRNA-treated HEK293T cells were infected with VSV-G-pseudotyped HIV-1 that had either mutant or native NF-κB binding sites in the LTR (Figure 2D). We found that knocking down *BIRC2* by siRNA treatment had little effect on HIV-1 expression when the NF-κB binding sites were inactivated by mutation. Consistent with these findings, mutating the NF-κB binding sites in the LTR abrogated the effects of SBI-0637142 upon HIV-1 transcription (Figure 2E). Moreover, overexpression of LTβR or CD40, both members of the TNF receptor superfamily that stimulate the noncanonical NF-κB pathway, increased the expression of the viral luciferase reporter gene upon HIV-1(VSVg) infection (Figure 2F). Taken together, these results indicate that BIRC2 affects HIV-1 LTR-dependent transcription through regulation of NF-κB signaling.

## BIRC2 Antagonists Act as Latency-Reversing Agents

Since transcriptional regulation has been implicated in the maintenance of HIV-1 latency, we investigated whether antagonism of BIRC2 can lead to reactivation of latent infection. Treating the latently infected Jurkat cell line JLat 10.6 with SBI-0637142 led to a dose-dependent reactivation of the provirus with negligible effects on cell viability (Figure 3A). The extent of viral latency reversal was found to be proportional to the depletion of BIRC2 and the activation of the noncanonical NF-κB pathway, as indicated by the accumulation of NIK and the processing of p100 to p52. Importantly, we found that three additional Smac mimetics, which have previously been evaluated in clinical trials (Bai et al., 2014), also showed LRA activity in a Jurkat latency model (Figure S2A). This indicates that latency reversal is not limited to individual compounds, but that Smac mimetics more generally represent a novel class of LRAs.

We also monitored the activation kinetics of NF-κB signaling upon treatment with SBI-0637142. After exposing JLat cells to the Smac mimetic, we observed degradation of BIRC2 within 15 min, followed by the stabilization of NIK after 1 hr, and subsequent increases of the p52 protein levels (Figure 3B), indicating the activation of the noncanonical NF-κB pathway. However, we did not observe a reduction in the levels of IκBα by western blot, which is a hallmark of canonical NF-κB signaling activation (Figure 3B). The kinetics of viral RNA (vRNA) expression following the treatment of latently infected Jurkat cells with SBI-0637142 correlated with the observed induction of NIK-dependent NF-κB signaling (Figure S2B).

To determine whether the observed activation of HIV transcription by Smac mimetics is solely mediated through the NIK signaling axis, we generated a *NIK* knockout in the latently infected 2D10 cell line using a CRIPSR-based approach (Figure S2C). The loss of NIK abrogated the reactivation of latent HIV-1 through SBI-0637142 treatment, while not impinging upon the activity of PKC agonist bryostatin, an activator of canonical NF-κB signaling (Figure 3C). To further investigate the mechanism by which induction of the noncanonical pathway results in the LTR-dependent transcriptional activation of HIV-1, we have analyzed the nuclear translocation of NF-κB transcription factors upon treatment of CD4+ T cells with SBI-0637142 (Figure 3D). We found that both p52 and RELB translocate to the nucleus following BIRC2 depletion and NIK accumulation. In addition, low levels of RELA translocation were detected as well. Importantly, the translocation of RELA paralleled the delayed kinetics of NF-κB activation via the noncanonical NIK-p100 signaling axis, in contrast to the rapid nuclear accumulation resulting from TNFα stimulation. This suggests that activation of RELA was likely due to pathway crosstalk initiated after the induction of noncanonical signaling.

