Combinatorial Latency Reactivation for HIV-1 Subtypes and Variants

Running Title: HIV-1 Combinatorial Anti-latency Strategies

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Abstract

The eradication of HIV-1 will likely require novel clinical approaches to purge the reservoir of latently infected cells from a patient. We hypothesize that this therapy should target a wide range of latent integration sites, act effectively against viral variants that have acquired mutations in their promoter regions, and function across multiple HIV-1 subtypes. By using primary CD4+ and Jurkat-based in vitro HIV-1 latency models, we observe that single-agent latency reactivation therapy is ineffective against most HIV-1 subtypes. However, we demonstrate that the combination of clinically promising drugs – namely prostratin and suberoylanilide hydroxamic acid (SAHA) – overcomes the limitations of single-agent approaches and can act synergistically for many subtypes including A, B, C, D, and F. Finally, by identifying the proviral integration position of latent Jurkat clones, we demonstrate that this drug combination does not significantly enhance the expression of endogenous genes nearest to the proviral integration site, indicating that its effects may be selective.
Introduction

HIV-1 post-integration latency poses the greatest barrier to complete eradication of the virus from a patient (25). Latent infections have low or no transcriptional activity and fail to generate viral progeny, rendering them untreatable with current antiretroviral treatments that only target actively replicating virus (78). Moreover, latent infections, which persist in resting memory CD4+ T-cells with a half-life of up to 44 months (79), provide a permanent reservoir for reactivation and reseeding of the replicating virus (83). Therapeutic reactivation of latent infections, combined with antiretroviral treatments, may accelerate the depletion of latent reservoirs (reviewed in (29)). However, such latency reactivation strategies have yielded variable results in recent clinical trials (49, 80, 82), underscoring the difficulties associated with purging latent infections. Therefore, the complexities of latency warrant the further development of *in vitro* analytical and screening assays that can model the conditions of latency and test potential therapies (9).

After viral entry and integration of the viral genome into the host chromosome, the HIV 5′ long terminal repeat (LTR) promoter recruits RNA polymerase II (RNAPII) and other host factors to regulate viral gene expression. Initially, low basal transcription generates primarily abortive transcripts – due to the stalling of RNAPII – and a small fraction of fully elongated viral transcript that is initially spliced to generate mRNA encoding a positive regulator, the transcriptional activator (Tat) (47). Tat protein interacts with cellular positive transcriptional elongation factor B (P-TEFb) (99), and the resulting Tat:P-TEFb complex binds to the trans-activation response (TAR) element at the 5′ end of nascent viral transcripts (3). Here, P-TEFb phosphorylates the C-terminal domain (CTD) of stalled RNAPII to enhance the efficiency of
elongation (98). This Tat-mediated transactivation thereby amplifies viral gene expression nearly 100-fold, yielding a strong positive feedback loop (23).

In addition to Tat and host elongation factors, other cellular activating and repressing mechanisms control transcription and the local chromatin environment of the integrated virus (52, 86). Viral transcription correlates with the recruitment of histone acetyltransferase (HAT) proteins to the LTR and the subsequent acetylation of both histone tails (57) and the Tat protein (41). Alternatively, gene silencing and heterochromatin assembly are driven by the removal of acetyl moieties from histone tails by histone deacetylases (HDACs) (36, 92). Within the U3 enhancer region of viral 5' LTR, numerous cis-binding elements recruit positive and negative factors that regulate histone modifications and chromatin structure (Figure 1B). Among these elements, NFAT and AP-1 sites recruit activating factors (11, 53), while YY1 sites recruit silencing factors including HDACs (72). Furthermore, κB and Sp1 binding sites promote either transcriptional silencing via HDAC recruitment (58, 92) or activation through recruitment of HATs and transcription factors (2, 26). Collectively, these sites play significant and sometimes synergistic roles in the decision between viral replication vs. the establishment of latency (10, 68).

*In vitro* models of HIV latency, often composed of an integrated HIV-1-based vector in CD4+ Jurkat cells, have revealed that nonproductive transcription after viral integration may result from repressed chromatin (37, 92), transcriptional interference from nearby genes (50), the absence of elongation factors (94), and insufficient levels of the viral protein Tat for viral transactivation (89, 90). Using the HIV-1 based *LTR-GFP-IRES-Tat (LGIT)* lentivirus in Jurkat cells, we have previously demonstrated that – due to stochastic fluctuations in Tat concentration – clonal populations with single integrations of *LGIT* may phenotypically bifurcate (PheB) into...
inactive (Off) and active (Bright) populations (89). Moreover, inactivating point mutations introduced into each of the Sp1 or κB elements in the HIV LTR of the LGIT model have revealed that each of these elements uniquely contributes to the recruitment of repressing and activating factors and to the overall stabilities of the Off and Bright expression modes (10). In particular, mutation of κB site I (mut1 NF-κB) decreases recruitment of the NF-κB activating heterodimer p50-RelA, while mutation of Sp1 site III (mutIII Sp1) impedes the recruitment of both p50-RelA and the HAT p300 (10). Since different HIV-1 subtypes and circulating recombinant forms (CRFs) throughout the world have sequence variability within the same Sp1 and κB elements, as well as in other domains throughout U3, these results suggest that subtypes may access distinct latency mechanisms and raise the problematic possibility that each may require a distinct, “tailored” reactivation strategy.

Due to the complex nature of latency, the success of clinical efforts to purge latent reservoirs will depend on the ability to reverse one or more of the possible latency mechanisms (reviewed in (29)). Resting CD4+ T-cells, which maintain low levels of activating factors NF-κB and NFAT (27, 42), provide a cellular environment that favors silenced proviral gene expression and latent infections. In vitro studies suggest that activation of resting CD4+ T-cells with proinflammatory cytokines would also reactivate latent infections (63). However, in vivo activation of resting CD4+ T-cells with proinflammatory cytokines IL-2 and IFN-γ or the anti-CD3 monoclonal antibody OKT3 results in long-term depletion of all CD4+ T-cells and fails to measurably purge the latent reservoir (46, 87). Moreover, T-cell activation therapies are ineffective against latent infections in actively dividing cells (35) and are unlikely to stimulate latent infections attributed to chromatin silencing or transcriptional interference (reviewed in (93)).
As a potentially more effective alternative to T-cell activation with cytokines, latency reactivation therapies may utilize pharmacological agents that directly target latency mechanisms. For example, direct activation of the NF-κB pathway with the cytokine tumor necrosis factor alpha (TNF-α) increases the nuclear concentration of the activating p50-RelA heterodimer and induces viral NF-κB-dependent gene expression (19). Similarly, phorbol myristate acetate (PMA) and prostratin – a non-tumor-promoting phorbol ester – stimulate a portion of latent infections by activating the NF-κB and PKC pathways to enhance the recruitment of activating factors and P-TEFb to the LTR (84, 91). However, like T cell activation therapies, these mitogens may fail to target latent infections that arise from transcriptional interference or chromatin silencing (93). To reverse the effects of chromatin silencing, latent infections may require the treatment with HDAC inhibitors such as trichostatin A (TSA) (86) and clinically tested HDAC inhibitors suberoylanilide hydroxamic acid (i.e. SAHA or Vorinostat) (1, 17, 21, 40) and valproic acid (49). Stimulation of the PI3K/Akt pathway by either SAHA or the clinically tested chemotherapeutic hexamethylene bisacetamide (HMBA) (75) may alleviate latency by triggering the localization of P-TEFb to the LTR to enhance viral transcriptional elongation (14, 16, 17). Transcriptional elongation can also be enhanced with okadaic acid, the non-clinical inhibitor of protein phosphatases type 1 (PP1) and 2A (PP2A) (24), which increases the levels of phosphorylated RNAPII (94). The polyphenol resveratrol, which activates both growth receptor Egr1 (70) and class III HDAC SIRT1 (6), may enhance viral gene expression by upregulating Egr1-dependent growth factors (44) or by promoting the deacetylation of Tat protein via SIRT1 (66), although the precise mechanisms of SIRT1 activation are unclear and contested (65). Finally, the clinically viable DNA methylase inhibitor 5-aza-2’-deoxycytidine (i.e. 5-aza-dC) may have promise, as genomic silencing of latent
infections may be regulated by DNA methylation (34, 35). Since each of these drugs targets a distinct mechanism contributing to latency, complete reactivation of the latent reservoir – a heterogeneous population regulated by a variety of distinct mechanisms – may not be feasible with a single agent and instead require multifaceted combinatorial strategies (69).

In this proof-of-concept study, the following criteria are adopted to evaluate the effectiveness of reactivation strategies that could potentially eradicate latent HIV-1 reservoirs. First, it must be capable of reactivating in a Tat-independent manner, since low or zero levels of Tat exist in latent cells (37, 38). Second, the mechanisms of reactivation must target a wide range of integration sites within latently infected cells, as latency may arise from transcriptional interference from nearby expressing genes or from viral integration in regions of dynamic chromatin or heterochromatin (30, 31, 50, 91). Third, the regimen must stimulate multiple and complementary pathways to maintain effectiveness against potential viral variants and maximize the possible synergy between mechanisms, which could potentially decrease the dosage level and toxicity of each component. Fourth, the therapeutic regimen should optimally target most or all HIV-1 subtypes and CRFs, such that a strategy could be broadly implemented and that individuals with quasispecies infections would be unlikely to develop escape mutants.

