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The mTOR complex controls HIV Latency

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SUMMARY

A population of CD4 T lymphocytes harboring latent HIV genomes can persist in patients on antiretroviral therapy, posing a barrier to HIV eradication. To examine cellular complexes controlling HIV latency, we conducted a genome-wide screen with a pooled ultracomplex shRNA library and in vitro system modeling HIV latency and identified the mTOR complex as a modulator of HIV latency. Knockdown of mTOR complex subunits or pharmacological inhibition of mTOR activity suppresses reversal of latency in various HIV-1 latency models and HIV-infected patient cells. mTOR inhibitors suppress HIV transcription both through the viral transactivator Tat as well as via Tat-independent mechanisms. This inhibition occurs at least in part via blocking the phosphorylation of, CDK9, a p-TEFb complex member that serves as a cofactor for Tat-mediated

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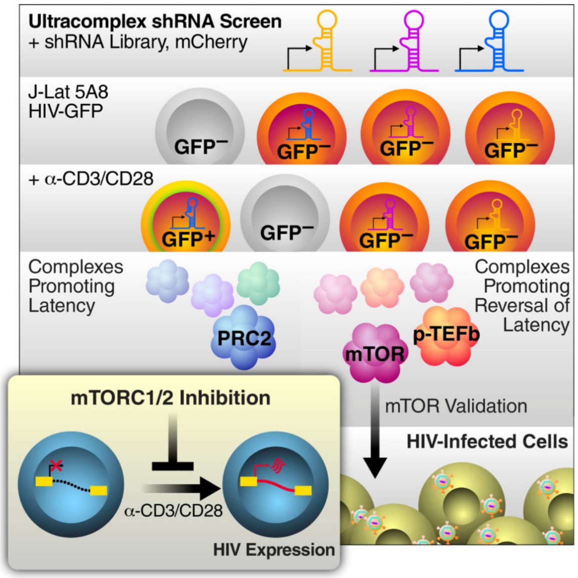
AUTHOR CONTRIBUTIONS

E.B., S.H., M.K., H.W.L, M.B., and E.V. designed the experiments. E.B., S.H., M.K., N.H., A.M., H.W.L., J.P.S, A.G, R.C, E.B. and J.C. performed the experiments. E.B., S.H., M.K., N.H., A.M., H.W.L., E.V., N.K., J.P.S, A.G, R.C, E.V. analyzed the data. E.B., S.H., M.K., N.H., A.M., H.W.L., E.V., E.B., W.G., N.K., R.F.S., J.S.W. J.P.S, A.G, R.C, and E.V. drafted and revised the manuscript.

transcription. The control of HIV latency by mTOR signaling identifies a pathway that may have significant therapeutic opportunities.

Graphical abstract



INTRODUCTION

Remarkable progress has been made in treating HIV infection due to the development of specific inhibitors of HIV replication. However, current therapies are not curative, and patients must remain on antiretroviral drugs for life. HIV persists in treated patients due to the existence of transcriptionally silent HIV in resting CD4 T cells and possibly other cell types (Ruelas and Greene, 2013; Shan and Siliciano, 2013). This long-lived reservoir becomes established very early during acute infection (Chun et al., 1998) and can reseed the infection upon the cessation of anti-retroviral therapy.

Several molecular mechanisms have been proposed to explain HIV latency (Hakre et al., 2012). For example, the transcriptional activity of the HIV promoter is governed by a combination of cis- and trans-effects. Cis-effects reflect the variety of chromatin environments at different sites of integration within the host cell genome, and trans-effects reflect the combination of trans-acting transcription factors in CD4 T cells and their regulation by T cells (van der Sluis et al., 2013). As an example of a cis-acting effect, heterochromatin is tightly packed and not permissive to transcription factor binding to the viral promoter (Hakre et al., 2011; Taube and Peterlin, 2013). This represses viral gene expression and promotes silencing of the HIV promoter. Repressive histone marks mediated by histone methyl transferases, such as G9A, SUV39H1, and Polycomb Repressive Complex 2 (PRC2), maintain the HIV promoter in a heterochromatic state and promote gene silencing (du Chene et al., 2007; Friedman et al., 2011; Imai et al., 2010; Marban et al., 2007). Histone deacetylases (HDACs), a family of chromatin-associated proteins that regulate histone acetylation and the accessibility of DNA to transcription factors, appear to be associated with the latent HIV genome, and remarkably, inhibition of HDACs is sufficient to reactivate a fraction of latent HIV in a variety of experimental systems (Shirakawa et al., 2013). Similarly, methylation of CpG islands within the HIV promoter is correlated with a strong repressive state of the viral promoter, and inhibition of DNA methylation is associated with enhanced reactivation of latent HIV (Blazkova et al., 2009; Kauder et al., 2009). Transcriptional interference between the HIV promoter and cellular promoters at the site of integration is another mechanism that can cause HIV transcriptional silencing (Lenasi et al., 2008). RNA polymerase II initiated from an upstream host promoter can displace transcription factors from the HIV promoter and suppress its activity.

The dynamic interplay between inactive and active p-TEFb complex, a critical cofactor for the HIV transactivator Tat, in resting versus activated cells CD4 T cells is an example of trans-acting effect. p-TEFb subunits, cyclin T1 and CDK9, are expressed at low levels in resting T cells (Sung and Rice, 2006), and their expression and activity increase strongly upon cellular activation. Similarly, the activity of NFκB, a transcription factor that strongly activates the HIV promoter activity, is tightly linked to T-cell activation and HIV reactivation from latency by activating transcription initiation (Williams et al., 2004). Much of HIV cure research focuses on the “shock and kill” approach that aims to force reactivation of latent HIV and eliminate latently infected cells via cytopathic effects or immune recognition. Different latency reversal agents (LRAs) are studied for their ability to reactivate HIV latency, such as HDAC inhibitors (Panobinostat, Romidepsin) and BET Bromodomain inhibitors (JQ1), or phorbol 12-myristate 13-acetate (PMA) and 3-caproyl-ingenol (Ingenol-B), both targeting NFκB and protein kinase C (PKC), and CD3/CD28 co-stimulation to activate T cells.

However, despite many proposed mechanisms, we do not fully understand what controls latency from a mechanistic standpoint.

Here, we conducted a human genome-wide analysis with an ultracomplex shRNA screen to reveal mechanistic insights of HIV latency (Bassik et al., 2013; Kampmann et al., 2013; Matheny et al., 2013). The large number of shRNAs per gene and the negative controls enable us to detect hit genes in a genome-wide screen with high sensitivity and specificity (Kampmann et al., 2013). We identified several complexes potentially involved in HIV latency, including the mTOR complex (mTORC). mTOR is an evolutionarily conserved serine/threonine kinase complex that integrates diverse environmental and cellular cues, such as growth factors, hormones and nutrients, into coordinated cellular growth responses (Zoncu et al., 2011). mTORC1 regulates biological processes, such as lipid metabolism, cap-dependent mRNA translation, autophagy and mitochondrial biosynthesis, and mTORC2 regulates cell proliferation, survival and actin polymerization (Laplante and Sabatini, 2012; Zoncu et al., 2011). We showed that mTOR inhibitors, Torin1 and pp242, suppressed the reactivation of latent HIV via T-cell stimulants both in the Bcl-2 HIV latency primary cell model and in CD4 T cells from patients on highly active anti-retroviral therapy (HAART). Further mechanistic dissection revealed that the mTOR inhibitors abrogated Tat-independent and Tat-dependent transactivation of the HIV promoter in a dose-dependent manner and reduced the global CDK9 phosphorylation in CD3/CD28-stimulated CD4 T cells from uninfected donors. These results provide mechanistic insights into the role of mTOR in controlling HIV latency and open possible therapeutic opportunities for the management of latent HIV in patients.

RESULTS

An Ultracomplex Pooled shRNA Library to Study the Reversal of HIV Latency by TCR Co-Stimulation

To identify genes controlling the activation of latent HIV, we used a cell line that contains a single integrated latent HIV-GFP reporter genome: J-Lat 5A8 (Chan et al., 2013; Jordan et al., 2003; Ruelas et al., 2015). Under basal conditions, the HIV genome in J-Lat 5A8 is transcriptionally silent, and few cells expressing GFP levels are detected (less than 0.5%). However, when subjected to T-cell stimuli, such as crosslinking with antibodies against CD3/CD28 or in response to phorbol esters, latent HIV is reactivated, and this reactivation can be monitored by FACS analysis of the induced GFP expression (Figure 1A). In a published comparison of in vitro models for HIV latency, J-Lat 5A8 clustered very close to a patient cell outgrowth assay for HIV latency (Spina et al., 2013).

