Cyclin-dependent kinase 7 (CDK7)-mediated phosphorylation of the CDK9 activation loop promotes P-TEFb assembly with Tat and proviral HIV reactivation

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ABSTRACT

The HIV trans-activator Tat recruits the host transcription elongation factor P-TEFb to stimulate proviral transcription. Phosphorylation of Thr186 on the activation loop (T-loop) of cyclin-dependent kinase 9 (CDK9) is essential for its kinase activity and assembly of CDK9 and cyclin T1 (CycT1) to form functional P-TEFb. Phosphorylation of a second highly conserved T-loop site, Ser175, alters the competitive binding of Tat and the host recruitment factor bromodomain containing 4 (BRD4) to P-TEFb. Here, we investigated the intracellular mechanisms that regulate these key phosphorylation events required for HIV transcription. Molecular dynamics simulations revealed that the CDK9/CycT1 interface is stabilized by intramolecular hydrogen bonding of pThr186 by an arginine triad and Glu96 of CycT1. Arginine triad substitutions that disrupted CDK9/CycT1 assembly accumulated Thr186-dephosphorylated CDK9 associated with the cytoplasmic Hsp90/Cdc37 chaperone. The Hsp90/Cdc37/CDK9 complex was also present in resting T cells, which lack CycT1. Hsp90 inhibition in primary T cells blocked P-TEFb assembly, disrupted Thr186 phosphorylation, and suppressed proviral reactivation. The selective CDK7 inhibitor THZ1 blocked CDK9 phosphorylation at Ser175, and \textit{in vitro} kinase assays confirmed that CDK7 activity is principally responsible for Ser175 phosphorylation. Mutation of Ser175 to Lys had no effect on CDK9 kinase activity or P-TEFb assembly but strongly suppressed both HIV expression and BRD4 binding. We conclude that the transfer of CDK9 from the Hsp90/Cdc37 complex induced by Thr186 phosphorylation is a key step in P-TEFb biogenesis. Furthermore, we demonstrate that CDK7-mediated Ser175 phosphorylation is a downstream nuclear event essential for facilitating CDK9 T-loop interactions with Tat.
INTRODUCTION

HIV patients require continuous antiviral therapy to prevent viral rebound from latent reservoirs (1-3). Dissecting the host and viral mechanisms that are utilized by HIV to emerge from transcriptional latency is therefore crucial for the development of strategies to eliminate HIV from infected individuals.

HIV transcription is auto-regulated by the viral trans-activator protein, Tat. In the absence of Tat, weakly processive transcription initiation complexes assembled at the proviral promoter synthesize short transcripts of ~60 nucleotides, due to promoter proximal pausing by RNA polymerase II (RNAP II) in a region adjacent to the 5'-nucleotide TAR RNA coding sequence (4-6). Tat efficiently stimulates HIV transcription elongation by recruiting P-TEFb in complex with a super elongation complex (SEC) to RNAP II as it transcribes through the TAR RNA hairpin (7-11).

P-TEFb is a nuclear heterodimer complex comprising the CDK9 serine/threonine kinase and a cyclin T subunit. Although cyclin T1 (CycT1) is the only cyclin capable of interacting with HIV Tat, the alternative cyclin T splice variants T2a and T2b might support transcription of cellular genes (12,13). Recruitment of CDK9/CycT1 at TAR enables CDK9 to phosphorylate the E subunit of the repressive NELF complex which causes NELF to dissociate from TAR and the paused polymerase complex (14-16). CDK9 also extensively phosphorylates the CTD of RNAP II mainly at Ser2 residues of the heptad Y-S-P-T-S-P-S repeats and C-terminal repeats (G-S-Q/R-T-P) of hSpt5 subunit of DSIF at Thr4 residues (17-21). The overall effect of these phospho-modifications by P-TEFb is to remove the blocks to elongation imposed by NELF and DSIF and to stimulate efficient elongation and co-transcriptional processing of proviral transcripts.

The P-TEFb that is assembled following T-cell activation is incorporated into a nucleoplasmic 7SK snRNP complex. Within this RNP complex, 7SK snRNA provides a three dimensional molecular scaffold that binds two molecules of P-TEFb to a homodimer of its inhibitory protein HEXIM1 (22-25). 7SK snRNP localizes to nuclear speckles found in proximity to genomic sites of active transcription (26-29). Thus, the 7SK snRNP complex provides an exchangeable pool of P-TEFb that can be readily availed to stimulate gene transcription (30). In a recent proteomics study designed to identify modifications on HEXIM1 that regulate the sequestration of P-TEFb by 7SK snRNP, we found that 7SK snRNP also serves to retain P-TEFb in the nucleus, suggesting that exchange of P-TEFb subunits between the nucleus and the cytoplasm is a regulatory feature of this system (31).