Our overall strategy is to explore the ability of single and combinatorial compounds to activate latent HIV-1 in different in vitro latency models. Moreover, to assess the efficacy of such reactivation therapies, we have tested these drugs across a range of conditions, including model virus containing enhancer elements from numerous HIV subtypes, virus with sequence variations in key host transcription factor binding sites, lentiviral vectors that model the Tat feedback loop, and Tat-deficient latent virus. In both PBMC and Jurkat-based systems, our results indicate that certain HIV-1 subtype and promoter mutants that could arise naturally may
be resistant to reactivation with any individual anti-latency drug. However, we demonstrate that
a combination of the NF-κB/PKC activator prostratin with the HDAC inhibitor SAHA – both
clinically tested pharmacological agents – synergistically reactivates latent infections across a
variety of integration sites, promoter mutants of κB and Sp1 binding sites, and distinct HIV-1
subtype and CRF isolates. Importantly, our results indicate the majority of different subtype
promoters in either Jurkat or primary CD4+ T cell latency models are synergistically reactivated
by the combination of prostratin and SAHA.

Methods and Materials

Lentiviral latency models in Jurkats

Construction of LG and LGIT HIV-1 based plasmids and LGIT variants containing two
(for κB mutants) or three (for Sp1 mutants) inactivating point mutations have been previously
detailed (10, 89). Lentiviral plasmids for LG (pCLG) and LGIT (pCLGIT) were packaged and
harvested in HEK 293T cells using 10 μg of vector, 5 μg pMDLg/pRRE, 3.5 μg pVSV-G, and
1.5 μg pRSV-Rev, as previously detailed (89). Culture media were replaced after 12 hours, and
24 hours later viral supernatant was passed through a 0.45 μm filter to remove cell debris. Virus
was then loaded onto a 20% (wt/wt) sucrose cushion and concentrated by ultracentrifugation in
an SW41 rotor (Beckman Coulter, Fullerton, CA) for 1.5 hours at 25,000 rpm (107,000 × g) and
4°C. The viral pellet was resuspended in 100 μl of 4°C phosphate-buffered saline (PBS, pH 7.0)
to yield typically between 10⁷ and 10⁸ infectious units/ml. An estimated 10³-10⁶ infectious units
of concentrated virus were used to infect 3 ×10⁵ Jurkat cells. Six days after infection, titering
curves were constructed by incubating cells with a combination of 5 mM HMBA, 20 ng/ml TNF-
α, 400 nM TSA, and 12.5 µg exogenous Tat protein for 18 hours and then analyzing GFP expression by flow cytometry to obtain specific titer values. A unique titering curve for LG and each LGIT variant was used to attain the desired MOI (~0.05-0.10).

Primary cell latency model

Whole blood from three healthy, anonymous donors (9.1, 9.2, and 9.3) was obtained from the City of Hope Donor Apheresis Center (Duarte, CA). Primary blood mononuclear cells (PBMCs) were isolated using Histopaque 1077 (Sigma-Aldrich). Naïve CD4+ T cells were further purified using the Naïve CD4+ T cell Biotin-Antibody Cocktail II, Anti-Biotin MicroBeads, and MACS LS Columns (Miltenyi Biotec). Cells were activated with 30 U/ml rIL-2 (NIH AIDS Reagent Program) and Human T-Activator CD3/CD28 Dynabeads (Invitrogen). One week after isolation, 10^5 infectious units of concentrated LGIT (subtypes A2, A, B, C, C', D, and F) virus were used to infect 1 ×10^6 CD4+ T cells. Primary cells were cultured in RPMI media supplemented with 10% AB-Human Serum (Invitrogen).

Pharmacological treatments

To determine the theoretical limits of latency reactivation for each Jurkat-based lentiviral model, all pharmacological agents were tested at saturating levels for in vitro conditions. In particular, the following drugs were tested at the specified concentrations: 12.5 µg exogenous Tat protein per 3 ×10^5 cells (NIH AIDS Reagent Program), 20 ng/ml tumor necrosis factor-α (TNF-α, Sigma-Aldrich), 10 nM phorbol myristate acetate (PMA, Sigma-Aldrich), 400 nM trichostatin A (TSA, Sigma-Aldrich), 5 mM hexamethylene bisacetamide (HMBA, Sigma-Aldrich), 1 µM prostratin (LC Laboratories), 30 nM okadaic acid (Sigma-Adrich), 4 µM SAHA...
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(Toronto Research Chemical), 5 mM valproic acid (Sigma-Aldrich), 1 µM 5-aza-deoxycytidine (Sigma-Aldrich), 500 µM (±)-S-Nitroso-N-acetylpenicillamine (SNAP, Calbiochem), 425 µM DETA-NONOate (Cayman Chemical), 20 µg/ml phytohemagglutinin (PHA, Sigma-Aldrich), 30 µM resveratrol (Sigma-Aldrich), and 500 mM sorbitol (Sigma-Aldrich). After 30 minute incubation with 0.5 M sorbitol, Jurkat cells were washed with media and analyzed by flow cytometry six hours later. Incubation with either resveratrol or 5-aza-deoxycytidine was performed for 48 hours prior to flow cytometry analysis. All other drugs were incubated with Jurkats or primary CD4+ T cells for 24 hours prior to GFP analysis. For the most efficacious agents (prostratin, SAHA, HMBA, and the combination of prostratin+SAHA), cell viability after drug treatment was analyzed by MTS cell viability assay or by propidium iodide (Sigma-Aldrich) and Hoeschst 33342 (Invitrogen) staining using flow cytometry (Supplemental Figure S1).

Flow cytometry analysis

To phenotype primary T cells, 5 ×10^5 cells were stained with the following monoclonal antibodies (Invitrogen): Pacific Blue-anti-CD4, APC-anti-CD45RA, PerCP-anti-CD45RO, and APC-Alexa Fluor 750-anti-CD27. Stained cells were analyzed by flow cytometry using the CyAn ADP 9color flow cytometer (Dako) with three laser excitation sources (405 nm, 488 nm, and 635 nm).

Jurkat cells infected with the LGIT or LG lentivirus were sorted with a DAKO-Cytomation MoFlo Sorter based on GFP fluorescence. GFP analysis for Jurkat cells was performed using a Beckman-Coulter FC500 flow cytometer. Analysis of flow cytometry was performed with FlowJo (Tree Star, Inc.).
Reagents

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: p93BR020.1, p90CF056.1, p92UG037.1, p93BR029, p94UG114.1, and p92NG003.1 from Drs. Beatrice H. Hahn and Feng Gao, and the UNAIDS Network for HIV Isolation and Characterization; p98CN009.8 from Drs. Cynthia M. Rodenburg, Beatrice H. Hahn, Feng Gao, and the UNAIDS Network for HIV Isolation and Characterization; p94CY017.41 from Drs. Stanley A. Trask, Feng Gao, Beatrice H. Hahn, and the Aaron Diamond AIDS Research Center; p93IN904 and p93IN999 from Dr. Kavita Lole, Dr. Robert Bollinger, and Dr. Stuart Ray; Tat protein from Dr. John Brady; and recombinant human IL-2 from Dr. Maurice Gately, Hoffmann – La Roche Inc.

Mapping of viral integration sites

An established method has been used for identifying human immune deficiency virus (HIV-1) integration sites (95). The genomic DNA of infected Jurkat cells was isolated using Qiagen mini DNA kit (QIAGEN) and then restricted by either HpyCH4III or MseI (New England Biolabs). The restricted DNA fragments were ligated to pre-annealed Hpy linker or Mse linker DNA (Hpy linker+, 5'-GTAATACGACTCATATAGGGCTCCGCTTAAGGGACN-3', Hpy linker-, 5' PO4-7); Mse linker+, 5' -PO4-7). The ligation products were then used as templates for
primary PCR with primers annealing to the HIV LTR and the linkers (HIV-LTR, 5’- AGTGCTTCAAGTAGTGTGTGCCCG-3’, linker primer, 5’- GTAATACGACTCACTATAGGGC-3’) under the following conditions: pre-incubation at 95°C for 2 min, then 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 2.5 min and the final extension of 72°C for 10 min. Samples of the initial PCR product were used for nested PCR with primers (HIV LTR nested, 5’- AAAAAGGATCCCGTCTGTGTGTGACTCTGGTAACT-3’, linker primer nested, 5’- AAATTAAGCTTAGGGCTCCGCTTAAGGGAC-3’) under the same conditions as primary PCR. The amplified viral-host genome junctions were cloned into pBS SK SP plasmid after restriction with Bam HI and Hind III (NEB) and then sequenced. The retroviral integration sites were mapped to the human genome (February 2009 assembly) using the BLAT program on the Ensembl genome browser website (www.ensembl.org). Based on the chromosomal locations, various genomic annotations for each retroviral integration site were made via genome browser websites (Ensembl and UCSC genome browser (www.genome.ucsc.edu)).

mRNA extraction and quantification by RT-PCR

RT-PCR was used to determine the LTR-driven gene expression and the expression of nearby genes for three LG clones (BB1, BC5, and DA4) after treatment with anti-latency drugs. For each LG clonal population, 2 ×10^6 Jurkat cells incubated for three hours with 5 mM HBMA, 1 µM prostratin, 4 µM SAHA, the combination of 1 µM prostratin and 4 µM SAHA, or DMSO (vehicle) control. Total mRNA was isolated using RNA STAT-60 Reagent (Tel Test) and total cDNA was generated M-MLV Reverse Transcriptase (Invitrogen). GFP and β-Actin primer sets are previously described (51), and primer sets for endogenous genes were obtained using
PrimerBank (http://pga.mgh.harvard.edu/primerbank/) (81). QPCR primer sequences are provided in Supplemental Table S1. For all samples and primer sets, QPCR conditions included an initial melting step (95°C, 3 min), followed by 35 cycles of melting (95°C, 20 sec), annealing (55°C, 30 sec), and extension (68°C, 20 sec) steps. All RT-QPCR measurements were performed in triplicate and melt curves were generated using the CFX96 Real Time PCR Detection System (Bio-Rad).