To identify genes that increase or suppress HIV activation from latency, we conducted the screen with a concentration of CD3/CD28 antibodies leading to half of the maximally attainable activation frequency in this system, approximately 15% GFP-positive cells (Figure 1A). The J-Lat 5A8 cell line was infected with a lentivirus vector at a low multiplicity of infection, leading to the stable integration of the shRNA library (Figure 1B). In this vector, shRNAs are expressed within a transcript that also encodes an mCherry marker for detecting shRNA-expressing cells via FACS. After CD3/CD28 stimulation, we isolated the J-Lat 5A8 cells expressing mCherry and GFP, indicative of shRNA uptake and activation of latent HIV. As a control, the whole unsorted cell population was also harvested (Figure 1C). Genomic DNA was isolated from both populations, the shRNA-encoding cassettes were PCR-amplified, and their frequencies determined by deep sequencing. We defined a quantitative phenotype (log2 enrichment) for each shRNA, as the log2 of the ratio of frequencies of cells expressing this shRNA in the double-positive versus the unsorted population (Figure 1C). If a gene knockdown promotes activation of latent HIV, shRNAs targeting this gene should be enriched in the double-positive population (positive log2 enrichment value) (the targeted gene is latency-promoting). Conversely, if gene knockdown suppresses activation of latent HIV, shRNAs should be relatively disenriched from the double-positive population (negative log2 enrichment value) (the targeted gene is latency-inhibiting) (Table S1). For each gene, log2 enrichment values for shRNAs targeting the gene of interest were compared to the distribution of log2 enrichment values of the negative control shRNAs and a p-value was calculated using the Mann Whitney test (Figure 1C) (Kampmann et al., 2013).

Based on a genome-wide screen carried out in duplicate, we identified genes whose knockdown had a strong, consistent and contrasting effect on the activation of latent HIV. For example, knockdown of FANCC, a member of the Fanconi anemia complex (Huard et al., 2014; Marathi et al., 1996), promoted HIV reactivation. Most of the 25 shRNAs for FANCC were relatively enriched in the double-positive population (i.e., FANCC is latency-promoting) (Figure 1D). On the other hand, knockdown of the protease Calpain 10 (CAPN10), involved in reorganization of actin cytoskeleton (Paul et al., 2003), suppressed

HIV reactivation with a significant number of the shRNAs targeting these genes disenriched from the double-positive population (i.e., CAPN10 is latency-inhibiting) (Figure 1E and Table S1). There were 1145 significantly enriched ($p<0.05$) genes, but the number of genes decreased when the threshold was more stringent. At $p<0.01$, 335 genes were significantly enriched (Figure 1F). Similarly, 950 genes were significantly disenriched ($p<0.05$). With a stringent threshold ($p<0.01$), 330 genes were significantly disenriched (Figure 1G).

Analysis of the shRNA Screen Uncover the mTOR Pathway as a Modulator of HIV Latency

A first examination of the list of the top enriched and disenriched genes (Table 1) identified chromatin regulators (Histones HIST2H3A, HIST1H2AH), proteins involved in inflammation and metabolism such as C1q/TNF-related protein 5 (C1QTNF5), Fbxl2 and tumor necrosis factor receptor-associated factors (TRAF) (Chen and Mallampalli, 2013), regulators of T-cell differentiation (CD46), regulators of actin cytoskeleton organization (CDC42EP5, ABI1) but no evidence for unique cellular pathways controlling latency.

To identify such pathways, we used two complementary methods to analyze enrichment of genes in our dataset. First, we investigated enriched pathways for enriched (latency-promoting) and disenriched (latency-inhibiting) genes separately using Ingenuity Pathway Analysis (IPA), selecting genes with $p<0.01$ and compared the selection to the reference set Ingenuity Knowledge Base (Table S2). Two pathways, Adenosine monophosphate-activated Protein Kinase (AMPK) signaling (8 disenriched genes) and Leucine Degradation I (2 enriched genes), were enriched with a $p<0.01$ (Table S2). At higher p-values (between 0.01 and 0.02) three canonical pathways linked to actin remodeling were identified (Signaling by Rho Family GTPases, RhoGDI Signaling, Actin Cytoskeleton Signaling).

IPA can also identify putative “upstream regulators” to explain observed changes in the dataset. This analysis revealed both Negative elongation factor A (NELFA) and Copper Metabolism (Murr1) Domain Containing 1 (COMMD1) as potential upstream regulators using the latency-promoting genes list ($p<0.001$) (Table S3). This is consistent with the described role of both factors as HIV-1 restriction factors. NELFA is a component of the NELF complex restricting transcription elongation at the LTR promoter in absence of Tat (Karn and Stoltzfus, 2012). COMMD1 is an HIV-1 restriction factor in primary resting CD4 lymphocytes (Ganesh et al., 2003) and can reinforce HIV-1 latency by attenuating NF-κB signaling in myeloid cells (Taura et al., 2015). Another potential identified upstream regulators, Transforming Growth Factor, Beta Receptor 1 (TGFBR1) is also interesting since the mTOR pathway is a downstream effector of TGF-β signaling and an upstream regulator of actin remodeling.

Our second approach was to interface our gene list (enriched and disenriched) onto the CORUM database (Ruepp et al., 2010), which describes a limited but high-confidence set of curated protein complexes. Briefly, we found 2468 unique genes that were described as part of 1728 protein complexes in the CORUM database (Figure 2A, Table S4). We found 75 “Latency-Promoting Complexes” ($p<0.05$), corresponding to 170 unique genes, and 82 “Latency-Inhibiting Complexes” ($p <0.05$), corresponding to 381 unique genes.

Several cellular complexes were identified as regulators of HIV latency (Figure 2B). The p-TEFb complex (p=0.003), a well-known regulator of HIV transcription and cellular cofactor for the HIV transactivator Tat, and the PRC2 complex (EED, EZH2 and YY1) (p=0.013), which is involved in the silencing of HIV genome, were found, as expected, as latency-inhibiting and latency-promoting, respectively (Friedman et al., 2011; Mbonye et al., 2013; Ott et al., 2011). These results confirm that our screen can identify latency-promoting and inhibiting complexes.

In addition to FANCC (Figure 1D), we found the FANC complex as promoting latency (Figure 2B, p=0.001). Interestingly some subunits showed an enhancing effect (as FANCC, FANCA and C17orf70), but others had an opposite effect (as RMI1 and FANCG).

In addition to CAPN10 (Figure 1E), member of the calpain proteins important for actin remodeling, we identified complexes regulating the actin cytoskeleton WAVE-2 (p=0.003) and COFILIN/ACTIN ($p=5.7\times10^{-5}$) as latency-promoting and inhibiting, respectively (Figure 2B).

The screen also identified the SKI-SKIL-SMAD4 pentameric complex (p=0.005) that is involved in TGF-β signaling, and the mammalian target of rapamycin (mTOR) complex (p=0.02) (Figure 2B). The proto-oncogene SKI functions as a repressor of TGF-β signaling and was significantly enriched in the screen (p=0.008), suggesting that inhibition of TGF-β signaling through SKI activity promotes latency. MLST8 (also called G protein b-subunit like protein (GβL)), a subunit shared by mTORC1 and mTORC2 complexes, was disenriched in the shRNA screen (p=0.003), suggesting that inhibition of mTOR signaling, like inhibition of TGF-β signaling, promotes HIV latency.

Interestingly, the mTOR pathway is a downstream effector of TGF-β signaling and an upstream regulator of actin remodeling. Since the mTOR pathway had not previously been identified as a regulator of HIV latency, we further investigated its molecular mechanism.

CRISPRi against mTORC Subunits Prevents Reactivation from HIV Latency in K562 Cells

Given that the mTOR complex was disenriched in the complex analysis, and particularly the MLST8 subunit (p=0.003), we first validated the effect of MLST8 knockdown on HIV latency reversal. We used the CRISPR interference (CRISPRi) K562 cell line (Gilbert et al., 2014) and a new second-generation dual color HIV virus (LTR-HIV-delta-env-nefATG-csGFP-EF1α-mKO2, unpublished, Figure S1A), derived from the R7/E-/GFP/EF1α-mCherry virus (R7GEmC) (Calvanese et al., 2013).

