







SUPPLEMENTAL FIGURE LEGEND

Figure S1. mTOR Inhibitors, Torin1, pp242 and rapamycin Suppress HIV Latency Reactivation in Bcl2-Transduced Primary Cell Latent Model, Related to Figure 3

(A) HIV_{GKO} reporter map: new version of dual-fluo virus R7/E-/GFP/EF1 α -mCherry (R7GEmC) (Calvanese et al., 2013), containing a codon switched eGFP (csGFP) and a distinct, unrelated fluorescent protein mKO2 still under the control of EF1 α .

(B) Latent CRISPRi K562 cells expressing NC or MLST8 sgRNAs were treated with Panobinostat, Romidepsin or JQ1 at the indicated concentrations. Percentage of GFP-positive cells after 24 hours of reactivation with PMA are indicated. Cells transduced with NC (negative control) sgRNA lentivirus were used as control. Mean and standard deviation from duplicate samples from 4 independent experiments are represented.

Figure S2. mTOR Inhibitors, Torin1, pp242 and Rapamycin Suppress HIV Latency Reactivation in Bcl2-Transduced Primary Cell Latent Model, Related to Figure 4

Torin1 (A, C) and pp242 (B, D) and rapamycin (E, F) were used in two additional human donors infected with latent GFP virus. GFP reactivation was assessed in the latent model treated with these mTOR inhibitors (top panels).

Cell viability in each donor after Torin1, pp242 and rapamycin treatment, respectively (bottom panels). Data are represented as mean \pm SD.

Figure S3. mTOR Inhibitor Torin1 Suppresses Tat-Dependent and Tat-Independent Reactivation of Latent LTR Promoter in J-Lat cells, Related to Figure 5

(A, B) J-Lat A2 (harboring LTR-Tat-IRES-GFP) (A) and A72 (harboring LTR-GFP) (B) cells were pre-treated with Torin1 at the indicated concentrations for 4 hours and then stimulated with PMA for 20 hours. Quantification of GFP-positive cells was done by flow cytometry to measure the reactivation of the latent LTR promoter. Percentage of latent HIV reactivation with 6.3ng/mL PMA is represented. Mean and standard deviation from duplicate samples of two independent experiments are indicated.

Table S1. Genes Identified in the shRNA Screen, Related to Figure 1 and Table 1

File is divided according to Gene ID, Symbol, Gene Info, Enriched versus Disenriched (E/D), pMann Whitney (pMW) values, log transformed signed values, and the subpool of the library (Chip) it is derived from.

Table S2. Top canonical pathway summary with Ingenuity Pathway Analyses, Related to Figure 1

List of top canonical pathways identified with Ingenuity Pathway Analysis for enriched genes or disenriched genes identified with a pMW<0.01.

Ratio is the number of molecules from the dataset (with pMW<0.01) in the selected pathway relative to the total number of molecules in that pathway.

Pathway p-value is calculated using Fisher's exact test.

Table S3. Top upstream regulators summary with Ingenuity Pathway Analyses, Related to Figure 1

List of top upstream regulators identified with Ingenuity Pathway Analysis for enriched genes or disenriched genes identified with a pMW<0.01.

Table S4. Protein Complexes Identified in the shRNA Screen, Related to Figure 2

Excel file showing the complex analysis conducted using the CORUM database. The list is divided according to complex id, complex name, the number of subunits and the major genes involved.

Besnard *et al*, Table S2. Top canonical pathway summary with Ingenuity Pathway Analyses, Related to Table 1 and Figure 1