Statistics for reactivation effectiveness

In the Jurkat experiments from Figure 2, the polyclonal, infected, Off cells were sorted by FACS at 21 days post-infection. The following day (Day 22 post-infection) the same cells were treated with the reactivation drugs. The reported % Reactivation was calculated by subtracting the percentage of Off cells after stimulation from the percentage of Off cells from an identical, unperturbed, Off-sorted sample, and then dividing this amount by the total percentage of Off cells in the same unperturbed control. The % Reactivation for the primary cell experiments in Figure 4 was calculated with the same method, but these cells were not FACS sorted prior to reactivation. Stimulated and unperturbed (vehicle control) samples were measured by flow cytometry at the same time, and all measurements were performed in triplicate. Reported values are the averages of triplicate measurements, and error bars are standard deviations of these replicates. Statistical significance was determined using both nonparametric (Mann-Whitney-Wilcoxon, MWW) and parametric (Student’s t-test) methods with a significance level of $\alpha = 0.05$ for both. Statistical significance is claimed only when confirmed by both MWW and t-test methods.
Statistical analyses for synergism

A combination of drugs may act synergistically if their combined activity exceeds the results obtainable by any of the individual components. This investigation employs the fractional product method to quantify whether various drug combinations synergistically reactivate latent infections (88). This simple and classic definition can be derived from the mass-action law principle using the assumptions of first-order behavior and mutually non-exclusive components (13). Both of these assumptions are supported by prior investigations of the co-administration of a NF-κB/PKC activator and an HDAC inhibitor (69) and the cooperative mechanisms of NF-κB and Sp1 factors (68). To evaluate whether synergy exists in a combinatorial drug treatment, consider the anti-latency strategies of prostratin (treatment “P”), SAHA (treatment “S”), and a combination of the two (treatment “PS”) at the following drug doses: \( d_P = 1.0 \mu M \) prostratin, \( 0 \mu M \) SAHA, \( 0 \mu M \) prostratin, \( d_S = 4.0 \mu M \) SAHA, and \( (d_P = 1.0 \mu M, d_S = 4.0 \mu M) \). Let \( \mu \) denote the measured% Reactivation (average of biological triplicates) after each drug treatment such that,

\[
\mu_P = \mu_P(d_P, 0); \quad \mu_S = \mu_S(0, d_S); \quad \text{and} \quad \mu_{PS} = \mu_{PS}(d_P, d_S).
\]

For the polyclonal WT LGIT (WT.OFF) samples, the measured values are as follows: \( \mu_P = 57.9\% \), \( \mu_S = 38.6\% \), \( \mu_{PS} = 76.6\% \), with standard deviations \( \sigma_P = 1.7\% \), \( \sigma_S = 0.7\% \), \( \sigma_{PS} = 0.9\% \), respectively. Using the fractional product method, a synergistic effect applies when:

\[
\mu_{PS} > 1 - (1-\mu_P) \times (1-\mu_S); \quad 0.766 > 1 - (1-0.579) \times (1-0.386) = 0.742
\]

Thus, for the WT.OFF sample, the combination of prostratin+SAHA is synergistic compared to the effects of the individual components. Statistical significance was determined using both nonparametric (Mann-Whitney-Wilcoxon, MWW) and parametric (Student’s t-test) methods.
with a significance level of $\alpha = 0.05$ for both. Statistical significance is claimed only when confirmed by both MWW and t-test methods.

### Results

Establishment of systems to assess reactivation of polyclonal and clonal HIV-1 latency models

Recent studies have revealed that combinations of drugs may act upon multiple latency mechanisms to provide synergistic reactivation of latent infections (5, 39, 71). Since synergistic reactivation by the combinatorial treatment of NF-$\kappa$B/PKC activators and HDAC inhibitors is likely mediated by the $\kappa$B and Sp1 sites within the HIV-1 LTR (68), we first investigated a system in which we could simultaneously explore the regulation of these sites in conjunction with the role of Tat and its feedback loop. In particular, we have previously found that a LTR-GFP-IRES-Tat (LGIT) lentivirus within Jurkat cells can exhibit active (Bright) and inactive (Off) gene expression modes, and in a process termed phenotypic bifurcation (PheB), clonal populations of LGIT-infected Jurkats with single viral single integration positions may give rise to both Off and Bright subpopulations (89). Furthermore, we have previously demonstrated that the Sp1 and $\kappa$B sites in the viral LTR differentially recruit activating and repressing factors, and that mutation of these sites may destabilize both Off and Bright modes and thereby result in an increased frequency of PheB (10). Since this phenotype is likely driven by stochastic fluctuations in the concentration of Tat (89) and competition between activating and repressing host factors at the promoter (10), such PheB clones can serve as a sensitive means to examine the efficacy of potential anti-latency drugs.
As natural variations occur within the Sp1 and κB elements, an individual patient may carry a swarm of LTR variants that can persist for years (62). Sequence variability within the LTR further increases across isolates of HIV-1 subtype B, in which polymorphisms occur throughout each Sp1 and κB element (www.hiv.lanl.gov, Supplemental Figure S2). Since Sp1 and κB sites differentially regulate latency (10), reactivation of latent infections may vary upon the particular configurations of these sites. To determine which Sp1 and κB sites are required for particular reactivating mechanisms, we utilized LTR variants of the LGIT lentivirus containing inactivating mutations in each of the Sp1 (mutI Sp1, mutII Sp1, and mutIII Sp1) and κB (mutI NF-κB and mutII NF-κB) binding sites (10).

Jurkat cells were infected with wild type LGIT or mutant variant lentivirus at low MOI (~0.05-0.10, Figure 1A). Six days after infection, gene expression was stimulated with exogenous Tat protein and HMBA to activate the population of “Infected but Off” cells that remain transcriptionally inactive after infection (Figure 1A, panel 2a). Eighteen hours after stimulation, FACS was utilized to isolate the polyclonal fraction of GFP+ cells from uninfected cells (Figure 1A, panel 3a). After sorting the infected fractions for wild type LGIT and each Sp1 and κB mutant, cells were cultured under normal conditions for two weeks during which substantial fractions of GFP+ cells relaxed Off, generating a bimodal gene expression profile (Figure 1A, panel 4a). Following this two week expansion period, FACS was again applied to isolate the polyclonal Off (GFP-) population, which represents the “latent” population of infected cells (Figure 1A, panel 5b). Similarly, after the same two week expansion, single cells were sorted and expanded for four weeks until three PheB clones for wildtype LGIT and each Sp1 and κB mutant were identified by flow cytometry (Figure 1A, panel 5a).
Reactivation of polyclonal and clonal LGIT populations with mutations in each Sp1 and κB binding element

After isolating the polyclonal and clonal LGIT populations, each was treated with a variety of pharmacological agents to reactivate latent infections. For both PheB clones and polyclonal Off sorts, the success of each treatment was evaluated by the percentage change of GFP- (Off) cells after stimulation, which corresponds to the percentage of latent infections that were reactivated (referred to as % Reactivation). While the PheB clones in this study represent an important subset of inactive integration sites that exhibit a sensitive Tat-dependent phenotype, the polyclonal Off-sorts include a larger latent subpopulation that likely encompasses the different phenotypes of PheB and fully silenced clones, such as J-Lats (37). Thus, by examining PheB clones and the polyclonal Off-sorts for the same Sp1 and κB mutants of LGIT, we aim to test the reactivation of each mutant within a system that will be sensitive to stochastic fluctuations in regulating factors but will also access a broad range of silent integration positions and latency mechanisms (Figure 1A, panels 6a-b).

Throughout this study, we use a variety of pharmacological agents to survey which drugs can effectively reactivate latent infections and whether certain promoter subtypes, mutants, or integration positions might restrict these drugs. In general, the potential clinically viable agents in this study include prostratin, HMBA, SAHA, and valproic acid. However, in many cases, we have also tested the efficacy of potent, non-clinical agents as a benchmark for the clinical alternatives. For example, TNF-α and PMA are tested alongside prostratin, while SAHA and valproic acid are evaluated with the more toxic HDAC inhibitor TSA. Thus, we can evaluate particular drugs, such as prostratin and SAHA, as well as larger classes of drugs (NF-κB/PKC activators and HDAC inhibitors) to evaluate the most effective anti-latency agents.
NF-κB and PKC activators require κB site I for latency reactivation

To determine the contribution of each κB and Sp1 site to reactivation via induction of NF-κB/PKC pathways, each polyclonal Off sort was treated with TNF-α, PMA, or prostratin. Treatment with TNF-α reactivated over half of the latent infections for the wild type LGIT Off sort (WT.OFF), while treatment with prostratin or PMA achieved approximately 60% reactivation for the same subpopulation (Figure 2A). Mutation to any Sp1 site did not diminish reactivation via the NF-κB/PKC pathways, as treatment with TNF-α, PMA, or prostratin strongly reactivated the polyclonal Off sorts (73%-93% reactivation) for mutI Sp1 (S1), mutII Sp1 (S2), and mutIII Sp1 (S3) (Figure 2A). This observation is consistent with our previous findings, that Sp1 mutants have a reduced occupancy by HDAC1 and, as a result, are more easily reactivated by TNF-α (10). In contrast, mutation of κB site I (N1) dramatically reduced reactivation by TNF-α, PMA, or prostratin for the polyclonal Off sort (4%-14% reactivation, Figure 2A), consistent with our previous observations that this mutant fails to sufficiently recruit RelA (10). Although mutII NF-κB (N2) populations were slightly more susceptible to reactivation by TNF-α than mutI NF-κB (20% reactivation), reactivation with either PMA (65%) or prostratin (58%) was statistically indistinguishable from wildtype subtype B LGIT (p>0.05, Figure 2A). Reactivation of the PheB clones for each LGIT NF-κB and Sp1 mutant with TNF-α, PMA, or prostratin closely resembled the trends of the polyclonal Off sorts (Supplemental Figure S3). Collectively, LGIT Off sorts and PheB clones revealed that κB site I (N1) – a relatively well-conserved element with mutations in 2.4% of the 127 subtype B isolates from the LANL database (www.hiv.lanl.gov, Supplemental Figure S2) – plays a critical role for the reactivation
with NF-κB and PKC activators. Moreover, these results indicate that prostratin is capable of reactivating latent infections comparable to potent, immunogenic (TNF-α) or toxic (PMA) agents.