We infected the CRISPRi K562 cells with the HIV reporter and sorted the latent cells (i.e. that expressed mKO2 only) (Figure 3A). For knockdown, we used single guide RNA (sgRNA) lentiviruses. We transduced the sorted latent K562 cells with a non-targeting sgRNA used as a negative control (NC) and three different sgRNAs targeting MLST8 (Gilbert et al., 2014; Horlbeck et al., 2016) (Figure 3A,B). Western blot analyses showed different degrees of MLST8 knockdown efficiency (Figure 3B), with a higher knockdown for MLST8-2 and MLST8-3 sgRNAs than for MLST8-1 sgRNA. We then tested the reversal of latency in cells lacking or not MLST8 using either PMA or Ingenol-B for 24 h (Figure

3C, D). MLST8 knockdown blocked latency reversal compared to NC sgRNA. The more efficiently MLST8 was knocked down, the more latency reversal was suppressed in response to both PMA or Ingenol-B (Figure 3C, D). In contrast, MLST8 knockdown does not repress reactivation induced by the LRAs Panobinostat, Romidepsin or JQ1 (Figure S1B), suggesting that repression of HIV latency reversal by mTOR inhibition depends on NFκB- and PKC-related pathways in latent K562 cells. To further confirm the effect of mTOR on HIV latency, we used the same approach to knockdown MTOR, the catalytic subunit of mTORC1 and mTORC2 (Figure 3E). As expected, knockdown of MTOR repressed latent HIV reactivation following PMA treatment (Figure 3F). To test which mTOR complexes were involved in the regulation of HIV latency, we used sgRNAs against RAPTOR, a specific subunit of mTORC1 or against RICTOR, a specific subunit of mTORC2. Both RICTOR and RAPTOR knockdown repressed latent HIV reactivation (Figure 3E, G, H), suggesting that both mTORC1 and mTORC2 regulate HIV latency reversal. Importantly, knockdown of TSC1, an inhibitor of mTORC1 activity, does not repress latent HIV reactivation (Figure 3E, I), suggesting that inhibition of mTOR complex by knocking down mTORC subunits specifically represses reactivation of latent HIV. Altogether these results confirm the role of both mTOR complexes in HIV latency reversal.

Inhibition of mTOR Signaling Prevents Reactivation from HIV Latency in a Model of Latently Infected CD4 T Cells

Given that knockdown of MLST8 prevented reactivation from latency, we predicted that inhibiting both mTORC1 and mTORC2 function with mTOR inhibitors would interfere with reactivation of latent HIV. To investigate this possibility, we tested the effect of three mTOR inhibitors (i.e. pp242, Torin1, and rapamycin), on HIV latency reversal. Both pp242 and Torin1 compete with ATP for its binding site and inhibit both mTORC1 and mTORC2 (Feldman et al., 2009), whereas rapamycin forms a complex with FKBP12 and binds to mTORC1 causing its inhibition (Dowling et al., 2010). mTORC2, however, is largely insensitive to rapamycin unless used for a prolonged time in certain cell types in which mTORC2 assembly is disrupted (Sarbassov et al., 2006). First, we tested whether Torin1 were repressing HIV latency reversion in latent K562 cells (Figure 4A). Upon PMA stimulation, Torin1 prevented reactivation from HIV latency at 100 and 200 nM concentration (Figure 4A). In the same experiment, we confirmed a suppression of latent HIV reactivation by sgRNA specific for MLST8 (Figure 4A). Importantly, the suppressive effect of the sgRNA on HIV reactivation became smaller in the presence of increasing concentrations of Torin1, consistent with the model that Torin1 inhibits latent HIV reactivation by inhibiting the mTOR complexes (Figure 4A).

Next, we tested whether mTOR inhibition affected reactivation from HIV latency in primary CD4 T cells. We first compared the effect of pp242, Torin1, and rapamycin on phosphorylation of mTOR regulators and substrates in resting primary CD4 T cells stimulated by CD3/CD28 antibodies for 30 minutes. We used a PathScan array based on the sandwich immunoassay principle to measure the level of the following phosphoproteins: AKT-Thr308, which is targeted by PDK1 and monitors PI3K activation and AKT activity towards the positive regulation of mTORC1 activity, AKT-Ser473 targeted by mTORC2, PRAS40-Thr246 substrate of AKT, both mTORC1 targets 4E-BP1-Thr37/46 and p70 S6

Kinase-Thr389, and the p70 S6K substrate S6-Ser235/236 (Figure 4B). As expected, CD3/CD28 co-stimulation increased phosphorylation of all 6 studied phosphosites in 4 different donors (Figure 4B). pp242 decreased the phosphorylation of all 6 mTOR-related proteins. Interestingly we observed that both Torin1 and pp242, and to a less extent rapamycin, globally repressed phosphorylation of most targets examined (Figure 4B). As expected, rapamycin strongly inhibited phosphorylation of the mTORC1 target p70 S6 Kinase-Thr389 and its substrate S6-Ser235/236.

To test the effect of these mTOR inhibitors on reactivation from HIV latency, we used an established model for HIV latency in primary human CD4 T cells, the Bcl2-transduced primary CD4 T-cell latent model (Yang et al., 2009). Cells with latent virus were treated with pp242, Torin1 and rapamycin. The ability of the reporter to reactivate latent HIV was assessed by measuring GFP expression by flow cytometry. We found that pp242 and Torin1 suppressed HIV reactivation in cells isolated from three human donors in a dose-dependent manner (Figure 4C,D and Figure S2A–D). Rapamycin also suppressed HIV reactivation but was not as effective (Figure 4E, Figure S2E–F). Importantly, pp242 and Torin1 did not affect cellular viability, whereas rapamycin induced a slight decrease of viability (Figure 4C–E and Figure S2). For these reasons, we used pp242 and/or Torin1 for the next experiments. These results show that inhibiting both mTORC1 and mTORC2 in CD4 T cells prevents the reactivation of latent HIV proviruses without affecting viability.

Inhibition of mTOR Signaling Represses Tat-Dependent and Tat-Independent Transcription of Latent HIV in CD4 T Cells

Next, we investigated the mechanism of mTOR action in HIV latency. Reactivation of HIV latency is dependent on HIV transcription and the cofactor Tat (Ott et al., 2011). To test whether mTOR inhibitors suppress Tat-mediated HIV gene expression, we transfected an HIV LTR-luciferase construct with a Tat expressing vector into Jurkat cells treated or not with the mTOR inhibitors, pp242, Torin1, and rapamycin. Each of these inhibitors suppressed Tat-mediated gene activation by roughly three-fold in a dose-dependent manner and had no visible effect on HIV promoter basal activity (Figure 5A–C). We also investigated whether mTOR inhibitors could repress the activity of an integrated LTR-Luciferase construct using the established TZM-bl cell line (Figure 5D). We found that both Torin1 and pp242 suppressed HIV promoter activity at several Tat plasmid concentrations (Figure 5D). In contrast, rapamycin did not suppress and actually increased Tat-mediated LTR transactivation (Fig 5D). In conclusion, Torin1 and pp242, but not rapamycin, suppress Tat-mediated LTR activity in the context of an integrated LTR construct in the presence of Tat.

The effect of mTOR inhibition on HIV promoter activity independently of Tat was further examined in J-Lat A72 cells (which harbors an LTR-GFP construct, Tat-independent) and in J-Lat A2 cells (which harbors an LTR-Tat-IRES-GFP construct). These two cell lines were treated with mTOR inhibitors and then activated with the phorbol ester PMA. First, we observed that in absence of any stimulation and Tat, pp242, rapamycin, and Torin1 repressed basal HIV promoter activity in A72 cells (Figure 5E). Second, following PMA stimulation, Torin1 repressed HIV promoter activity in both A72 and A2 cells (Figure S3), consistent with our observation in latent K562 cells (Figure 4A).

Altogether these results indicate that inhibition of mTOR prevents reactivation from HIV latency by blocking Tat-dependent and Tat-independent transcription of HIV.