To further investigate this result, we used chromatin immunoprecipitation (ChIP) to assess the physical occupancy of the HIV-1 LTR by NF-κB transcription factors after induction of noncanonical signaling (Figure 3E). We find that Smac mimetic treatment leads to an association of RELB with the viral LTR, suggesting that noncanonical NF-κB transcription factors can

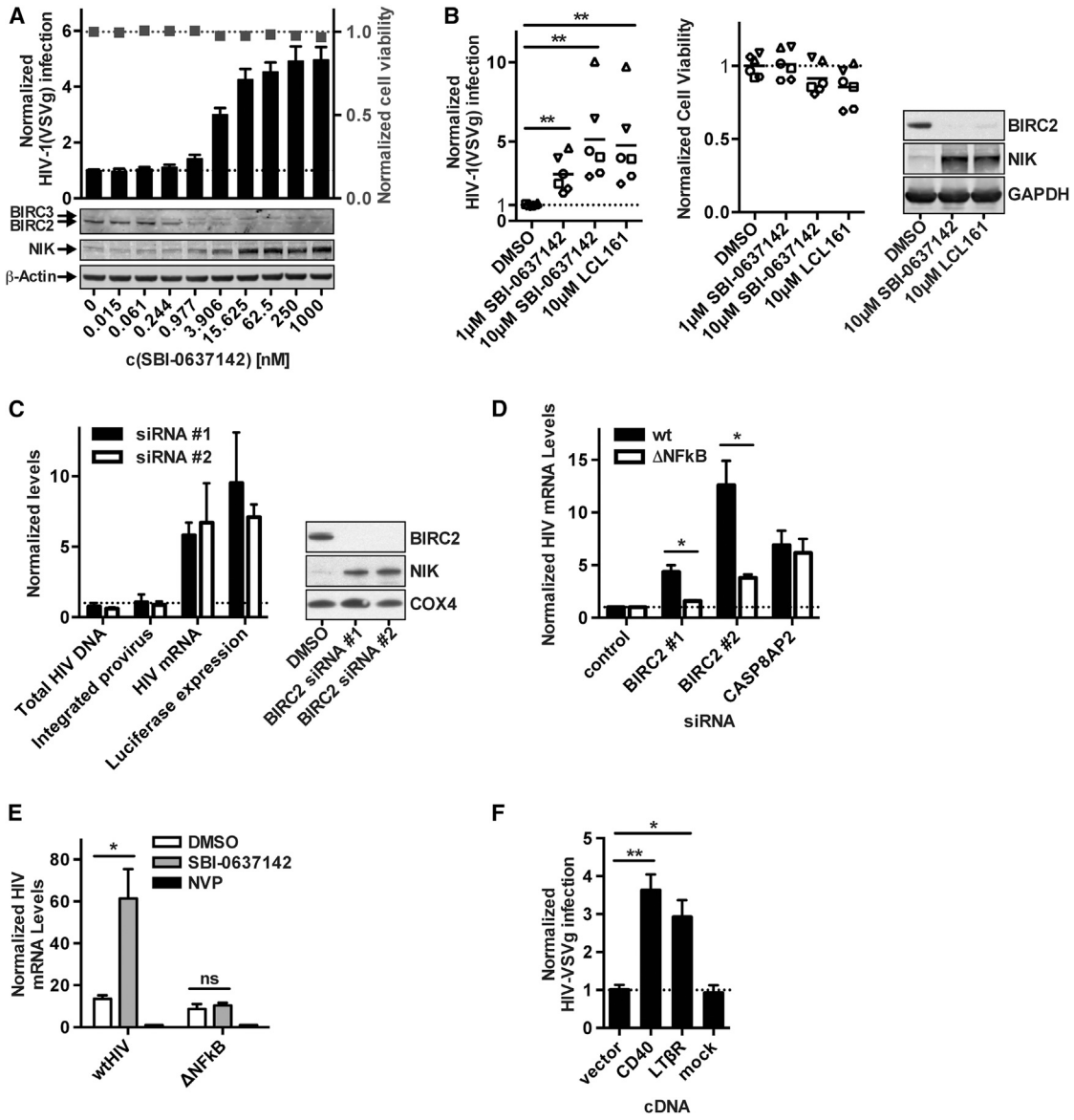


**Figure 2. Effects of BIRC2 Depletion on HIV-1 Transcription in HEK293T Cells and Primary Cells**

(A) HEK293T cells were treated with the BIRC2 antagonist SBI-0637142 at the indicated concentrations and infected with HIV-1(VSVg) for 24 hr. Levels of infection were evaluated by measuring luciferase reporter activity. Lysate of SBI-0637142-treated cells was evaluated for BIRC2 and NIK protein levels by western blot.