Latency reactivation with HDAC inhibitors is regulated by Sp1 site III

Previously, we have demonstrated that mutation in any of the three Sp1 sites decreases regulation by HDAC1, which suggests that these mutants may be desensitized to latency reactivation therapies involving HDAC inhibition (10). Treatment with either TSA or SAHA reactivated at least 40% of the WT polyclonal Off sorts and outperformed TNF-α for S1, S2, N1, and N2 (p<0.05, Figure 2B). However, S3, which responded with 88% reactivation to TNF-α and 90% to prostratin, only exhibited 28% reactivation with either TSA or SAHA. These results are consistent with our previous observations that Sp1 site III is particularly important for activation by HDAC inhibitors due to its role in the synergistic and potentially cooperative recruitment of activating factors p300 and RelA (10). Consistent with the polyclonal Off sorts, SAHA strongly reactivated all three WT LGIT clones and was similarly effective on PheB clones for both κB mutants, S1, and S2 (Supplemental Figure S3). However, like the polyclonal Off sorts for mutIII S1, each clone (S3.B3, S3.B6, and S3.C4) exhibited decreased sensitivity to SAHA, and none of the three clones displayed more than 60% reactivation after stimulation (Supplemental Figure S3). These results indicate that SAHA provides similar efficacy as the non-clinical HDAC inhibitor TSA, but that neither drug is effective in reactivating mutants in site III Sp1.

In addition to SAHA and TSA, we decided to explore the reactivation capabilities of the clinically viable HDAC inhibitor valproic acid, given the recent interest in this particular drug in
clinical studies (49, 80). Valproic acid reactivated all LGIT polyclonal Off sorts and PheB clones with markedly lower efficacy compared to SAHA (Figures 2B and S3), indicating that SAHA may serve as a more effective clinical alternative to valproic acid. Moreover, these results underscore the importance of Sp1 site III – a moderately conserved element that contains potentially disruptive polymorphisms in 10% of the subtype B isolates from the LANL database (www.hiv.lanl.gov, Supplemental Figure S2) – in reactivating strategies involving HDAC inhibitors.

Elongation agonists and DNA methylase inhibitors are weak activators of latent infections

In addition to a lack of transcriptional activation, latency may partially result from insufficient transcriptional elongation and Tat-transactivation (54). We therefore also utilized the Tat-driven LGIT system to examine the effects of the P-TEFb and PI3K/Akt agonist HMBA to determine whether promoting elongation could lead to Tat accumulation and subsequent strong transcriptional activation. HMBA reactivated 13% of WT Off-sorts and induced statistically equivalent responses from all mutant polyclonal populations except mutIII Sp1 (p>0.05, Figure 2B). Likewise, HMBA was modestly effective in reactivating WT PheB clones, but was virtually ineffective for mutIII Sp1 clones (Supplemental Figure S3). Therefore, like HDAC inhibitors, HMBA requires an intact site III Sp1 for maximum reactivation, but appears less effective than SAHA.

We anticipated that okadaic acid, which promotes elongation independently of NF-κB (94), may equally reactivate WT and κB mutant LGIT. However, this treatment reactivated merely 4% of WT LGIT-Off sorts, with statistically indistinguishable results between WT LGIT and all mutants (p>0.05, Supplemental Figure S5A), such that its effects were marginal.
Combinatorial Anti-latency Strategies

Compared to HDAC inhibitors and NF-κB/PKC activators. Treatment with resveratrol, which may enhance viral transcription by activating Egr1-dependent growth factors or by promoting the deacetylation of Tat protein via SIRT1, was ineffective on all PheB clones (Supplemental Figure S3) and statistically negligible for WT and mutant Off-sorts (p>0.05, Supplemental Figure S5A). Since treatment with either okadaic acid or resveratrol marginally reactivated WT LGIT or any κB or Sp1 mutant, both drugs were not further examined.

Recent investigations have associated latent proviral infections with CpG methylation (34, 39). Thus, the reversal of DNA methylation with the clinically tested methylase inhibitor 5-aza-dC may alleviate gene silencing and help promote reactivation independently of the roles of Sp1 and κB sites. Treatment with 5-aza-dC reactivated merely 4% of WT Off-sorts and yielded similarly weak reactivation for all other mutants, indicating that DNA methylase inhibition is not sufficient for latency reactivation in this particular model (Supplemental Figure S4).

Combinatorial therapies synergistically reactivate latent κB and Sp1 LTR mutants

Although individual treatments of NF-κB/PKC activators and HDAC inhibitors were effective in activating substantial fractions of Off cells for WT LGIT, the combination of multiple drugs may have synergistic effects. Importantly, co-stimulation of WT LGIT Off-sorts with prostratin+SAHA (77% reactivation, Figure 2C) or PMA+TSA (87% reactivation, Supplemental Figure S4) synergistically reactivated gene expression, resulting in approximately 30% greater activation than either prostratin or PMA and more than 2-fold greater clearance than SAHA or TSA alone. Although the combinatorial treatment of TSA+HMBA (50% reactivation) is not synergistic, it provides enhancement over individual treatment with either TSA or HMBA by 15% and 37% clearance, respectively. However, the combination of PMA+TSA+HMBA
(82% reactivation) provided no further activation relative to PMA+TSA (Supplemental Figure S4), suggesting that HMBA may either have additive or redundant effects to these other two agents or that PMA+TSA simply saturate the effects of HMBA. The inclusion of 5-aza-dC also provided no further reactivation when paired with TSA, PMA, or TSA+PMA (Supplemental Figure S4), suggesting that this clinically tested drug is not essential in latency reactivation.

Using both clonal and polyclonal LGIT models, we have demonstrated that mutations in Sp1 site III (S3) severely weaken the effectiveness of HDAC inhibitors, and mutation to κB site I (N1) abrogates the response to NF-κB/PKC activators and HDAC inhibitors (Figure 2A-B and Suppmental Figure S3). These results indicate that such individual therapy approaches risk the possibility of mutational evasion because relatively minor mutations in non-coding regions, which may readily arise in patients of subtype B infection (Supplemental Figure S2), may severely undermine drug efficacy. We hypothesize that these risks may be greatly tempered by reactivating latent infections with multiple agents, such as the potential synergistic combination of prostratin+SAHA.

For all LGIT mutant polyclonal Off-sorts, the combinations of prostratin+SAHA or PMA+TSA achieved between 59% (N1.OFF) and 99% (S1.OFF) reactivation and outperformed every individual component (Figures 2C and S4). Although N1.OFF was resistant to prostratin and PMA, and both S3.OFF and N1.OFF were desensitized to SAHA and TSA (Figures 2C and S4), the combination of prostratin+SAHA or PMA+TSA synergistically reactivated both populations and overcame the limitations of mutation to any κB or Sp1 site. Moreover, for N1.OFF, either drug combination provided at least 4-fold greater reactivation than prostratin or PMA alone and 2-fold greater reactivation than only SAHA or TSA. Although the effects were not synergistic for N2.OFF, the combination of either prostratin+SAHA or PMA+TSA...
reactivated almost 30% more latent cells than either SAHA or TSA alone, and approximately 10% more than either prostratin or PMA (Figures 2C and S4). As observed with WT LGIT, HMBA provided no further reactivation for any mutant when paired with TSA (TSA+HMBA) or when included with PMA and TSA (PMA+TSA+HMBA) (Supplemental Figure S4).

Collectively, these results demonstrate that, analogous to HAART, therapeutic reactivation of latent infections may require a combinatorial approach that performs effectively and often synergistically against different potential promoter architectures and minimizes the likelihood of mutational escape.

Latency reactivation of eleven distinct HIV-1 subtype and recombinant isolates

Although recent investigations examined the potential reactivation of latent reservoirs using pharmacological agents, these focused exclusively on isolates from subtype B – the subtype most prevalent in the United States and Europe (8, 14, 45, 49). However, due to significant sequence diversity in the LTR between HIV-1 subtypes (28, 56, 73), non-subtype B isolates may respond distinctly to these agents. In particular, subtypes with variable κB and Sp1 elements may exhibit resistance to drugs, similar to the phenotypes observed for mutIII Sp1 and mut1 NF-κB versions of LGIT (Figures 2A-B and S3-S4). To both examine strategies of latency reactivation for various HIV subtypes and identify potential limitations for divergent promoters, we generated LGIT variants containing U3 regions specific to the following subtypes and recombinants: A, A2, A/G, B, B/C, B/F, D, F, H, and two distinct isolates of C (Supplemental Table S2 and Figure S6). As this investigation focuses on the role of the cis-acting elements in reactivation from latency, model LGIT variants were constructed with the U3 regions of each subtype or CRF (Figure 1B), but with the R (including TAR), U5, and Tat regions of subtype B.
In addition to having diversity throughout the Sp1 and κB sites, these various U3 enhancer regions also differ in other cis-regulatory elements including AP-1, YY1, NFAT, COUP-TF, and ILF sites (Figure 1B). The use of eleven subtype enhancer sequences representing HIV-1 isolates from five continents may help elucidate whether certain subtypes and CRFs would require drug regimens to be “tailored” to their particular promoter architectures.