	Top Canonical Pathways	Pathway <i>p</i>-Value	Genes Ratio	Genes
For Enriched Genes	Leucine Degradation I	7.78E-03	2/9	BCAT1, AUH
	Signaling by Rho Family GTPases	1.01E-02	9/234	ARPC2, NOX1, ATM, MSN, CDC42EP5, RHOT1, GNG2, PIP4K2A, RND2
	Glioma Invasiveness Signaling	1.11E-02	4/57	ATM, TIMP3, RHOT1, RND2
	RhoGDI Signaling	1.72E-02	7/173	ARPC2, MSN, RHOT1, GNG2, WASF2, PIP4K2A, RND2
	Actin Cytoskeleton Signaling	1.82E-02	8/216	ARPC2, PDGFD, SSH3, ATM, MSN, BCAR1, WASF2, PIP4K2A
For Disenriched Genes	AMPK Signaling	5.29E-03	8/178	GNB1L, SMARCD1, CPT1C, FASN, PPP2R3B, MLST8, HNF4A, INS
	Palmitate Biosynthesis I (Animals)	2.95E-02	1/2	FASN
	Fatty Acid Biosynthesis Initiation II	2.95E-02	1/2	FASN
	Adenine and Adenosine Salvage I	2.95E-02	1/2	APRT
	Glutathione Redox Reactions I	3.19E-02	2/19	CLIC2, GPX4

Besnard *et al*, Table S3. Top upstream regulators summary with Ingenuity Pathway Analyses, Related to Table 1 and Figure 1

	Top Upstream Regulator Name	Gene Symbol	Gene <i>p</i>-value
For Enriched Genes	Negative Elongation Factor Complex Member A	NELFA	2.07E-04
	Copper Metabolism (Murr1) Domain Containing 1	COMMD1	3.57E-04
	Coagulation Factor VII (Serum Prothrombin Conversion Accelerator)	F7	1.10E-03
	Ribonuclease H2, Subunit B	RNASEH2B	1.22E-03
	Ring Finger Protein (C3H2C3 Type) 6	RNF6	1.22E-03
For Disenriched Genes	Zinc Finger Protein 202	ZNF202	2.62E-03
	Transforming Growth Factor, Beta Receptor 1	TGFBR1	3.04E-03
	Growth Hormone Secretagogue Receptor	GHSR	5.21E-03
	Aryl Hydrocarbon Receptor Nuclear Translocator	ARNT	5.74E-03
	Endoplasmic Reticulum to Nucleus Signaling 1	ERN1	7.32E-03

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture

J-Lat A2 and J-Lat A72 (NIH AIDS Reagent Program), Jurkat, J-Lat 5A8, CRISPRi K562 stable clones containing dCas9-BFP-Krab were cultured in RPMI-1640 (Life Technologies), supplemented with 10% FBS (Atlantic Biologicals), 1% pen/strep and 2 mM L-glutamine (Life Technologies). TZM-bl (NIH AIDS Reagent Program) and 293T cells were cultured in DMEM, supplemented with 10% FBS (Atlantic Biologicals) and 1% pen/strep (Life Technologies). Primary CD4 T cells were isolated from healthy donor blood (Blood Centers of the Pacific, San Francisco, CA) by negative selection with the RosetteSep Human CD4 T Cell Enrichment Cocktail (StemCell Technologies). Primary CD4 T cells were maintained in RPMI-1640 in 10% FBS (Gemini), 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 mg/ml) and 20 U/ml IL-2 (PeproTech).

Transduction of Cells with Pooled shRNA Libraries

The genome-wide RNAi screen was carried out using a pooled ultracomplex shRNA library that we developed earlier. The library targets each human-protein coding gene with 25 independent shRNAs on average and contains thousands of negative control shRNAs. It is divided into nine subpools of 55,000 shRNAs (2,200 human genes) each, expressed from the lentiviral vector pMK1047 (Bassik et al., 2013; Kampmann et al., 2013). Each subpool of the library was transfected into 293T cells using standard methods as described (Bassik et al, 2013). At 72 hours post-transfection, virus was filtered using a 0.45-micron PVDF filter (Millipore) and 50 ml unconcentrated virus was used to infect 35 million J-Lat 5A8 cells in 6-well plates (5 ml of virus/cell mixture per well) for 2 hours at 32 °C at 2500 rpm by spin infection. The infected cells were maintained and expanded in 10% FBS supplemented RPMI-1640. At 3 days post-infection, cells were treated with puromycin (Sigma) at a final concentration of 0.75 µg/ml for 2 days. After 2 days, cells were washed and allowed to recover for 3 days in fresh medium before treatment with anti-CD3/CD28. The cells were expanded in T-175 flasks to approximately 8 x10⁸ cells total before stimulation and sorting. Each subpool of library had a biological duplicate that was sorted independently.