(B) Primary activated CD4+ T cells isolated from six healthy donors were treated with SBI-0637142 or LCL161 at the indicated concentrations for 24 hr. Cells were subsequently infected with HIV-1(VSVg) for 48 hr before analysis of luciferase reporter activity. Cell viability was evaluated by measuring cellular ATP levels. Each data point indicates mean of biological triplicates from a single donor. Lines indicate mean of six donors. BIRC2 depletion and NIK accumulation were analyzed by western blot.

(C) HIV-1(VSVg)-infected HEK293T cells treated with siRNAs targeting *BIRC2* were analyzed for levels of total HIV-1 DNA, integrated provirus, and HIV-1 mRNA by qPCR. Luciferase expression levels were evaluated in parallel. All values are normalized to nontargeting control siRNAs. BIRC2 and NIK protein expression levels upon siRNA treatment were analyzed by western blot.

(D) siRNA-treated HEK293T cells were infected with VSVg-pseudotyped HIV-1 (WT) or a virus mutant lacking functional NF-κB binding sites (ΔNFκB). Viral mRNA was measured by qPCR 24 hr after infection, and values were normalized to nontargeting control siRNAs. *CASP8AP2*-targeting siRNAs are shown as control.

(E) HEK293T cells were treated with 1 μM SBI-0637142 and infected for 24 hr with VSVg-pseudotyped HIV-1 containing either a functional or mutated NF-κB binding site in the viral LTR. HIV-1 mRNA levels were quantified by qPCR and normalized to samples from cells treated with 5 μM nevirapine (NVP).

(F) HEK293T cells transfected with vectors expressing CD40 or LTβR were subsequently infected with HIV-1(VSVg) for 24 hr. Viral infection was quantified by measuring expression of the viral encoded luciferase reporter and normalized to cells transfected with an empty vector as negative control.

All data are represented as mean ± SD of three biological replicates (A, B, and D) or as mean ± SEM of at least three independent experiments (E–G). p values were calculated using an unpaired t test with *$p < 0.05$ and **$p < 0.01$.