Inhibition of mTOR Signaling Represses Global CDK9 Phosphorylation

Since efficient Tat-dependent transcription requires an active p-TEFb complex, we next wondered whether suppressing Tat-mediated gene activation by these inhibitors affected CDK9, a component of the p-TEFb complex. Protein extracts from primary CD4 T cells from uninfected donors treated with increasing doses of pp242 and either left unstimulated or stimulated with CD3/CD28 for 120 minutes were run on an SDS PAGE gel. Western blot analysis revealed that CD3/CD28 crosslinking induces mTOR activity as seen by the induction of phospho-S6-Ser240/244, consistent with the electrophoretic mobility shift of S6. Second, western blot analyses with a CDK9-specific antibody revealed that CD3/CD28 signaling induces an electrophoretic mobility shift of the 42-kDa form of CDK9, showing at least three distinct upper bands (Figure 5F, one representative of three donors). CDK9 is regulated by post-translational modifications, such as ubiquitination and phosphorylation (Cho et al., 2010; Mbonye et al., 2013; Nekhai et al., 2014). Interestingly, treatment of CD3/CD28-stimulated CD4 T-cell extracts with Antarctic phosphatase shows that the gel mobility shift is phosphatase sensitive (Figure 5G), suggesting that the upper bands in Figure 5D correspond to phosphorylated CDK9 forms. The phosphatases PP2A and PP1α dephosphorylate CDK9 (Chen et al., 2008; Cho et al., 2010; Nekhai et al., 2014). Okadaic acid inhibits PP2A when used at 10 nM and both PP2A and PP1α when used at 1 μM (Ammosova et al., 2011). To further confirm that these bands correspond to the phosphorylated forms of CDK9, we treated the unstimulated and CD3/CD28-stimulated cells with increasing doses of okadaic acid. Treatment with okadaic acid resulted in a dose-dependent increase in expression and shift in mobility of the 42-kDa form of CDK9, thus ensuring that these gel bands are indeed phosphorylated forms (Figure 5H).

In cells from three independent donors, treatment with pp242 prevented the phosphorylation of the downstream effector S6 on Ser240/244 in a dose-dependent manner (Figure 5F, one representative donor), showing that pp242 treatment efficiently blocks mTOR activity in these conditions. Moreover, treatment with pp242 prevented the mobility shift of CDK9. High doses of pp242 (200 and 1000 nM) suppressed the presence of high mobility shift bands of CDK9 in stimulated CD4 T cells. These data support the model that suppression of latency reversion by mTOR inhibitor occurs via the downregulation of phosphorylation of the 42-kDa form of CDK9, which may prevent CDK9 activation and hence the efficient Tat-dependent transcription.

Inhibiting mTOR Prevents HIV Latency Reversal in HIV-Infected Patient Cells

Next, we wanted to confirm that the mTOR inhibitors suppressed HIV reactivation in the most physiologically relevant experimental system, latent cells from HIV-infected patients. To test this, we treated CD4 T cells from three HIV-infected patients, on antiretroviral therapy for at least 6 months and with undetectable viral loads (<50 copies/ml), with two concentrations of pp242 (10 and 200 nM) and Torin1 (20 and 200 nM). We harvested these treated cells and performed qPCR with primers complementary to the HIV genome as described (Shan et al., 2013). HIV mRNA quantification is shown either as fold-change of reactivation, compared to unstimulated cells (Figure 6A), or as percentage of the maximal reactivation after CD3/CD28 stimulation (Figure 6B). Both representations clearly show that increasing doses of pp242 and Torin1 strongly suppressed reactivation of these latently infected cells and thereby support the role of mTOR in regulating latent HIV.

DISCUSSION

This study identified protein complexes as regulators of HIV latency. The main cellular pathways uncovered here included TGF-β signaling, actin remodeling and mTOR signaling. These three pathways are linked together: the mTOR pathway is downstream of the TGF-β signaling and upstream of actin remodeling. We have confirmed a role of mTOR in HIV-1 latency reversal.

mTOR inhibitors were recently reported to suppress acute viral HIV replication in humanized mice (Heredia et al., 2015). INK128, an inhibitor of mTORC1 and mTORC2, inhibited transcription of HIV in U1 cells treated with PMA. While this report did not address the mechanisms behind the suppression, these observations support our data and show that an inhibitor of both mTORC1 and mTORC2 can suppress HIV-1 reactivation. In addition to an effect in J-Lat and K562 cell lines, we report here that mTOR inhibitors targeting both mTORC1 and mTORC2 strongly suppressed the reactivation of latent HIV-1 virus in a latency model of primary CD4 T cells as well as in HIV-infected patient cells following TCR co-stimulation.

We also found that inhibition of mTORC1 and mTORC2 downregulates CDK9 phosphorylation induced by TCR co-stimulation in CD4 T cells. The findings that mTOR inhibition regulates CDK9 activity is of particular interest. Despite the identification of kinases (i.e. CDK2, CDK7, CaMK1D, CDK9 itself) and phosphatases targeting CDK9 (i.e. PP1α, PP2A, PPM1A, PPM1G) *in vitro* and *in vivo*, the regulation of CDK9 dephosphorylation and phosphorylation remains poorly understood (Cho et al., 2010; Mbonye et al., 2013; Nekhai et al., 2014).

mTORC1 and mTORC2 having pleiotropic effects, it is possible that inhibition of mTORC1/2 triggers other mechanisms in addition to our proposed mechanism. For instance, inhibition of mTORC1 activates autophagy. Sagnier and colleagues have recently reported that inducing autophagy by inhibiting mTOR with Torin1 represses HIV-1 virion production in CD4 T lymphocytes by selectively degrading Tat (Sagnier et al., 2015). Their results are consistent with our observations.

Our results support an essential role for both mTORC1 and mTORC2 in HIV latency. Indeed, dual inhibitors for mTORC1 and mTORC2 such as Torin1 and pp242 are significantly more potent against HIV than a more specific mTORC1-specific inhibitor such as Rapamycin. We also found that knockdown of MLST8 or MTOR, two subunits shared by mTORC1 and mTORC2, prevented HIV reactivation from latency more strongly than knockdown of RAPTOR or RICTOR, alone, two subunits that are unique to either mTORC1 or mTORC2, respectively.

Our observation that MLST8 knockdown prevents HIV reactivation upon PMA stimulation but not following BET inhibitor and HDAC inhibitor treatment suggest that PKC-dependent NF-κB activation might be an important target of mTOR in relation to its effect on latent HIV. Indeed, the ability of PMA to reactivate latent HIV is dependent on PKC activity (Yang et al., 2009) and mTORC1 and mTORC2 play key roles in integrating TCR/CD28 signaling and PKC-dependent NF-κB activation (Lee et al., 2010; Yang et al., 2013).

Much of HIV cure research has thus far focused on the “shock and kill” approach. The aim of this approach is to force reactivation of latent HIV and eliminate latently infected cells via either cytopathic effects or immune recognition (Archin et al., 2012). However, significant concerns have been raised about the feasibility of this approach (Bullen et al., 2014). Also, early clinical trials aimed at reactivating the latent reservoir have highlighted possible problems with this approach (Chun et al., 2015).

In contrast, our data opens up an alternative approach based on the stable suppression of HIV expression in latently infected cells. Other recent reports have identified other targets, such as Tat inhibitors (Mousseau et al., 2015) or Hsp90 inhibitors (Anderson et al., 2014) that might be used along with mTORC1/2 inhibitors to “block and lock” the latent reservoir.

EXPERIMENTAL PROCEDURES

Ultracomplex shRNA screen

The genome-wide RNAi screen was carried out using a pooled ultracomplex shRNA library (Kampmann et al., 2013) in 5A8 cells stimulated with CD3/CD28 antibodies and sorted as reported in the Supplemental Experimental Procedures. The shRNA frequencies in sorted populations were quantified using deep sequencing.

HIV Latency Model in the CRISPRi K562 Cell Line

K562 cell lines expressing dCas9-BFP-KRAB under the SFFV promoter were constructed as described (Gilbert et al, 2014). Cells were infected with the HIV dual fluorescence reporter (HIV virus pseudotyped with the envelope G glycoprotein of the vesicular stomatitis virus (VSV-G), (unpublished) to a final infection rate of 5–20% (3 days post-infection). “Productive infection” was measured by the expression of the LTR-driven codon-switched GFP (csGFP) reporter. Latent infection was reflected by the expression of the LTR-independent marker mKO driven by an EF-1α promoter. Four days post-HIV infection, latent cells were sorted by BD FACS AriaII flow cytometry for stable mKO-only expression.