Although NF-κB/PKC activators stimulated sizable fractions of Off-sorts for each subtype or CRF (including greater than 50% reactivation of subtype B Off-sorts), interesting differences emerged among them (Figure 2A). Activation of NF-κB/PKC with PMA reactivated at least 50% of latent infections for subtypes and CRFs with deviations in Sp1 sites II or III (A2, A, A/G, D, F, and H) (Figures 1B and 2A), consistent with the clonal and polyclonal analyses of LGIT Sp1 mutants. Furthermore, among all subtypes and recombinants tested, the LGIT version for A2, which contains three nucleotide deviations in Sp1 site II (Supplemental Figure S6), reactivated most strongly to TNF-α, PMA, and prostratin (68%-81% reactivation, Figure 2A). In contrast, subtype C, which contains an additional κB site, exhibited the weakest response to TNF-α, PMA, and prostratin (38%-47% reactivation, Figure 2A). Interestingly, these results indicate that Sp1 sites are not necessary for effective latency reactivation with PMA or prostratin, but an additional κB site could actually restrict latency reactivation with these NF-κB/PKC activators.

Similarly to NF-κB/PKC stimulation, reactivation with HDAC inhibitors TSA and SAHA effectively reactivated subtypes and CRFs containing nucleotide disparities from subtype B in Sp1 site II (A, A2, A/G, Figure 2B). Subtype recombinant B/F, which shares identical κB and Sp1 sequences to subtype B, and the three aforementioned subtypes and CRFs (A, A2, and A/G), all displayed reactivation over 60% with TSA and at least 50% with SAHA. Interestingly,
all subtypes that contained deviations in Sp1 site III (D, F, and H) failed to achieve greater than 51% reactivation with TSA or 45% reactivation with SAHA (Figure 2B), consistent with the observation that mutation of this site (mutIII Sp1) weakens the stimulatory effects of HDAC inhibitors (Figure 2B). However, stimulation with either TSA or SAHA also failed to reactivate at least 50% of Off-sorts for CRF B/C and subtypes C and B, indicating that subtypes and recombinants with an intact Sp1 site III may also fail to strongly respond to HDAC inhibition (Figure 2B). For all subtypes and CRFs, reactivation with valproic acid was dramatically weaker than TSA and SAHA, consistent with other LGIT polyclonal and clonal results (Figures 2B and S3). Interestingly, stimulation with HMBA yielded similar trends as the HDAC inhibitors and reactivated subtypes and recombinants A, A2, A/G, and B/F by greater than 30% reactivation, but failed to reactivate B/C, C, C’, and B beyond 20% (Figure 2B). Collectively, these results highlight the importance of Sp1 site III for reactivation with HDAC inhibitors, since TSA and SAHA more strongly reactivate subtypes and CRFs with deviations in Sp1 site II (A2, A, and A/G) than those with deviations in Sp1 site III (D, F, and H).

**Combinatorial treatment of prostratin and SAHA synergistically reactivate some LGIT subtypes**

In similarity to models for subtype B (Figures 2C and S4), co-stimulation of an NF-κB activator and an HDAC inhibitor produces a synergistic effect for many LGIT subtypes and CRFs compared to the individual components. The combination of prostratin+SAHA reactivated at least 85% of “latent” cells for A, A/G, B/F, and F. Moreover, this combination exhibited synergistic reactivation on 6 of 11 (55%) subtype isolates including C’, A, A/G, B, B/F, and F (p<0.05, Figure 2C). These collective results reveal that reactivation of latent infections with individual drugs will likely vary across subtypes and CRFs and that the utilization of only
the canonical subtype B as a model for latency may miss the behavior of different subtypes and recombinants.

Direct inhibition of YY1 or activation of AP-1 fails to reactivate most HIV-1 subtypes

Despite the extensive evidence for the regulation of latency by Sp1 and κB sites (10, 36, 57, 92), the roles of other cis-acting factors such as YY1 and AP-1, are less defined (33, 96). These unspecified roles are confounded by the distinct genotypic differences in the positions and sequences of YY1 and AP-1 sites across different HIV-1 subtypes and CRFs (Figure 1B). Since YY1 may promote transcriptional silencing and latency by recruitment of HDAC1 (18), treatment with HDAC inhibitors including TSA, SAHA, and valproic acid in the LGIT system would likely reverse these effects (Figure 2B). Likewise, treatment of LGIT system with TNF-α would likely activate AP-1 (85), but any specific regulatory roles of AP-1 might be overshadowed by NF-κB (Figure 2A). For this study, however, we also aimed to determine whether the broad discrepancies in YY1 and AP-1 binding sites across subtype and CRF isolates (Figure 1B) may impact latency regulation and specific reactivation strategies.

We thus employed the subtype and CRF variants of LGIT to test whether inhibition of YY1 with either DETA or SNAP may reverse latency for any variant. In contrast to the direct inhibition of HDACs with SAHA, inhibition of the YY1 pathway with either DETA or SNAP marginally reactivated latent infections for all subtypes and CRFs. Although SNAP outperformed DETA for all subtypes and recombinants, it only reactivated at least 5% Off-sorts from four of the eleven subtypes and CRFs (A2, A, A/G, and B/F, Supplemental Figure S5B). Interestingly, higher reactivation occurred specifically for subtypes and CRFs containing a YY1 site at the same position (~229-235) within the LTR (Supplemental Figures S5B and S6).
To determine whether AP-1 specifically plays a distinct role in latency reactivation, we have tested each LGIT subtype with sorbitol, which induces hyperosmotic shock and thereby leads to increased binding activity of AP-1 (85). Although activation of both NF-κB and AP-1 with TNF-α moderately activated expression for all subtypes and CRFs (Supplemental Figure S5B), treatment with sorbitol failed to reactivate any LGIT variant beyond 3% reactivation. Therefore, neither mild activation of AP-1 with sorbitol nor inactivation of YY1 with DETA or SNAP appears sufficient to reactivate latency for any HIV-1 subtype or CRF model in this study.

Generation of in vitro model for HIV-1 latency

In addition to the Jurkat model for HIV-1 latency described in Figure 2, we have also used human CD4+ primary cells to better model the physiological conditions of latency. Generation of this model begins with naïve CD4+ T cells isolated from whole blood of healthy patients, similar to other systems (7, 39, 59). Consistent with the properties of human naïve CD4+ T cells, isolated cells are positive for CD4, CD45RA, and CD27 surface antigens, and largely negative for CD45RO (Figure 3A-D). After isolation, cells are activated with CD3/CD28 Dynabeads (Invitrogen) and expanded in activating conditions for one week (Figure 3E). At day 7, cells are infected with the LGIT lentivirus (MOI~0.5-0.10), including the same subtype LGIT variants A, A2, B, C, C’, D, and F used in the Jurkat experiments (Figure 2). After one more week in activating conditions (day 14 post-isolation), cells are transferred to minimal media with low levels of interleukins (1 ng/ml IL-7 and 10 U/ml IL-2) to maintain cell viability in resting conditions (Figure 3E). Cells are cultured for two weeks in resting conditions (until post-isolation day 28), at which anti-latency drugs are used to reactivate latent LGIT infections.
Latency reactivation is quantified by flow cytometry by measuring the percentage change in GFP+ cells 24 hours after drug treatment. Additionally, all procedures for isolation, infection, and reactivation were performed using CD4+ cells from three different donors (9.1, 9.2, and 9.3) to evaluate potential donor variability.

Latency reactivation in primary CD4+ T cells

At one week after infection (day 14 post-isolation), 10^5 cells from each LGIT subtype and donor were strongly stimulated using CD3/CD28 Dynabeads, 1 ng/ml IL-7, and 30 U/ml IL-2 to determine the total percentage of GFP+ cells in T cell activation conditions. This percentage was set as the baseline to normalize the subsequent latency reactivation following a two week period of resting culture conditions (Supplemental Figure S7A-B). After the two week resting period (day 28 post-isolation), LGIT infections were reactivated with HMBA, prostratin, SAHA, or the combination of prostratin+SAHA (Figure 4A). Consistent with the observations in the Jurkat system (Figure 2B), HMBA was only moderately effective in reactivating latent infections for all LGIT subtypes and reactivated no more than 12% of latent infections for any variant except subtype A (A9.1: 27% and A9.3: 19% reactivation, Figure 4A). In contrast, prostratin reactivated at least 15% of latent infections in cells from at least one donor for all subtypes (Figure 4A). Interestingly, treatment with SAHA accomplished at least 15% reactivation of all subtypes in at least one donor, except for the two variants of subtype C (C9.1: 3.7%, C9.2: 4.9%, C9.3: 1.1% and C'9.1: 6.7%, C'9.2: 3.8%, C'9.3: 2.2% reactivation, Figure 4A). These results are strikingly similar to the Jurkat-based experiments (Figure 2), which reveal that subtype C (C and C') are poorly reactivated by SAHA in comparison to prostratin (Table 1).
Importantly, the combination of prostratin+SAHA reactivated latent infections in resting CD4+ primary cells substantially better than either drug alone. In fact, for 17 of 21 total conditions (seven LGIT subtypes in three different donors), reactivation with prostratin+SAHA was more effective than either individual component. Furthermore, for 10 of 21 total conditions, including for at least one donor from every LGIT subtype variant except LGIT A, prostratin+SAHA exhibited synergistic reactivation (p<0.05, Figure 4A). Subtype A, though not synergistically reactivated by prostratin+SAHA, was strongly reactivated by prostratin alone (A9.1: 98%, A9.2: 26%, and A9.3: 89% reactivation, Figure 4A). When paired with the Jurkat experiments, which also indicate strong reactivation by prostratin for subtype A (Figure 2A), these results suggest that this particular subtype may not require a combinatorial drug therapy for effective latency reactivation (Table 1). In contrast, subtype variants C and C’ are poorly reactivated by an individual drug and appear to require a combination drug therapy for synergistic reactivation (Table 1).