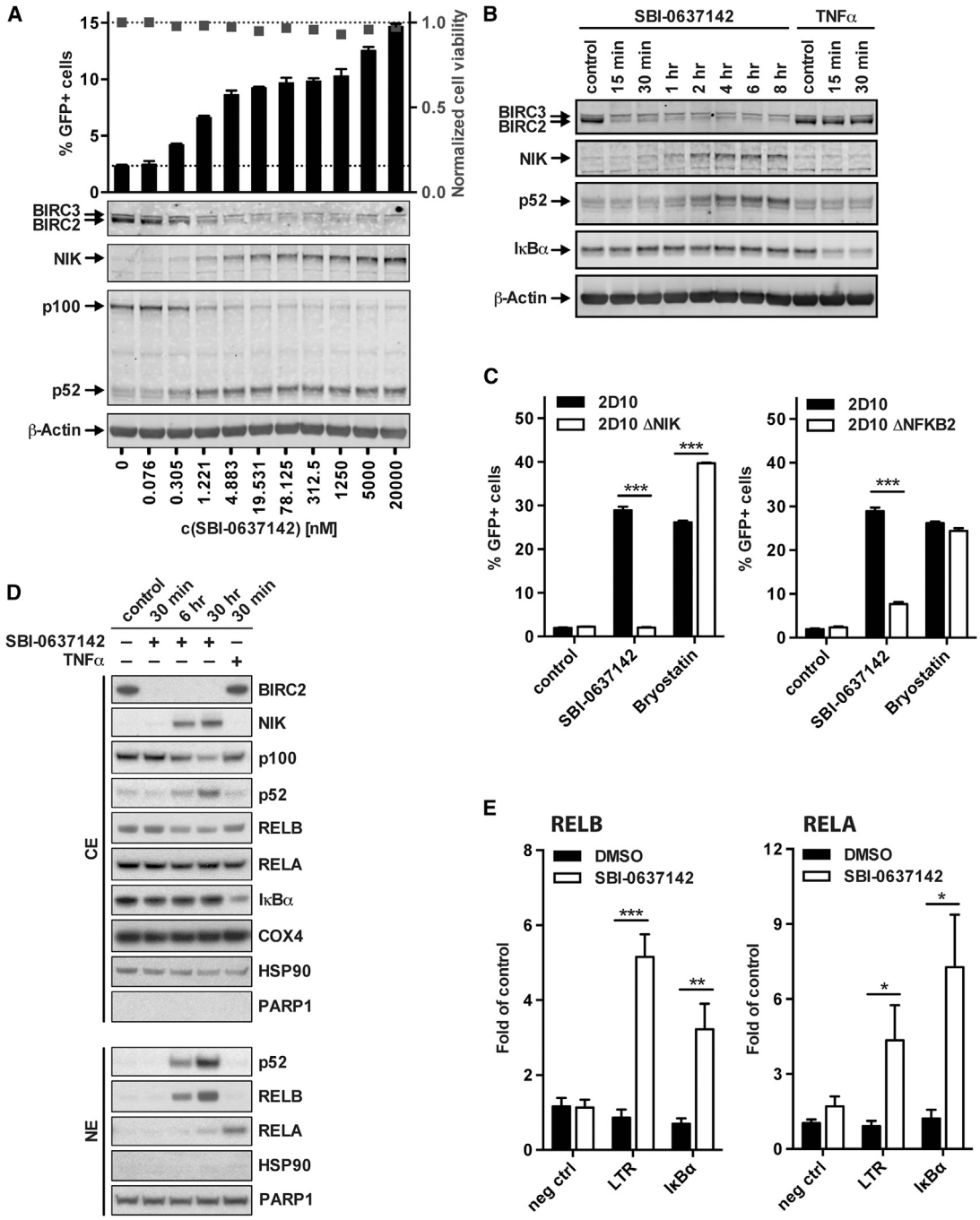


**Figure 3. Smac Mimetic-Mediated BIRC2 Depletion Leads to the Reactivation of Latent HIV-1 in JLat Cells**

(A) JLat 10.6 cells were treated with increasing amounts of SBI-0637142 for 36 hr. Reversal of latency was determined by FACS analysis of GPF expression. Depletion of BIRC2, accumulation of NIK, and processing of p100 to p52 were analyzed by western blot. FACS data are represented as mean ± SEM of three experiments.

(B) Kinetics of NF-κB activation upon treatment of JLat 10.6 cells with 1 μM SBI-0637142 were assessed by western blot. Treatment with 10 ng/ml TNFα served as positive control for canonical NF-κB pathway activation.

(C) 2D10 cells and clones with a knockout of *NIK* or *NFKB2* were incubated with 1 μM SBI-0637142 or 30 nM bryostatin for 36 hr. GFP expression was analyzed by FACS. Data are represented as mean ± SD of three biological replicates.

(D) CD4+ T cells from healthy donors were treated with 1 μM SBI-0637142 or 10 ng/ml TNFα. Cytoplasmic (CE) and nuclear (NE) extracts were analyzed by western blot. HSP90 and PARP1 served as control for cytoplasmic and nuclear proteins, respectively.

(E) 2D10 cells were treated with 1 μM SBI-0637142 for 9 hr prior to ChIP analysis using antibodies against RELA, RELB, or IgG as control. RELA- and RELB-specific association with the HIV-1 LTR and the IκBα gene promoter region, or an intergenic region upstream of the *PABPC1* gene not known to contain NF-κB binding sites as negative control, was analyzed by qPCR using specific primers and is shown as fold enrichment over IgG control. Data are represented as mean ± SEM of at least three experiments. *p < 0.05, **p < 0.01, ***p < 0.001, determined by unpaired t test.