Transduction of sgRNA in the Latent CRISPRi K562 Cell Line

Individual sgRNAs were cloned into lentiviral expression vectors as described (Gilbert et al, 2014). The latent CRISPRi K562 cell line was spinoculated with individual sgRNA lentivirus for 2 h at 2,000 rpm at 32°C to a final infection rate at 5–25% or nucleofected with sgRNA plasmid using Amaxa Cell Line Nucleofector program T-016. Three days post-transduction, CRISPRi K562 cells expressing the individual sgRNAs were selected with

0.65 μg/mL puromycin for at least 4 days. Cells were harvested after BFP-positive enrichment, washed once with PBS, and cell pellets were snap frozen for western blot experiments.

Reversal of HIV Latency with Drugs

Latent CRISPRi K562 cells expressing individual sgRNA were plated at 50,000 cells/well in round bottom 96-well plates in presence of reactivation drugs (PMA or Ingenol-B or vehicle (DMSO)). Torin1 was supplemented at the same time than reactivation drugs. After 21–24 h of drug treatment, cells were fixed at a final concentration of 2% PFA. Flow cytometry analysis was performed using a LSRII flow cytometer (BD Biosciences). J-Lat A2 and A72 cells were pre-treated 4 h with mTOR inhibitors and then treated with PMA for 20 h.

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) and simulated with CD3/CD28 antibodies as reported in the Supplemental Experimental Procedures.

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 αCD3/αCD28 activating beads (Life Technologies) (10 μL beads per million cells) for 2 h maximum.

Treatment of CD4 Latent T Cells from Patients with pp242 and Torin1

Three independent donors were used for the assay. All enrolled patients were on treatment for > 6 months with undetectable viral load (< 50 copies/ml). For the assay, plates were coated overnight with immobilized CD3 at a concentration of 10 μg/ml. CD28 was added to the cells at 1 μg/ml. pp242 and Torin1 were added at the time of CD28 stimulation. Cells were treated with respective inhibitors and stimulated with CD3/CD28 for 24 h. HIV-specific qPCR was conducted as described (Shan et al., 2013). Each qPCR well represents over 600,000 cells. qPCR was performed on ViiA7- Real Time PCR instrument (LifeTech).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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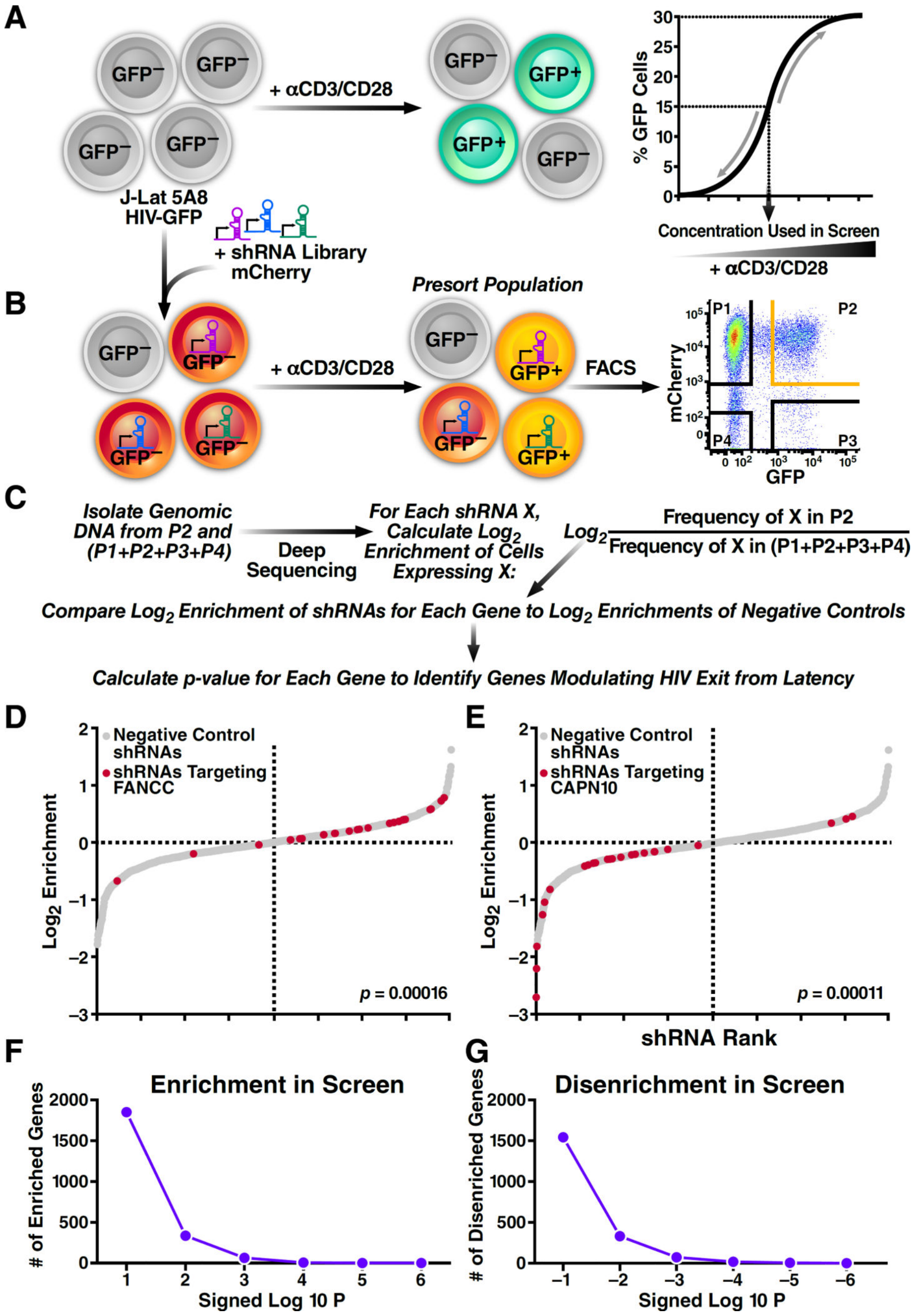

Figure 1. High Complexity shRNA Screen to Identify Genes that Control HIV Latency

(A) Schematic of strategy used to stimulate J-Lat 5A8 cells with CD3/CD28 to promote HIV exit from latency

(B) Strategy to introduce human genome-wide mCherry-tagged shRNA library into J-Lat cells, and then stimulate cells with a 3 μg/ml CD3/CD28 to yield 15% double-positive cells. GFP and mCherry represent J-Lat 5A8 HIV-GFP, and shRNA expression, respectively.

(C) Calculations conducted on samples that are deep sequenced to obtain p-values to identify genes involved in HIV latency.

(D) FANCC, an example of a gene that promotes latency.

(E) CAPN10, an example of a gene that inhibits latency.

(F, G) Graphs depicting number of enriched (F) or disenriched (G) genes plotted as a function of signed log 10 P values.