In addition to the reactivation of latent infections, as measured by GFP expression, cells were stained for specific surface markers to verify that the reactivated LGIT infections occurred exclusively in memory CD4+ T cells. After treatment with prostratin+SAHA, antibody staining revealed that all GFP+ cells were also positive for CD4 and CD45RO, a marker for memory T cells (Figures 4B-C and 4E-F). Additionally, we observed significant populations of GFP+ cells either positive or negative for CD27, indicating that this latency reactivation strategy is effective for memory (CD4+CD45RO+CD27+) and effector (CD4+CD45RO+CD27−) T cells (Figures 4D and 4G).
Single agent and combinatorial reactivation strategies for Tat-deficient \( \text{LG} \) clones

To this point, we have examined the reactivation of latency using the \( \text{LGIT} \) provirus, which enables strong Tat-feedback upon transcriptional activation. However, recent studies suggest that some latent infections may arise from HIV-1 variants with impaired Tat-transactivation (97). Moreover, since transcriptional silencing during latency precludes the production and accumulation of Tat, we hypothesize that latency reactivation strategies should be effective in Tat-deficient conditions. To model the Tat-deficient conditions of HIV-1 latency, we infected and generated clonal populations of Jurkat cells harboring single integrations of the \( \text{LTR-GFP (LG)} \) lentivirus, which drives GFP expression from the HIV-1 subtype B LTR (37, 38, 89). Similarly to the infections with the \( \text{LGIT} \) lentivirus, Jurkat cells were infected at low multiplicity of infection with the \( \text{LG} \) lentivirus (MOI ~0.05-0.10) prior to FACS sorting (Figure 1A, panels 1b-3b). Single GFP+ cells were then sorted by FACS and cultured under normal conditions for four weeks to generate clonal populations of \( \text{LG} \) cells (Figure 1A, panel 4b). Over this period, a substantial fraction of infected \( \text{LG} \) cells relaxed into the low GFP- region (Off), likely due to the decay of Tat and NF-\( \kappa \)B after stimulation.

Five \( \text{LG} \) clones that exhibited a variety of GFP distributions prior to stimulation (gray filled histograms, Supplemental Figure S8A-C) were selected to examine the reactivation of anti-latency drugs in the absence of Tat. However, first, to verify that each \( \text{LG} \) clone is susceptible to Tat activity, and thus would be capable of viral activation, we have infected each with a Tat-expressing lentivirus. The resulting proviral construct constitutively expresses both Tat and \text{mOrange} from the human ubiquitin promoter (\( Ub^P \)-mOrange-IRES-Tat, \( \text{OrIT} \)) to enable the identification of Tat-expressing cells by the mOrange fluorescent protein. Each \( \text{LG} \) clonal population was infected with the \( \text{OrIT} \) lentivirus (MOI ~ 0.15-0.20) and monitored by flow
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cytometry for both expression of mOrange (Tat) and GFP (LTR-driven expression). Each of the five LG clones responds to Tat-transactivation, as indicated by the GFP+/mOrange+ subpopulations for each two-parameter histogram (Figure 5A). Thus, each of these five LG clones (without OrIT lentivirus) will be further examined for reactivation by anti-latency agents.

Since each LG clone exhibits a distinct monomodal gene expression profile in the absence of the OrIT lentivirus, the fractions of GFP+ cells (Total % On) before and after stimulation for each clone are reported (Figure 5B, LG.BB1: 28%, LG.BC5: 0.1%, LG.DA4: 0.0%, LG.DD1: 54%, and LG.DD2: 61% On). The NF-κB inducing cytokine TNF-α significantly enhanced gene expression of all clones except LG.DA4 (Figures 5B and S8A).

Similarly, activation of both NF-κB and PKC pathways with the phorbol ester PMA significantly increased expression for clones LG.BB1, LG.BC5, and LG.DD2. However, treatment with prostratin only enhanced expression in clones LG.BB1 and LG.DD2 (Figure 5B). Therefore, stimulation of the NF-κB and PKC pathways in Tat-deficient clones failed to activate all five LG clones and thus appeared to preferentially enhance expression only at particular integration sites in the absence of Tat.

Treatment of the LG clones with TSA significantly enhances expression of only three clones (LG.BB1, LG.BC5, and LG.DD1), while SAHA activates all clones except LG.DA4 (Figures 5B and S8B). In contrast, treatment with P-TEFb agonist HMBA provides no stimulation for any clones except LG.BB1 (Figure 5B), suggesting that enhancing elongation is insufficient to reactivate many integration positions. Collectively, these results suggest that HDAC inhibition may provide a more potent reactivation mechanism than enhancing elongation, but that neither strategy can reactivate at all integration positions.
In similarity to the LGIT experiments in Jurkats and PBMCs (Figures 2C and 4A), combinatorial reactivation strongly activated expression of all LG clones, achieving between 68-99% GFP+ cells with prostratin+SAHA (Figures 5B and S8C), including synergistic reactivation for three clones (LG.BC5, LG.DA4, and LG.DD2). Therefore, the combination of clinically safe drugs prostratin and SAHA effectively activates expression in Tat-deficient systems and dramatically outperforms individual agents.

Integration site analysis for LG clones

Although we have demonstrated that treatment with anti-latency drugs is capable of activating latent HIV-1 promoters (Figures 2-5), the possibility remains that the same drugs will nonspecifically and uncontrollably perturb the expression of endogenous genes near the proviral integration site. As a final analysis to examine this possibility, we have identified the integration sites of three LG clones (Figure 6, LG.BB1, LG.BC5, and LG.DA4). The LG-integration site of clone BB1 lies outside an area of known transcripts between genes Spn (Gene ID: 6693) and QPRT (Gene ID: 23475, Figure 6A). In contrast, the integration site for clone BC5 lies within exon 9 of ATXNL2 (Gene ID: 11273), in the opposite orientation of that gene (Figure 6C). Finally, clone DA4 has an integration site within an intron of the ZMYM2 (Gene ID: 7750), also in the opposite direction of the gene (Figure 6E). RT-PCR analyses was then designed to quantify the change of HIV-1 expression as well as the expression of the nearest endogenous genes (Supplemental Table S1). Each clone was treated with HMBA, prostratin, SAHA, or the combination of prostratin+SAHA and then prepared for RT-PCR three hours after drug perturbation. For clone BB1, we have quantified the expression of the two nearest genes, while...
for clones BC5 and DA4, we examined the expression levels of the same genes in positions upstream and downstream of the proviral integration site.

For the LG clone BB1, treatment with HBMA increased levels of GFP mRNA by 1.6-fold, with a 25% decrease in expression of Spn and a 33% increase of QPRT (Figure 6B). Treatment with prostratin enhanced GFP mRNA by nearly 2-fold, but resulted in a substantial reduction in the expression of Spn (48%) and a lesser decrease in QPRT (8%). In contrast, SAHA enhanced expression of GFP, Spn, and QPRT by at least 46%. However, treatment with prostratin+SAHA significantly increased GFP mRNA expression by 3.1-fold (p<0.05) without having any significant effect in the expression of either Spn or QPRT (p>0.05, Figure 6A).

Reactivation of LG clone BC5 with either HMBA or prostratin resulted in negligible changes in expression for GFP and the upstream and downstream regions in ATXNL2 (Figure 6C). Similarly, treatment with SAHA did not enhance the expression of GFP or either ATXNL2 region. However, prostratin+SAHA elevated GFP expression by 1.75-fold (p<0.05), while resulting in statistically insignificant decreases in the expression of the upstream and downstream sites (p>0.05, Figure 6C).

Similarly to BB1, treatment with either HMBA or prostratin on LG clone DA4 resulted in a significant increase in GFP expression (p<0.05), but no increase in the host gene expression upstream or downstream of the integration site (p>0.05, Figure 6F). However, treatment with SAHA did not statistically enhance the expression of GFP or the endogenous gene ZMYM2 (p>0.05, Figure 6F). Like clones BB1 and BC4, treatment with prostratin+SAHA synergistically activated expression of GFP without increasing the expression of the endogenous gene (Figure 6F). However, in clones BC5 and DA4, which have integration sites within and in the opposite direction of endogenous genes, the increase of GFP expression appears to loosely correlate with
the decrease of expression of the endogenous gene. Though this observation might be the result of transcriptional interference from two opposing promoters, our results would also indicate that the combination of prostratin+SAHA could overcome the effects of transcriptional interference as a mechanism for HIV-1 latency (20, 31, 50).

**Discussion**

In this proof-of-concept study, we have considered the potential design criteria for effective drug regimens to reactivate latent HIV-1 infections. These results demonstrate that a cocktail therapy, principally composed of clinically viable components SAHA and prostratin, can provide synergistic activation (Figures 2 and 4-6), perform effectively against different HIV-1 subtype enhancers (Figures 2 and 4), exhibit robustness against viral mutants (Figure 2), target a wide range of silenced integration sites (Figures 2 and 4-6), and function independently of Tat protein (Figures 5-6).