Cell Stimulation and Sorting for Screen

T-175 flasks were coated overnight at 4°C with CD3 antibody (UCSF Hybridoma Core) diluted in 1XPBS at a concentration of 3 µg/ml. The next morning flasks were washed with twice with 1XPBS and cells were stimulated with 1 µg/ml CD28 (e-Bioscience) for 24 hours. 5×10^8 cells were prepared for sorting by centrifugation, washing, filtering through a cell strainer, and resuspending in sort buffer (1xPBS with 2% FBS). 5×10^7 stimulated but unsorted cells were frozen using 90% FBS and 10% cell-culture grade DMSO (Sigma) for subsequent genomic DNA extraction once sorted cells were obtained. The BD FACS Aria sorter was used. An 85-µm nozzle was used with an approximate psi of 45 KHz. An 8-hour sort was conducted on each replicate of the subpool. On average, approximately 2.4×10^8 cells were sorted in 8 hours, yielding approximately 1.2×10^6 GFP and mCherry double-positive cells. GFP and mCherry represent J-Lat 5A8 HIV-GFP, and shRNA expression, respectively.

Analysis of Genome-Wide Screen Results

The frequencies of cells expressing each shRNA in the unsorted versus the double-positive populations were quantified using deep sequencing as described (Bassik et al., 2013; Kampmann et al., 2013). Briefly, genomic DNA was extracted from the cell populations and the shRNA-encoding cassette was PCR amplified, introducing Illumina adapter sequences for sequencing with an Illumina HiSeq-2000 platform. Comparison of frequencies between both populations enabled the calculation of a quantitative phenotype, log2 enrichment, for each shRNA, as defined in (Kampmann et al., 2013). Phenotypes for each shRNA were averaged between two independent experimental replicates comparison of phenotypes for shRNAs targeting each gene with the phenotypes of negative control shRNAs was the basis for calculating p values for genes, using the Mann-Whitney U test (Kampmann et al., 2013).

shRNA screen data were analyzed through the use of QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity).

Analysis of Protein Complexes

The log-transformed Mann-Whitney p-values of all genes in our screen were mapped to the members of each complex in the CORUM database based on their Entrez GeneIDs. A composite score for every complex was computed by averaging these values. For all complexes of k

subunits, an empirical cumulative distribution function was derived by randomly sampling n ($n=1000$) complexes of k subunits and computing the average score of its members. The value of each composite score in the empirical cumulative distribution function approximated a p-value for each actual complex. Finally, the set of p-values was adjusted for multiple comparisons by applying the Benjamini-Hochberg correction, which controls the false discovery rate. Complexes with an adjusted p-value < 0.05 were considered to contain an overrepresentation of either enriched or disenriched subunit genes in our shRNA screen.

Transduction of sgRNA in the Latent CRISPRi K562 Cell Line

Individual sgRNAs were cloned into lentiviral expression vectors as described (Gilbert et al, 2014). Protospacer sequences of sgRNAs (5' to 3') used in this study are reported in the Supplemental Experimental Procedures.

were previously designed and used in CRISPRi screens: Negative Control (NC):

GAACGACTAGTTAGGCGTGTA, MLST8-1: GGCAGGGGGGAGTCACGCGC (Gilbert et al, 2014), MLST8-2: GCGGAGCCGCCCCGTAAGGTA, MLST8-3:

GGGCCTCCGTACCTTACGGG, MTOR-1: GTCCCGGCTTAGAGGACAGC, MTOR-2:

GGGACAGCGGGGAAGGCGGG, MTOR-3: GGCCTGGCACGACCCCTCTA; RAPTOR-1:

GTGGGGGCGCTAGGAAATG, RAPTOR-2: GCCTCAGCAGTTCCGAAGAC, RAPTOR-

3:GCGAGAGCAGGGTCATCGTG, RICTOR-1: GAGCGGGCTTACCTCGTACT, RICTOR-

2: GCCTGCGAGCCAATCCTAAT, TSC1-1: GGGCCTTGGCCCTTTCACGA, TSC1-2:

GCTGTGAGGTAAACAGCTGA, TSC1-3: GCTGAGGGGGAGGAGACGGT (Horlbeck et al., 2016).