See also Tables S1, S2 and S3

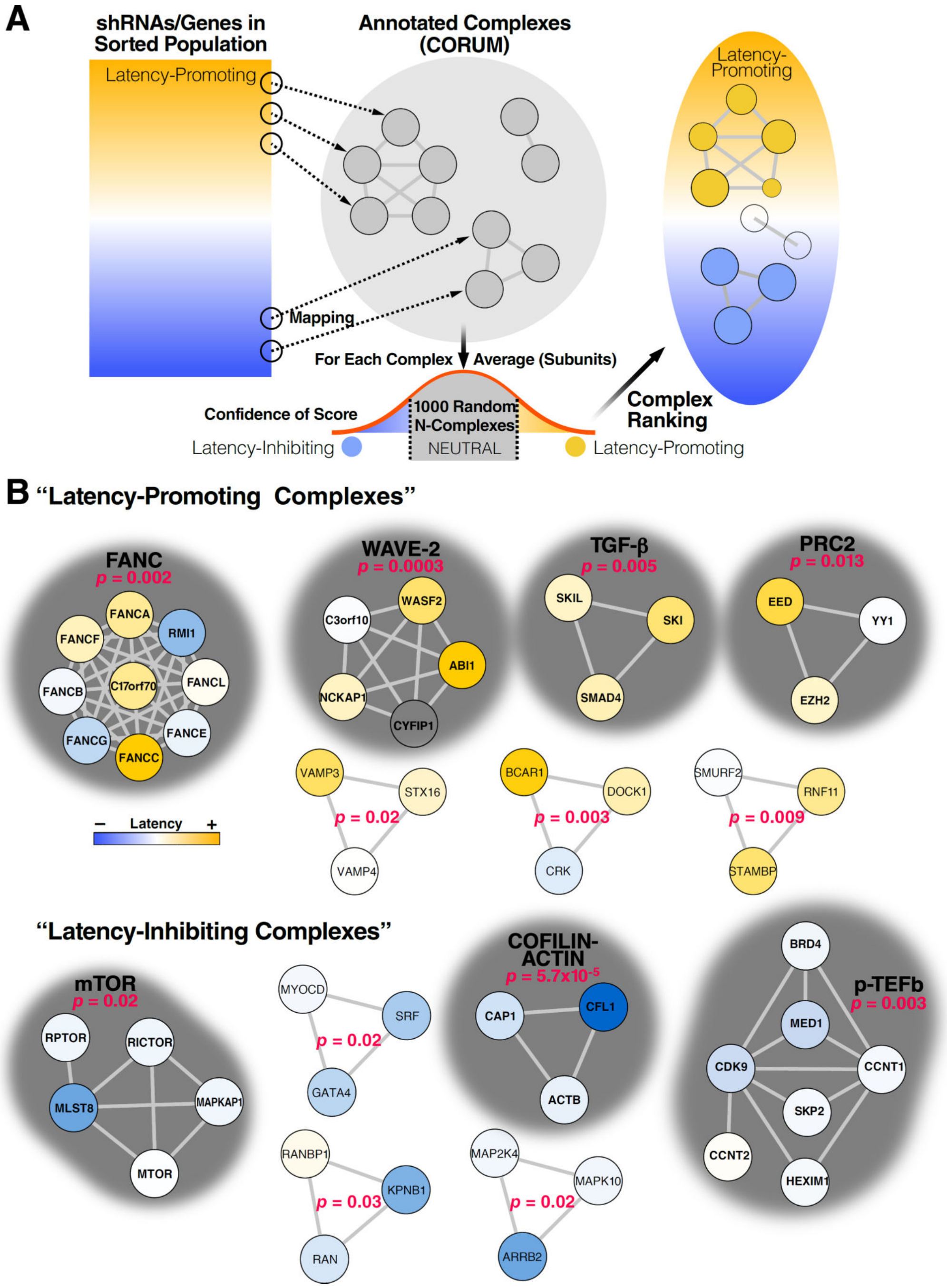

Figure 2. CORUM Analysis Results in the Identification of Several Interesting Complexes

(A) Schematic of procedure used to calculate and identify latency-promoting complexes and latency-inhibiting complexes in CORUM using p-values.

(B) (Top) Latency-promoting and (Bottom) latency-inhibiting complexes (see text for details).

See also Table S4

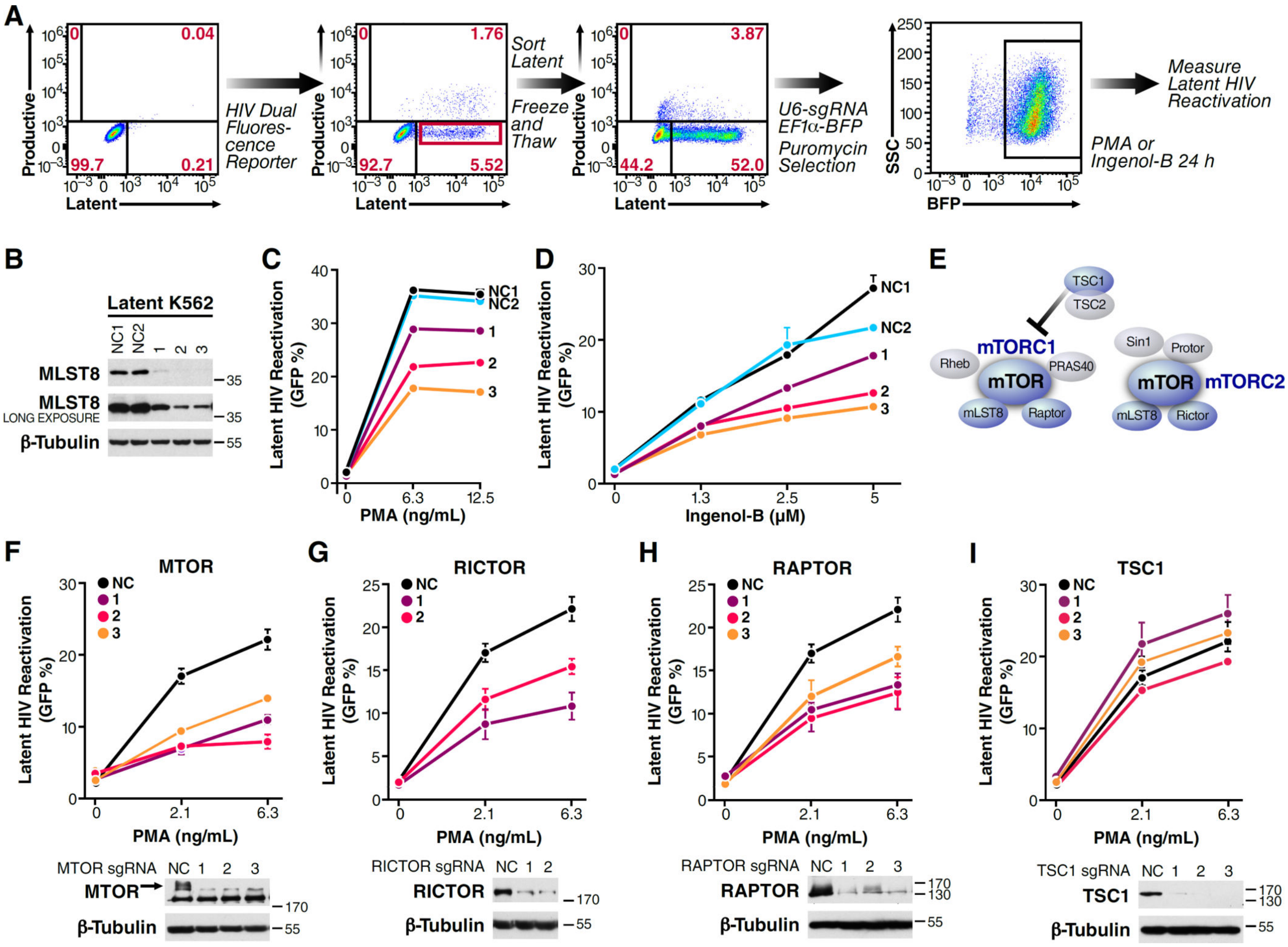

Figure 3. CRISPRi against MLST8 in latent K562 cells prevents reversal of HIV latency by LRAs

(A) Procedure to obtain latent CRISPRi K562 cells and transduce them with sgRNA lentiviruses and select by puromycin. LRAs were added to test reactivation of HIV.

(B) Efficiency of MLST8 knockdown with three different sgRNAs checked by western blot. Cells transduced with NC (negative control) sgRNA lentiviruses done in duplicate (NC-1 and NC-2) were used as control.

(C, D) Percentage of GFP-positive cells 24 h after reactivation with PMA (C) and Ingenol-B (D). Data are represented as mean +/− SD of triplicate values, representative of two independent experiments.

(E) Simple scheme representing the mTORC1 and mTORC2 subunits and regulator that were knockdown by CRISPR interference.

(F,G,H,I) Latent CRISPRi K562 cells were transduced with sgRNA lentiviruses targeting MTOR (F), RICTOR (G), RAPTOR (H), TSC1 (I) and selected by puromycin. Percentages of GFP-positive cells 21–24 h after reactivation with PMA are indicated on the upper panels. Efficiency of knockdown for each gene is shown on the lower panels with western blot. Cells transduced with NC (negative control) sgRNA lentivirus were used as control. Data are represented as mean +/− SEM of at least three independent experiments.