In this study, we have employed both Jurkat and PBMC-based latency models to establish reversible, transcriptionally silent infections in order to test for reactivation upon treatment of various potential drug therapies. The primary cell latency model in this study was adapted from recently reported *in vitro* systems that allow for long-term culturing of resting memory CD4+ T cells (7, 39, 59). Likewise, we employ LGIT and LG Jurkat-based models, similar to other *in vitro* systems that have proven useful for studying HIV-1 expression and latency (1, 37, 50, 67, 91). Collectively, both *in vitro* systems enabled the examination of anti-latency agents against an assortment of mutant and subtype LTR variants. Interestingly, this approach revealed significant promoter-dependent limitations of each drug that were previously undetected in models that exclusively employed viral isolates from subtype B (17, 45, 91).
Latency reactivation via HDAC inhibitors and NF-κB/PKC activators results from mechanisms that utilize the Sp1 and κB sites (Figure 1B), and likely involves the removal of repressing HDAC complexes (e.g. HDAC1) and the subsequent recruitment of activating factors (e.g. RelA and p300) (10). These observations suggest that variability in these binding elements, either due to mutations within a subtype or to considerable differences in these elements between subtypes (Figure 1B), may have a strong impact on emergence from latency. In agreement with our previous findings (10), we have identified the particular importance of each Sp1 and κB element by demonstrating that specific mutants exhibit severely desensitized responses to NF-κB/PKC activators (mutI NF-κB) and HDAC inhibitors (mutIII Sp1) (Figure 2). Importantly, however, the simultaneous treatment of prostaglandin and SAHA effectively reactivated “latent” cells for all Sp1 and κB mutants and subtype configurations, suggesting that a combinatorial strategy with these clinically viable drugs may target a broad arrangement of promoter elements and raise the bar for potential viral mutational escape.

Although the mutated versions of LGIT were created by direct mutagenesis, rather than isolated from a patient, these models expose limitations in reactivation therapies that target Sp1 and κB-dependent mechanisms. The capacity of Sp1 and κB mutants in LGIT to generate PheB clones that can bifurcate into Bright and Off populations demonstrates that these mutant promoters could likely establish both active and latent infections. Although natural mutation to any of these sites may weaken the magnitudes of transcriptional activation (74) and decrease viral replication rates (12, 32), such mutant viruses would likely remain functionally viable and may ultimately serve as a reservoir for progressive infection after periods of long-term latency (15, 22). Furthermore, even if such weakening polymorphisms arose, it is possible that these unfavorable mutations may be functionally offset by virally advantageous mutations elsewhere.
in the genome to restore viral expression and fitness (51). Thus, we have employed versions of
LGIT with point mutations in the Sp1 and κB sites to highlight the specific roles of these sites
and elucidate the potential limitations of individual drug therapies for similar clinical variants.

No individual drug effectively reactivated all latency models; however, combinations of
pharmacological agents overcame the limitations of single agents by acting via synergistic
mechanisms (Supplemental Table S3). For example, treatment with prostratin+SAHA
dramatically activated Off cells for all five LG clones, with synergistic effects for three of the
five (Figure 5B). Importantly, this drug cocktail strongly reactivated each Sp1 and κB mutant
version of LGIT, despite our findings that mutation of Sp1 site III diminished SAHA efficacy
and mutation of κB site I severely weakened reactivation with prostratin (Figure 2). Strikingly,
in both Jurkat and PBMC-based systems, this potentially therapeutic strategy also synergistically
stimulated latent infections across subtype and CRF versions of LGIT, despite the diverse
arrangements of cis-regulatory binding elements including Sp1, κB, YY1, NFAT, and AP-1 sites
(Figure 1B).

Although combinatorial treatments with prostratin and SAHA provide synergistic effects
for more than half of the subtypes and CRFs in both Jurkat and primary CD4+ T cell systems,
the extent of reactivation differs among them (Figures 2C and 4A). Based on our observations in
mutant versions of LGIT, subtle yet important deviations in the promoter sequence and
architecture, particularly within the Sp1 and κB domains, likely determine the response to either
prostratin or SAHA. In particular, HIV-1 genotypes with weakening polymorphisms in Sp1 site
II (A, A2, A/G) are strongly reactivated with individual and combinatorial drug treatments in the
Jurkat system (Figure 2), suggesting that the latent populations of these subtypes and CRFs are
destabilized. Similar trends are observed in the primary cell experiments, in which subtype A is
strongly reactivated by prostratin alone, while prostratin+SAHA synergistically reactivates latent infections from all three donors infected with LGIT A2 (Figure 4A). In agreement with the distinct role that Sp1 site III plays in reactivation via HDAC inhibitors (Figure 2B), subtypes with mutations in Sp1 site III (D, F, and H) responded less favorably to HDAC inhibitors than to NF-κB/PKC activators (Figure 2B). These trends are consistent with our previous analysis demonstrating that mutation to any Sp1 site in subtype B dramatically destabilized the latent population and that mutIII Sp1 exhibited weakened TSA stimulation (10). Moreover, the dependency on a functional Sp1 site III for effective reactivation by either SAHA or HMBA (Figure 2B) is interesting, considering that both agents may enhance transcription via stimulation of the PI3K/Akt pathway (16, 17). In general, stimulation of this pathway leads to the phosphorylation of p300 (55), the activation of RelA (61), and the recruitment of P-TEFb to the LTR (17). However, since mutIII Sp1 is deficient in the recruitment of both p300 and RelA (10), the effects of SAHA or HMBA induced stimulation of the PI3K/Akt pathway on the LTR are likely minimal for this particular mutant.

Subtype and CRF variants B/C, C, and C' each contain an additional κB or Sp1 site that may strengthen the recruitment of repressive complexes via the p50-p50 homodimer and Sp1 in resting T cell conditions, thereby stabilizing latent infections at particular integration positions. Interestingly, in the Jurkat system we observe that each of these subtype promoters is more susceptible to reactivation with prostratin than with SAHA (Figure 2A-B). Similarly, in the primary cell system, prostratin is more effective than SAHA for all three donors in LGIT C and for two of three donors in LGIT C'. Furthermore, LGIT C', but not C, is synergistically reactivated by the combination of prostratin+SAHA in the Jurkat system (Figure 2C), and the combination of prostratin+SAHA synergistically reactivates C' for all three donors in the primary
cell system (Figure 4A). These results suggest that mere inhibition of HDACs, without coincident activation of PKC/NF-κB pathways, is insufficient in latency reactivation for subtypes with an additional Sp1 or κB site.

A number of recent studies have revealed that latent infections may frequently arise for integration sites near or within actively expressed genes (20, 30, 31, 50). One particular mechanism to explain this phenotype is transcriptional interference from the nearby gene, which inhibits expression from the viral promoter (48). By indentifying the proviral integration sites for three LG clones, we have determined that all lie in the opposite orientation of the nearest host gene, including two clones with integration sites within the reading frame of the host gene (Figure 6). These results suggest that transcriptional interference may play a role in the transcriptional silencing of these infections, but that the combination of prostratin+SAHA is still sufficient to overcome this possible mechanism. Moreover, we have previously confirmed that LGIT PheB clones are often integrated near actively expressed genes (89), yet that the regulation of active and inactive gene expression states is partially due to differences in the local LTR occupancy of transcription factors and chromatin-modifying factors (10). Therefore, the LG and LGIT PheB clonal latency models both support the notion that latent viruses integrated near actively expressed genes may be significantly reactivated by treatment with the combination of prostratin+SAHA.

To further elucidate the mechanisms required for latency reactivation, we have tested several promising compounds, including the natural phorbol ester prostratin and the clinical chemotherapeutic SAHA. However, these drugs have yet to be clinically tested for reactivation of HIV-1 latency, and their individual or combined in vivo effects on global T cell activation are not fully known. These concerns are strongest with prostratin, which does not induce T cell
proliferation by itself, but can provide a secondary signal in T cell activation that could lead to inflammation and apoptosis of T cells (8, 43). However, prostratin could inhibit HIV-1 infection in CD4+ T cells at both entry and post-entry steps, which may reduce the risk of new infections after latency reactivation (4, 76). Finally, recent studies have identified synthetic PKC activators and HDAC inhibitors that may provide higher efficacy with reduced toxicity and cost compared to prostratin and SAHA (60, 64, 71, 77). Thus, with the development of such new anti-latency compounds, the prospects of activating latency across different subtypes and promoter mutants should be explored.

This investigation demonstrates a rigorous in vitro examination of latency reactivation strategies using multiple clonal, polyclonal, and primary cell latency models. We have postulated that the combination of multiple agents may synergistically reactivate latent infections, maintain high efficacy across integration sites, preempt potential mutational escape, and target numerous subtype isolates. Furthermore, in the development of these strategies, we have utilized a number of clinically viable agents, such as prostratin and SAHA, though the simultaneous administration of these agents has not been clinically tested. Therefore, this study provides results that may aid in the design of future in vivo and pre-clinical studies, and further supports the concept of multi-agent clinical therapies that aim to reactivate and eradicate the latent reservoir of HIV infected cells.

Acknowledgments

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Figure 1. Lentiviral latency system and HIV-1 LTR of various subtypes.

(A) FACS sorting procedure for polyclonal and clonal populations of LGIT and LG infections. Jurkat cells were infected at low MOI with LGIT (panel 1a) or LG (panel 1b) lentivirus. Six days post-infection, gene expression was strongly stimulated with exogenous Tat protein and HMBA for LGIT-infected cells (panel 2a) or Tat protein, HMBA, and TNF-α for LG-infected cells (panel 2b). Eighteen hours after stimulation, single GFP+ cells were sorted from LG infections (panel 3b) and cultured for four weeks to generate LG clones (panel 4b). Similarly, eighteen hours after stimulation, polyclonal FACS isolation of GFP+ LGIT-infected cells removed uninfected cells (panel 3a). After sorting, GFP+ LGIT cells were cultured under normal conditions for two weeks, during which substantial fractions of GFP+ cells relaxed Off (panel 4a). FACS was again applied to isolate polyclonal fractions of “Infected but Off” (GFP-) cells, and these fractions are used as models for latent infections (panel 5b). Likewise, single cells were sorted and expanded to generate LGIT clones, and after four weeks of culturing, PheB clones were identified (panel 5a).