PathScan analysis

The PathScan analysis was conducted with the Akt Signaling Antibody Array Kit (Cat. N. 9700, Cell Signaling). Arrays were probed according to the manufacturer instructions with 100 μ L extract of CD4 T cells (1×10^6 cells per treatment). A fluorescent image of the slide was captured with an Odyssey scanner (Li-Cor) and spot intensities were background-subtracted, quantified and averaged on duplicates using Image Studio Lite (Li-Cor).

Treatment of Bcl-2 Transduced CD4 T Cells with pp242 and Torin1

Bcl-2 transduced latent CD4 T cells were obtained as described (Spina et al., 2013; Yang et al., 2009). Upon establishment of Bcl-2-transduced latent CD4 T cells, plates were coated overnight with immobilized CD3 antibody: 2.5 µg/ml of CD3 was used in the quarter dose conditions, and 10 µg/ml CD3 was used in the “full dose” conditions. The next day, plates were washed, and 60,000 latent Bcl-2 transduced T cells were used in each condition and stimulated with 0.65 µg/ml CD28 antibody. pp242 and Torin1 were added at the same time as CD28, and cells were stimulated at 37°C for 48 hours. As a positive control, cells were stimulated with PMA/ionomycin. Each experiment was done in duplicates. Virus reactivation was measured via GFP expression and flow cytometry. 60,000 cells were used in each condition. Each inhibitor was tested in three donors, each in duplicate per assay.

Luciferase Assays in Jurkat and TZM-bl cells

Transient transfections were carried out in Jurkat cells with the Trans-IT Jurkat Transfection reagent (Mirus Bio LLC) using manufacturer’s instructions. The HIV LTR luciferase construct (LTR-Luc; pEV229) has been described before (Van Lint et al., 1997). The pEF-BOS elongation factor 1 alpha (EF-1α) and the EF-1α Tat constructs (pMO522) (Pagans et al., 2010) were a kind gift from Melanie Ott’s laboratory (Gladstone Institutes). In 24-well plates, 200,000 cells were transfected with a total of 500 ng of DNA per well: 200 ng of HIV LTR-luc, EF-1α Tat with increasing concentrations (0, 0.05, 0.25 and 1.25 ng) and empty pEF-BOS EF-1α vector to normalize amount of DNA used in each transfection condition. pp242, Torin1 and rapamycin (Tocris Bioscience) were used at concentrations of (0, 62.5, 250, and 1000 nM), (0, 15.6, 39.1, and 97.7 nM) and (0, 0.4, 2, 10 nM), respectively. Cells were treated with respective drugs at the time of transfection. At 24 hours post transfection, cells were lysed in 1X passive lysis buffer (Promega), and luciferase activities were read on BD Monolight Luminometer using manufacturer’s instructions (Luciferase Assay System, Promega). Relative light units (RLU) were normalized to total protein content determined by BCA assay (Pierce). Data are represented as mean +/- SD of triplicate values of relative luciferase units (RLU) normalized by protein content (representative of at least two independent experiments).

Exponentially growing TZM-BI cells were seeded at a density of 100,000 cells/mL and

transfected 24h later with indicated amounts of Tat plasmid using PolyJet (Signagen) according to the manufacturer's instructions. Empty vector plasmid was used to normalize DNA concentrations among samples for transfection. Cells were treated with two concentrations of each drug or vehicle control. 24h post-treatment, cells were lysed in passive lysis buffer (Promega) and luciferase activity was measured using the Luciferase Assay system (Promega) and Analytical Luminescence Laboratory Monolight 2010. Protein concentration was measured using the Bio-Rad Protein Assay with A590 determined using the EnSpire Multimode Plate Reader (PerkinElmer). Data are represented as fold change of RLU normalized by protein content (mean +/- SEM of duplicate values from three independent experiments).