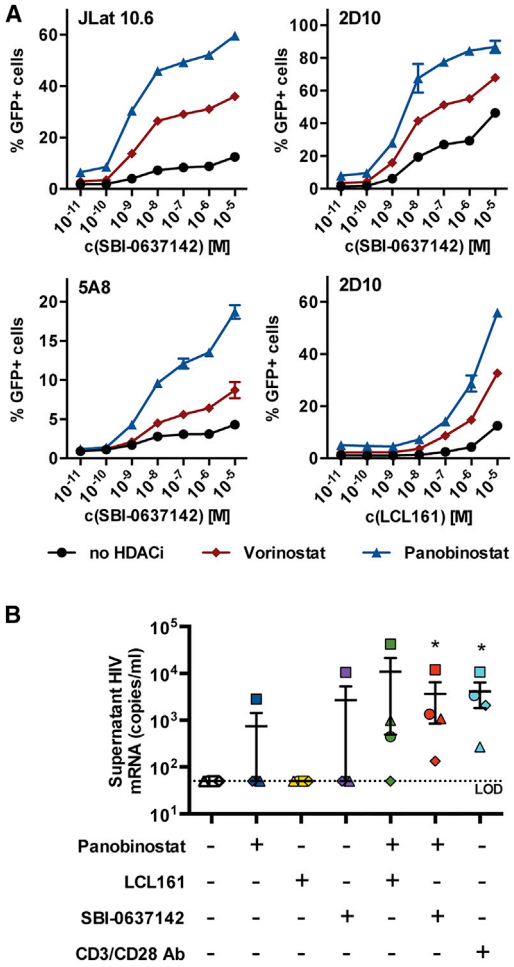


**Figure 4. Combined Treatment with Smac Mimetics and HDAC Inhibitors Reverses HIV-1 Latency in Jurkat-Based Latency Models and Patient-Derived Resting CD4+ T Cells**

(A) Latently infected Jurkat cell lines were treated with increasing amounts of SBI-0637142 alone or in combination with panobinostat or vorinostat. JLat 10.6 and 5A8 cells were treated with 10 nM panobinostat and 500 nM vorinostat, where indicated. 2D10 cells were treated with 5 nM panobinostat and 250 nM vorinostat. GFP expression was evaluated after 36 hr by FACS. Data are represented as mean ± SD of three biological replicates.

(B) Resting CD4+ T cells from HIV-1-infected patients under ART were treated with 100 nM panobinostat, 10 μM LCL161, 10 μM SBI-0637142, or a combination thereof for 48 hr. Viral production was subsequently evaluated by detection of viral mRNA in cell supernatants using qPCR. Data are represented as mean ± SEM of four donors. Significance of treatments was evaluated using a ratio paired t test. *$p < 0.05$.

directly influence HIV transcription. Moreover, we also detected binding of RELA to the HIV LTR, implicating a concerted action of both canonical and noncanonical transcription factors in HIV-1 activation upon Smac mimetic treatment. TNFα treatment, by contrast, resulted in RELA binding to the LTR but did not lead to a significant interaction with RELB (Figure S2D). To discern the functional contribution of the noncanonical RELB:p52 heterodimer to LRA activity after Smac mimetic treatment, we created a cell line with a knockout of the *NFKB2* gene, resulting in a loss of p100 expression (Figure S2E). In the absence of p100, and thereby p52, the reversal of HIV latency by Smac mimetics was reduced by ~75% (Figure 3C). Taken together, these results indicate that the LRA activity of Smac mimetics is solely initiated through the NIK-dependent noncanonical NF-κB signaling pathway. Induction of this pathway activates both the RELB:p52 heterodimer and, to a lesser extent, the RELA:p50 heterodimer, and results in the activation of LTR-dependent transcription that is primarily, but not exclusively, regulated by the noncanonical NF-κB transcription factor heterodimer.

### BIRC2 Antagonist Treatment Acts Synergistically with HDAC Inhibitors to Reverse HIV-1 Latency

LRAs, such as PKC activators and HDAC inhibitors, have been shown to activate latent provirus synergistically (Burnett et al., 2010; Laird et al., 2015). We thus investigated the effects of Smac mimetics together with HDACis in three Jurkat-derived latency models—JLat 10.6, 2D10, and 5A8 cells (Figures 4A and S3A) (Pearson et al., 2008; Sakane et al., 2011). In each of the three cell lines tested, the combination of the Smac mimetic SBI-0637142, with either of the HDAC inhibitors panobinostat or vorinostat, reactivated latent provirus synergistically (Figure S3B). Although less potent, the Smac mimetic LCL161 showed similar synergy.