See also Figure S1

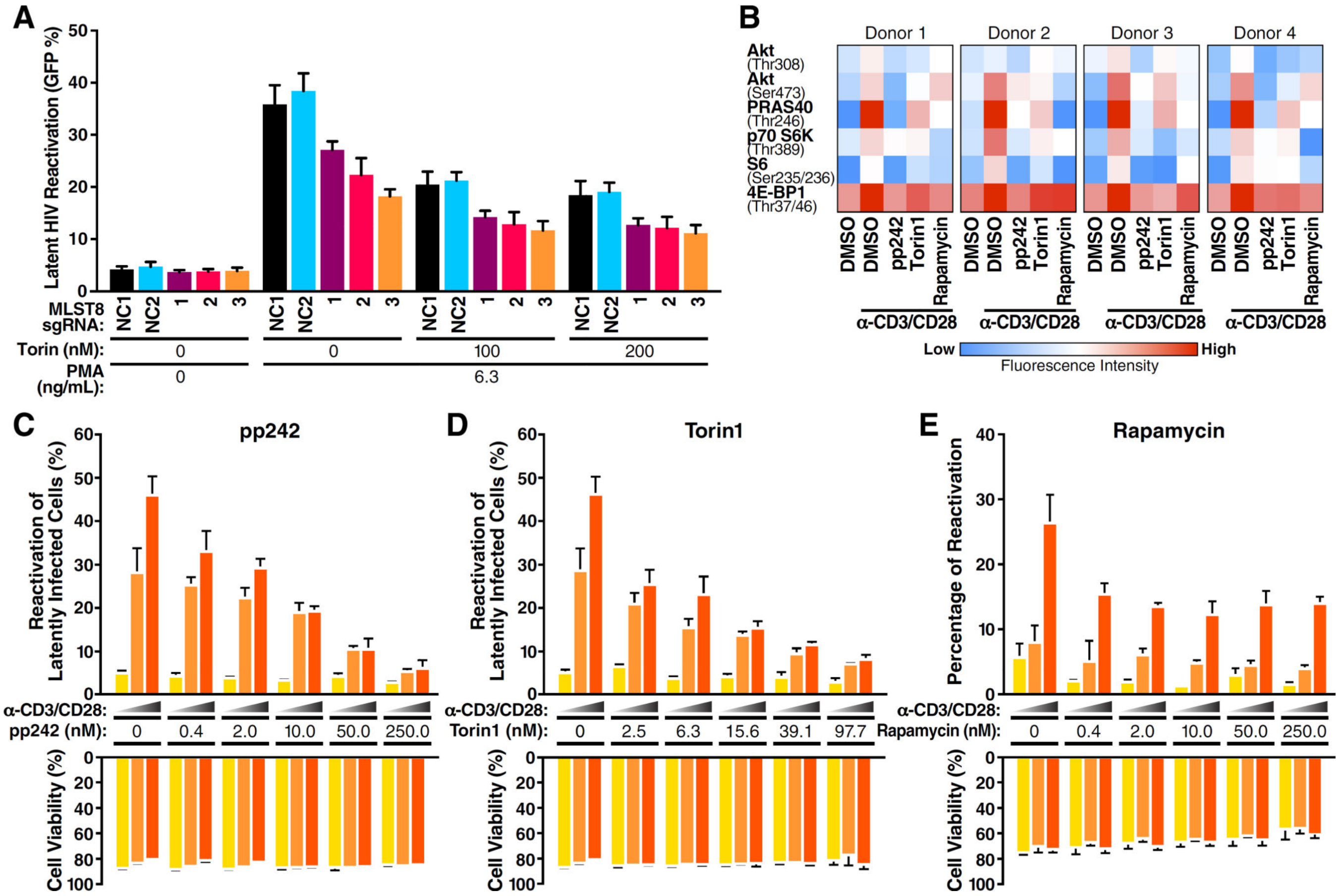

Figure 4. mTOR Pathway Inhibitors Suppress Reactivation of Latent HIV in Human CD4 T Cells

(A) Percentage of GFP-positive K562 cells expressing NC or MLST8 sgRNAs 21–24 h after PMA stimulation with or without simultaneous Torin1 treatment. Data are represented as mean +/− SD of four independent experiments.

(B) Detection of selected phosphorylated proteins in CD4 T cells from four independent donors using PathScan analysis. CD4 T cells were treated with either 0.01% DMSO or incubated for 30 minutes with 25μL of αCD3/αCD28 activating beads with DMSO, 250nM pp242, 97.7nM Torin1 or 10nM rapamycin. The amount of phosphorylated proteins is scaled internally to each donor.

(C–E) Bcl-2 transduced latently infected cells were either unstimulated (yellow) or stimulated with CD3/CD28 antibodies (2.5 μg/ml CD3 and 0.65 μg/ml CD28 (orange); 10 μg/ml CD3 and 0.65μg/ml CD28 (red)) for 48 h to reactivate HIV in the presence of increasing concentrations of pp242 (C), Torin1 (D) and rapamycin (E). Reactivation of HIV was assessed by measuring GFP by flow cytometry, and the percentages of reactivation were calculated for each batch of latently infected cells by maximum activation with PMA and Ionomycin (top panels) (Yang et al., 2009). Percentage of live cells in each sample is shown (bottom panels). Data are represented as mean +/− SD.

See also Figure S2

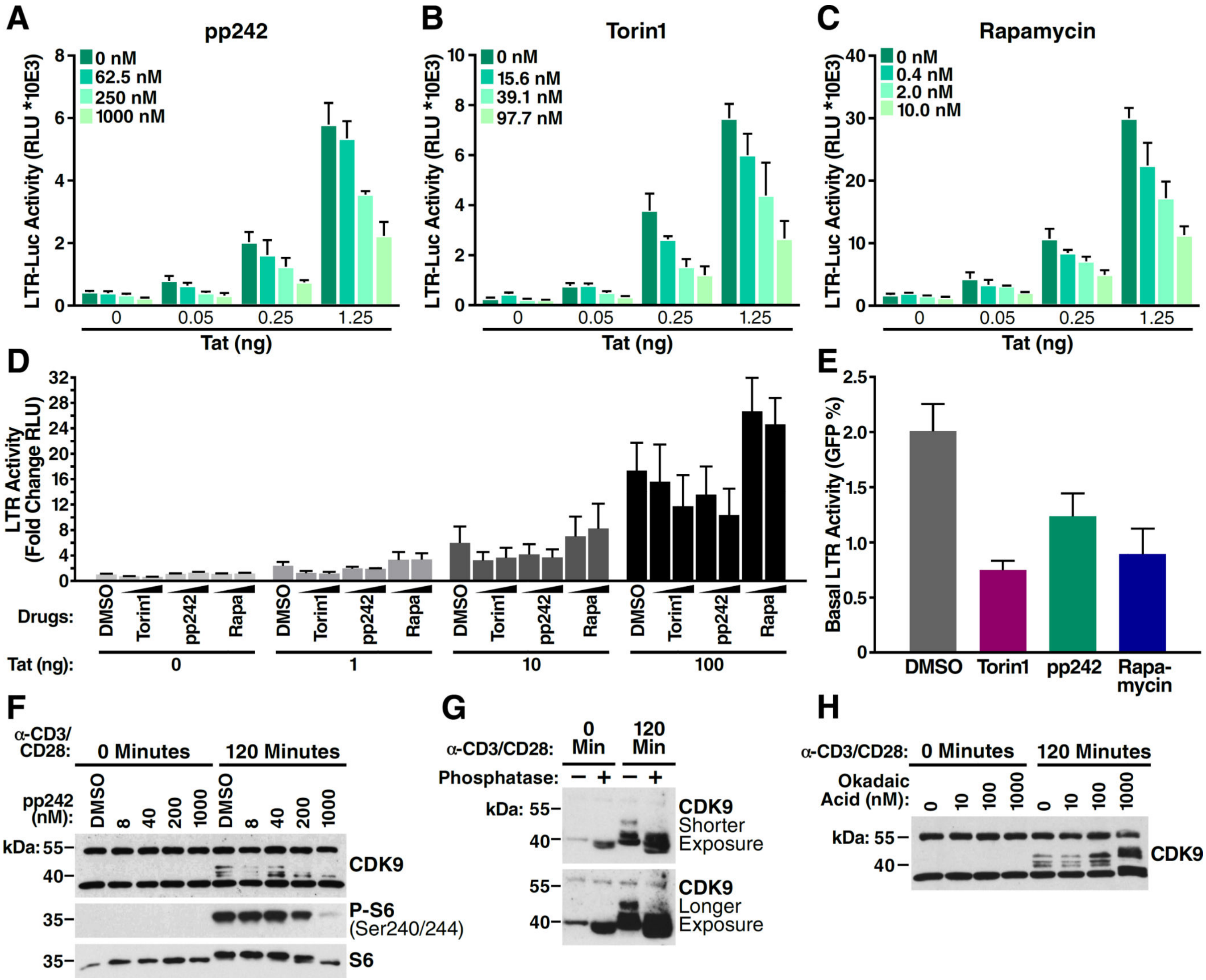

Figure 5. mTOR Inhibition Suppresses Tat-Independent and Tat-Dependent HIV LTR Activation and CDK9 Phosphorylation

(A–C) pp242 (A), Torin1 (B) and rapamycin (C) suppress Tat-dependent HIV LTR activation after 24h in a dose-dependent manner in luciferase assays with the HIV LTR construct in Jurkat cells. Tat was used at increasing doses (0, 0.05, 0.25 and 1.25 ng). 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).