Analysis of CDK9 Phosphorylation

Primary CD4 T cells were isolated from healthy donor blood. In 96-well round-bottom plate, 1 million CD4 T cells per well (5×10^6 cells/mL) were treated with pp242 (8, 40, 200, 1000 nM), Okadaic acid (10, 100, 1000 nM; Abcam) or vehicle (0 nM, DMSO) 30 minutes before adding human α CD3/ α CD28 activating beads (Life Technologies) (10 μ L beads per million cells) for 2 hours maximum. Cells were harvested right before adding the beads (0 minutes) and 120 minutes after adding the beads. Cells with or without beads were then washed once with 1XPBS, and cell pellets were frozen on dry ice.

To dephosphorylate CDK9, proteins were extracted with RIPA buffer without SDS (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton, 1% Na-deoxycholate, Protease Inhibitor Cocktail (P8340 Sigma)). Then 10X Phosphatase buffer was added to a final 1X concentration. Antarctic Phosphatase (NEB) was added to half of the obtained extract, and phosphatase-treated and untreated extracts were incubated at 37°C for 30 minutes, then cooled on ice before sample preparation for western blotting.

Western Blots

Western blots were done as reported in the Supplemental Experimental Procedures. Proteins were extracted from cells with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton, 1% Na-deoxycholate) with Halt Protease and Phosphatase Inhibitor (Pierce), 0.5 mM EDTA (Pierce), 25 μ M NAM, and 10 nM TSA. Protein content was measured by BCA assay (Pierce), and Laemmli 4X buffer was added to prepare the SDS PAGE samples. Whole-cell

lysates were run on homemade 10% polyacrylamide gels and transferred to nitrocellulose membrane. Protein levels were assessed using the following antibodies: MLST8 (CST #3274), TOR (CST #2983), RICTOR (CST #9476), TSC1 (CST #6935), RAPTOR (CST #2280), β -tubulin (ab6046), CDK9 antibody (CST #2316), p-S6-Ser240/244 (Millipore #07-2113) and S6 antibody (CST #2217). Blots were developed using Chemiluminescent Western Blot detection ECL (Thermo Fisher).

SUPPLEMENTAL REFERENCES

Bassik, M.C., Kampmann, M., Lebbink, R.J., Wang, S., Hein, M.Y., Poser, I., Weibezahn, J., Horlbeck, M.A., Chen, S., Mann, M., *et al.* (2013). A systematic mammalian genetic interaction map reveals pathways underlying ricin susceptibility. *Cell* 152, 909-922.

Calvanese, V., Chavez, L., Laurent, T., Ding, S., and Verdin, E. (2013). Dual-color HIV reporters trace a population of latently infected cells and enable their purification. *Virology* 446, 283-292.

Horlbeck, M.A., Gilbert, L.A., Villalta, J.E., Adamson, B., Pak, R.A., Chen, Y., Fields, A.P., Park, C.Y., Corn, J.E., Kampmann, M., *et al.* (2016). Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. *Elife* 5. eLife 2016;10.7554/eLife.19760

Kampmann, M., Bassik, M.C., and Weissman, J.S. (2013). Integrated platform for genome-wide screening and construction of high-density genetic interaction maps in mammalian cells. *Proc Natl Acad Sci U S A* 110, E2317-2326.

Pagans, S., Kauder, S.E., Kaehlcke, K., Sakane, N., Schroeder, S., Dormeyer, W., Trievel, R.C., Verdin, E., Schnolzer, M., and Ott, M. (2010). The Cellular lysine methyltransferase Set7/9-KMT7 binds HIV-1 TAR RNA, monomethylates the viral transactivator Tat, and enhances HIV transcription. *Cell host & microbe* 7, 234-244.

Spina, C.A., Anderson, J., Archin, N.M., Bosque, A., Chan, J., Famiglietti, M., Greene, W.C., Kashuba, A., Lewin, S.R., Margolis, D.M., *et al.* (2013). An in-depth comparison of latent HIV-1 reactivation in multiple cell model systems and resting CD4⁺ T cells from aviremic patients. *PLoS Pathog* 9, e1003834.

Yang, H.C., Xing, S., Shan, L., O'Connell, K., Dinoso, J., Shen, A., Zhou, Y., Shrum, C.K., Han, Y., Liu, J.O., *et al.* (2009). Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. *J Clin Invest* 119, 3473-3486.