We next evaluated Smac mimetics for their ability to reverse latency in resting CD4+ T cells collected from HIV-1-infected patients undergoing ART (n = 4). Cells were treated with SBI-0637142 or LCL161 alone, or in combination with the HDACi panobinostat, and release of viral genomic RNA to the supernatant was measured by the recently described REVEAL (rapid ex vivo evaluation of antilatency) assay (Spivak et al., 2015). While none of the small molecules were found to activate latent provirus after 48 hr when used individually, both LCL161 and SBI-0637142 in combination with panobinostat were found to reactivate latent HIV-1 at levels comparable to those achieved upon treatment with antibodies against CD3 and CD28 (Figure 4B). Importantly, these results reach statistical significance ($p < 0.05$) for SBI-0637142 and CD3/CD28 antibody treatment, and no significant activation of resting CD4+ T cells was observed upon treatment with SBI-0637142 (Figure S3C).

## DISCUSSION

Using a targeted siRNA screen, we identified *BIRC2*, a negative regulator of noncanonical NF-κB signaling, as a regulator of viral transcription. Critically, we observed that induction of noncanonical signaling results in both RELA:p50 and RELB:p52 heterodimeric transcription factors binding to the HIV-1 LTR, with genetic loss-of-function studies indicating that the transcriptional activation by RELB:p52 is the predominant regulator of this activity. Taken together, these results support an unappreciated role for the noncanonical NF-κB signaling machinery, and specifically RELB:p52, in the regulation of HIV-1 LTR-dependent transcription.

There have been considerable efforts focused on understanding the molecular basis of HIV-1 latency and devising pharmacological strategies to activate the latent provirus. Ideally, an LRA should only target cell types that are latently infected so as to avoid toxicity or widespread immune activation. In contrast to

strategies targeting canonical NF-κB signaling, it may be preferable to target components of the noncanonical NF-κB pathway, as it is active in a more restricted set of cell lineages. Smac mimetics are considered a promising new class of cancer therapeutics that are well tolerated in vivo, and primarily through their antagonism of XIAP, they promote apoptosis in tumor cells, while normal tissue remains unaffected (Fulda and Vucic, 2012). Many Smac mimetics can also trigger the noncanonical NF-κB signaling pathway through the depletion of BIRC2 or BIRC3. Although constitutive activation of the noncanonical NF-κB pathway through somatic mutations in mice has been associated with hematogenous malignancies including B cell lymphomas (Keats et al., 2007), proposed latency-reversing "shock and kill" approaches entail acute treatment regimens, largely mitigating these risks associated with chronic activation. In fact, six Smac mimetics, including LCL161, have been evaluated in clinical trials and have favorable safety and pharmacokinetic/pharmacodynamic (PK/PD) profiles (Bai et al., 2014). Unlike broadly acting Smac mimetics that target multiple proteins of the IAP family including the caspase inhibitor XIAP, certain compounds, such as SBI-0637142, preferentially target BIRC2 or BIRC3, the only known regulators of noncanonical NF-κB signaling among the IAP proteins (Finlay et al., 2014). Future studies will be required to investigate whether the observed increased potency of SBI-0637142 as an LRA may be related to its selectivity profile.

Many current strategies for reactivating latent HIV-1 focus on the use of PKC agonists to stimulate canonical NF-κB signaling. Due to toxicity risks, safety concerns have dampened enthusiasm for the use of these canonical NF-κB activators as LRAs (Morgan et al., 2012). HDACi have also shown promise as LRAs, with multiple compounds being evaluated in clinical trials (Rasmussen et al., 2013). Although HDACis increase intracellular levels of HIV-1 mRNA both in vitro and in vivo, the level of viral outgrowth induced by these compounds is likely insufficient to purge the viral reservoir (Bullen et al., 2014; Wei et al., 2014). Therefore, it is expected that a safe and effective drug regimen to reverse HIV-1 latency will require the combination of multiple agents (Xing and Siliciano, 2013), much like ART. Some HDACi have been shown to synergize with different classes of LRAs including PKC agonists (Laird et al., 2015), indicating that combinatorial use could increase efficacy while reducing required dosage. Consistently, we see similar levels of synergy between Smac mimetics and HDACi as levels reported for combinatorial treatment with HDACi and PKC agonists (Wong et al., 2014).