(D) pp242, Torin1 and rapamycin treatment differentially affects Tat-dependent HIV LTR activation in the context of integrated provirus (TZM-bl cells). Tat was used at increasing doses (0, 1, 10 and 100 ng plasmid). TZM-bl cells were transfected with indicated amounts of Tat plasmid and treated with DMSO, Torin 1 (100, 200nM), pp242 (0.5, 1µM), or Rapamycin (10, 50nM). 24h post-treatment, cells were lysed and HIV transcription was measured via luciferase activity. Data are represented as mean +/− SEM of fold change of RLU normalized by protein content.

(E) pp242, Torin1 and Rapamycin reduce basal LTR activity in A72 Jurkat cells (integrated LTR-GFP) measured by percentage of GFP-positive cells.

(F–H) Primary CD4 T cells were isolated from a donor’s blood and either pre-treated with pp242 (0, 8, 40, 200 and 1000 nM) for 30 minutes (F), left untreated (G), or pre-treated with Okadaic acid (0, 10, 100 or 1000 nM) for 30 minutes (H). The cells were co-stimulated with CD3/CD28 antibodies for 2 h. Cells were harvested for protein extraction right before (0 min) and after (120 minutes) adding the CD3/CD28 beads. Proteins were extracted as explained in Experimental Procedures. Whole cell protein extracts from (G) were either phosphatase-treated or not. Extracts were run on an SDS-PAGE gel and immunoblotted for the indicated proteins (CDK9, phospho-S6 (Ser240/244) and S6).

See also Figure S3

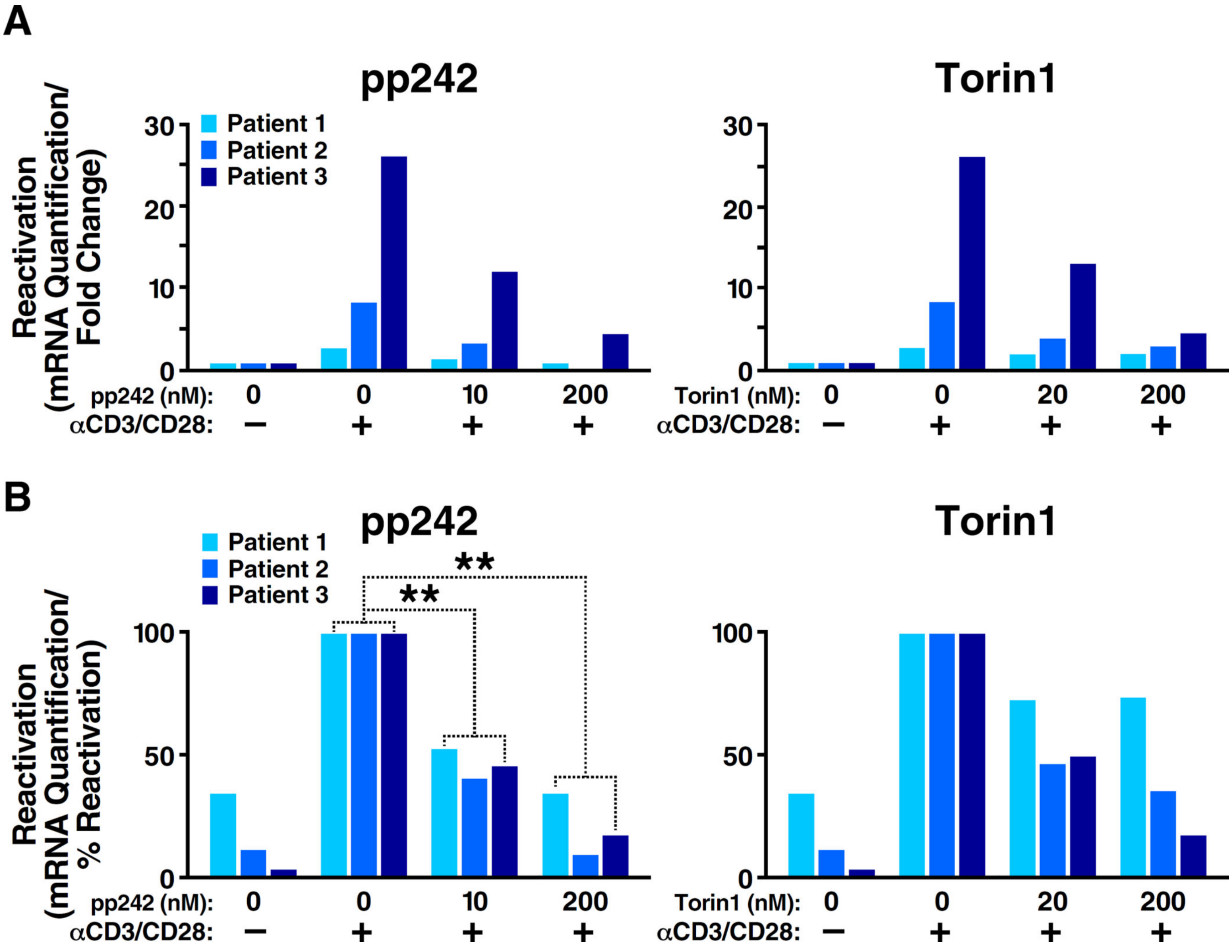

Figure 6. mTOR Inhibition Causes Suppression of Reactivation of Latently Infected Cells in the Patient Model

(A) pp242- and Torin1-treated CD4 T cells in three HIV-infected patients on anti-retroviral therapy with undetectable viral load led to a drastic decrease in fold change of HIV mRNA. (B) Patient samples in (A) were regraphed to show the percentage reactivation of HIV mRNA. Significant p-values with t-test (two-sample unequal variances) are shown (**: p<0.01)

Table 1

Top Hit Genes Identified in the shRNA Screen

The top 10 enriched and disenriched genes with Gene ID, Symbol, Gene Info and log transformed signed P Mann Whitney-value are shown.

	#GeneID	Symbol	GeneInfo	Signed log10 P
Top 10 Enriched Genes	387264	KRTAP5-1	Keratin Associated Protein 5-1	6.516
	148170	CDC42EP5	CDC42 Effector Protein (Rho GTPase Binding) 5	4.515
	333932	HIST2H3A	Histone Cluster 2, H3a	4.51
	25827	FBXL2	F-box and Leucine-rich Repeat Protein 2	4.442
	25794	FSCN2	Fascin Homolog 2, Actin-bundling Protein, Retinal (*Strongylocentrotus purpuratus*)	4.328
	147381	CBLN2	Cerebellin 2 Precursor	4.015
	2176	FANCC	*Fanconi anemia*, Complementation Group C	3.795
	85235	HIST1H2AH	Histone Cluster 1, H2ah	3.751
	10006	ABI1	Abl-interactor 1	3.749
	4179	CD46	CD46 Molecule, Complement Regulatory Protein	3.732
Top 10 Disenriched Genes	148252	DIRAS1	DIRAS Family, GTP-binding RAS-like 1	−13.326
	114902	C1QTNF5	C1q and Tumor Necrosis Factor Related Protein 5	−5.766
	1193	CLIC2	Chloride Intracellular Channel 2	−5.666
	390667	PTX4	Pentraxin 4, Long	−5.466
	51005	AMDHD2	Amidohydrolase Domain Containing 2	−5.301
	3630	INS	Insulin	−4.797
	220004	PPP1R32	Chromosome 11 Open Reading Frame 66	−4.755
	5473	PPBP	Pro-platelet Basic Protein (Chemokine (C-X-C Motif) Ligand 7)	−4.69
	284353	NKPD1	NTPase, KAP Family P-loop Domain Containing 1	−4.686
	50632	CALY	Calcyon Neuron-specific Vesicular Protein	−4.554

See also Tables S1, S2 and S3