(B) Schematic of alignments and DNA-binding elements of U3 regions for subtypes in this study. Binding sites were identified using Transcription Element Search System (TESS, http://www.cbil.upenn.edu/cgi-bin/tess/tess). Gray ovals indicate deviations in Sp1 sequence that likely compromise the function of Sp1 site II (for subtypes A2, A, and A/G) and Sp1 site III (for subtypes D, F, and H). Full U3 subtype sequences are supplied in Figure S6. Two distinct isolates of subtype C were analyzed throughout this investigation, and the notation C identifies AF067157, and C’ identifies AF067154.
Figure 2. Latency reactivation for LGIT mutants and HIV-1 subtypes in the Jurkat system. (A) Infection and serial FACS sorting were performed to isolate the infected, Off populations for variants of LGIT the following mutants of subtype B (mutI Sp1 (S1), mutII Sp1 (S2), mutIII Sp1 (S3), mutI NF-κB (N1), and mutII NF-κB (N2)) or with U3 regions isolated from the following subtypes: B, A2, A, A/G, B/C, C', C, B/F, D, F, and H (as in Figure 1B). One day after FACS sorting (Day 22 post-infection, Figure 1A, panels 5b and 6b), polyclonal Off-sorts for WT LGIT mutant and HIV-1 subtype variants were treated with the following pharmacological agents to reactivate latent infections: NF-κB/PKC activators TNF-α (white bars), PMA (gray bars), or prostratin (black bars). Data represent the averages of three independent measurements for each drug perturbation, and error bars are standard deviations. For the LGIT mutants of subtype B, upward (↑) and downward (↓) arrows indicate statistically significant deviations from the wildtype subtype B LTR configuration of LGIT (p<0.05). A dashed gray line at 50% reactivation is drawn as a reference marker. (B) Same as in (A) for latency reactivation by TSA (white bars), SAHA (light gray bars), valproic acid (dark gray bars), or HMBA (black bars). (C) Same as in (A) for latency reactivation using the combination of prostratin+SAHA. Asterisks denote statistical synergism by the combination of drugs, relative to the reactivation by either individual agent. Refer to the Methods and Materials for the quantitative treatment of synergy.

Figure 3. Isolation and infection of human primary CD4+ T cells.
(A-D) Naïve CD4+ T cells were isolated from human whole blood from three donors (9.1, 9.2, and 9.3). Six days after isolation cells were analyzed by flow cytometry for expression of surface receptors CD4 (A), CD45RA (B), CD45RO (C), and CD27 (D). Histogram overlays include negative controls (shaded gray) and naïve CD4+ T cells (black outline).

(E) Naïve CD4+ T cells were activated with CD3/CD28 antigen-beads for three days after isolation to promote T cell activation and expansion. At seven days post-isolation, cells from each donor were infected by one of seven different LGIT subtype variants at a low MOI. Cells were cultured in activating conditions until day 14, at which cells were transferred to minimal growth medium (1 ng/ml IL-7 and 10 U/mL IL-2).

Figure 4. Reactivation of latent HIV-1 in primary memory CD4+ T cells.

(A) After 14 days of culturing in quiescent conditions (and 28 days after CD4+ T cell isolation), cells were treated with anti-latency drugs HMBA (H), prostratin (P), SAHA (S), or the combination of prostratin+SAHA (P+S). Presented data include each LGIT subtype for each of three independent donors. Asterisks indicate synergism of prostratin+SAHA, with respect to individual treatments of either drug. Refer to the Methods and Materials for the quantitative treatment of synergy.

(B-D) At day 21 post-infection (day 28 post-isolation), primary cells were examined for the proviral LTR expression of GFP (x-axis) and the surface expression (y-axis) of (B) CD4, (C) CD45RO, or (D) CD27.

(E-G) At days 21 post-infection (same as B-D), primary cells were treated with prostratin+SAHA for 24 hours and examined for proviral LTR expression of GFP (x-axis) and surface expression (y-axis) of (E) CD4, (F) CD45RO, or (G) CD27.
Figure 5. Latency reactivation of LG clones with subtype B LTR.

(A) Five HIV-1 subtype B LTR-GFP (LG) clones (BB1, BC5, DA4, DD1, and DD2) were isolated to examine latency reactivation for the Tat-deficient lentivirus (Figure 1A, panel 4b). Each Jurkat clonal population was subsequently infected with another lentivirus (OrIT) that constitutively expresses mOrange and HIV-1 Tat (subtype B) from the human Ubiquitin promoter (MOI ~ 0.15-0.20). Cells are analyzed for expression of mOrange (x-axis) and GFP (y-axis), as shown in 2-D histograms. Although cells are clonal with respect to the LG (GFP) infection, there are subpopulations that are either responsive (mOrange+/GFP+) or resistant (mOrange+/GFP-) to Tat-transactivation.

(B) LG clones, as isolated in Figure 1A, panel 4b, were reactivated with either Tat (OrIT infection) or anti-latency agents. As measured by flow cytometry, the percentage of GFP+ cells are indicated for the original LG clone (green bars), the LG population infected with the OrIT lentivirus (orange bars), and each LG clone stimulated with HMBA (yellow bars), prostratin (blue bars), SAHA (red bars), or the combination or prostratin+SAHA (black bars). The position of the Off gate is set for uninfected Jurkats (GFP-), whereas the On gate indicates GFP+ cells. All data are averages of biological triplicates, and error bars are standard deviations. Filled circles (•) indicate at least a 10% increase from the unperturbed samples and asterisks (*) indicate synergistic reactivation by drug combination. Drug concentrations are provided in the Methods and Materials and histograms are provided in the Supplemental Figure S8. Refer to the Methods and Materials for the quantitative treatment of synergy.
Figure 6. Integration site analysis for LG clones after anti-latency activation.

(A) The integration site of LG clone BB1 was identified at chr16:29684467 outside of a region of known transcripts, and positioned between and in the opposite orientations of the reading frames for SPN (Gene ID: 6693) and QPRT (Gene ID: 23475).

(B) LG clone BB1 was treated with DMSO (negative control), HMBA, prostratin, SAHA or the combination of prostratin+SAHA. After a three-hour incubation period, RT-PCR was performed on the untreated and treated cells to quantify the expression from the LTR (GFP), the nearest endogenous downstream gene (SPN), and the nearest upstream gene (QPRT). Using the \( \Delta\Delta C_t \) method, all data are first normalized by the respective expression of \( \beta \)-Actin and then by the relative expression of the unperturbed control. All control and drug perturbations were performed in biological triplicate, and data represent averages of three independent measurements. Error bars are standard deviations and upward (▲) and downward (▼) arrows indicate statistically significant deviations from the DMSO negative control (p<0.05). Asterisks denote statistically significant synergism of the prostratin+SAHA combination, with respect to the individual components. Refer to the Methods and Materials for the quantitative treatment of synergy.

(C) The integration position of LG clone BC5 was identified at chr16:28841942 inside the reading frame and in the opposite orientation of ATXNL2 (Gene ID: 11273).

(D) Same as in (B) for LG clone BC5. RT-PCR was performed on the untreated and treated cells to quantify the expression from the LTR (GFP), and the mRNA from the ATXNL2 gene upstream (“atxUS”) and downstream (“atxDS”) of the LG integration position.
(E) The integration position of LG clone DA4 was identified at chr13:20605604 inside the reading frame and in the opposite orientation the ZMYM2 (Gene ID: 7750).

(F) Same as in (B) for LG clone DA4. RT-PCR was performed on the untreated and treated cells to quantify the expression from the LTR (GFP), and the mRNA from the ZMYM2 gene upstream (“zmUS”) and downstream (“zmDS1” and “zmDS2”) of the LG integration position. Primers for two different downstream sites of ZMYM2 were used to examine different potential splice variants.
Figure A: Percent reactivation of subtype B LGIT mutants with different treatments.

- TNF-α
- PMA
- Prostratin

Figure B: Percent reactivation of subtype B LGIT mutants with different treatments.

- TSA
- SAHA
- Valproic Acid
- HMBA

Figure C: Percent reactivation of subtype B LGIT mutants with prostratin and SAHA treatment.

- Prostratin+SAHA
E. CD4+ cells

- Isolation of naive CD4+ T cells from whole blood
- Isolated naive CD4+ T cells (day 1 post-isolation)

A. Long-term culturing of CD4+ T cells (day 1 post-isolation)

B. Reactivation of latent infections of CD4+ T cells using antiviral agents

C. Latent infection at low MOI (0.05-0.10)

D. Pharmacological agents to promote latency
TABLE 1. Summary of latency reactivation with prostratin and SAHA in Jurkat and primary CD4+ T cell models

<table>
<thead>
<tr>
<th>LGIT subtypes or mutants</th>
<th>Off-sorted Jurkat cells (mean of biological triplicates) From Figure 2A-C</th>
<th>Primary CD4+ T cells (mean of three donors) From Figure 4A</th>
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<td>Prostratin</td>
<td>SAHA</td>
<td>Prostratin + SAHA</td>
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<tr>
<td>B</td>
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<td>38.6%</td>
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<td>A2</td>
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<td>29.9%</td>
</tr>
<tr>
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</tr>
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Shading Legend
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- 20.0% | 39.9%
- 40.0% | 59.9%
- 60.0% | 79.9%
- 80.0% | 99.9%