Given the scarcity of clinical data to date, the optimal strategy for reversing HIV-1 latency in patients is far from certain. Our results demonstrating that Smac mimetics, in conjunction with the HDACi panobinostat, can reverse latency in patient-derived resting CD4+ T cells suggest a promising clinical approach toward the development of a "cure" for patients with HIV-1. Importantly, the established clinical safety and pharmacodynamic profiles of Smac mimetics should enable this class of small molecule antagonists to be readily evaluated as a therapeutic strategy. Taken together, these data indicate that rapid preclinical development and clinical repositioning of Smac mimetics may help provide a safe and effective combinatorial therapeutic regimen to eradicate HIV-1.

## EXPERIMENTAL PROCEDURES

### siRNA Transfections and Infection with HIV-1

siRNA transfections of HEK293T cells and infections with a single-cycle envelope deleted, VSV-G-pseudotyped HIV-1 reporter virus (HIV-1[VSVg]), were performed as previously described (König et al., 2008). Cells were infected 48 hr after siRNA transfection, and luciferase expression levels were determined 24 hr after infection. Mapping to viral replication cycle stages was done by isolating mRNA and DNA from infected cells and quantifying proviral DNA content, total HIV DNA, and HIV mRNA levels by qPCR. Cells infected with VSV-G-pseudotyped HIV-1 containing either wild-type or mutant NF-κB binding sites in the LTR (Bosque and Planelles, 2009) were analyzed by measuring HIV mRNA levels by qPCR. Cell viability was analyzed using the ATPlite cell viability assay (Perkin Elmer).

### HIV-1 Infection of Human CD4+ T Cells

Following isolation, CD4+ T cells from healthy donors were activated with 4 μg/ml phytohemagglutinin-P (PHA, Sigma) for 48 hr. Activated CD4+ T cells were treated with compounds for 24 hr prior to infection with HIV-1(VSVg). Luciferase expression levels were normalized to mock-treated cells; mean of DMSO-treated cells was defined as 1.

### Jurkat HIV Latency Model

Latently infected Jurkat cells were treated with compounds and subsequently analyzed for GFP expression by flow cytometry. Analytical cytometry was performed in the Sanford Burnham Prebys Flow Cytometry Core. Cell viability was determined using the ATPlite cell viability assay.

### Chromatin Immunoprecipitation

2D10 cells were stimulated with 1 μM SBI-0637142 or DMSO for 9 hr prior to ChIP using antibodies targeting RELA and RELB.

### Treatment of Resting CD4+ T Cells from Aviremic HIV Patients

Resting CD4+ T cells isolated from aviremic HIV-1-infected patients on ART were cultured in the presence or absence of reactivating compounds for 48 hr. Culture supernatants were analyzed by two-step qPCR using a primer and probe set for conserved regions of the 3′ LTR of HIV-1 mRNA as previously described (Spivak et al., 2015).

Please refer to the Supplemental Experimental Procedures for additional information.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.chom.2015.08.009.

## AUTHOR CONTRIBUTIONS

L.P., J.A.T.Y., and S.K.C. conceived the study. L.P., J.M.M., Y.H., A.M.M., L.M., and R.K. conducted screening and validation experiments. L.P., M.S.D., and J.P.M. conducted mechanistic studies. A.M.S., L.J.M., and A.B. conducted patient cell assays. M.V., P.T., and N.D.P.C. developed and synthesized compounds. V.S., A.F.-S., N.D.P.C., F.D.B., J.A.T.Y., V.P., and S.K.C. supervised the studies. L.P. and S.K.C. wrote the manuscript.

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