Mechanisms in primary T cells that govern the activation of P-TEFb and promote its assembly with Tat for efficient reactivation of latent HIV are incompletely understood. Here we studied the impact of key phosphorylation events on the control of P-TEFb at two key steps:

Step 1: Exchange of CDK9 from an Hsp90/Cdc37 cytoplasmic complex to the 7SK RNP complex requires pThr186 phosphorylation. In resting memory CD4+ T cells, which form the bulk of the latent HIV reservoir (32), P-TEFb is not yet formed (33,34). In these quiescent cells, CycT1 is largely absent and CDK9 is found in the cytoplasm (35,36), where it is thought to be in an immature, inactive state. Here we demonstrated that in resting CD4+ T cells Hsp90/Cdc37 stabilizes the basal expression of CDK9 prior to the elevation of CycT1 protein levels. CDK9 bound to the Hsp90/Cdc37 complex has previously been observed in HeLa and Jurkat T cells but its role in P-TEFb regulation is not completely understood (36,37).

Using molecular dynamics simulations and mutagenesis approaches we demonstrated that an intramolecular R65/R148/R172 hydrogen bond network coordinated by pThr186 and extending to Glu96 of CycT1 is required for heterodimer formation. Dephosphorylation of pThr186 releases CDK9 from CycT1 and permits its interactions with the Hsp90/Cdc37 chaperone complex in the cytoplasm of T cells. Conversely, the disruption of the CDK9/Hsp90/Cdc37 complex is associated with phosphorylation of Thr186, reassembly with CycT1 and incorporation of P-TEFb into 7SK snRNP.

Step 2: Exchange of P-TEFb between the the 7SK RNP complex and Tat and/or Brd4 is regulated by CDK7 phosphorylation of Ser175. Although Tat forms the majority of its contacts with the CycT1 subunit, the N-terminal end of its activation domain can potentially form two hydrogen bonding interactions with the T-loop of
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CDK9 (38). These CDK9-Tat intermolecular interactions can potentially be further strengthened by an additional hydrogen bond between pSer175 and Lys12 of Tat (36).

We have previously identified Ser175 to be a CDK9 phosphorylation site marking activated P-TEFb in T cells that is excluded from 7SK snRNP (36). However, the kinase(s) responsible for this key regulatory event has not been identified. Here we present evidence that CDK7 is responsible for this modification based on cell-based studies in T cells using the selective inhibitor THZ1 and in vitro kinase assays.

Ser175 also mediates the interaction between P-TEFb and BRD4, a bromodomain extra-terminal protein that is a major recruiter of P-TEFb to cellular genes. Immunoprecipitation of P-TEFb from HIV-infected T cells and studies of the Ser175 to Lys mutation substantiated the hypothesis that pSer175 favors Tat’s interaction with P-TEFb over BRD4.

RESULTS

Stabilization of the CDK9-CycT1 interface by Thr186 phosphorylation.

We have found previously that blocking the phosphorylation of Thr186 by site-directed mutagenesis not only abrogates in vitro CDK9 kinase activity but also severely disrupts its heterodimer interactions with the CycT1 subunit (36). Our findings were inconsistent with reports that while mutations of Thr186 (T186A or T186E) clearly abolished incorporation of CDK9 into 7SK snRNP, they did not disrupt CDK9-CycT1 heterodimer assembly (22,39,40).

In all published P-TEFb X-ray structures, phosphorylated Thr186 is positioned to coordinate an intramolecular hydrogen bonding network containing three arginine residues (Arg65, Arg-148, and Arg-172). In addition, Arg65 and Arg-172 are positioned to form intermolecular salt bridge interactions with Glu96 of CycT1 (Fig. 1A). This hydrogen bond network, nucleated at the phospho-Thr, therefore extends to the CDK-cyclin dimer interface. A homologous interface is observed in the CDK2-cyclin A X-ray structure, where it was first proposed to be important for stabilizing heterodimer assembly (41).

To assess the role of Thr186 phosphorylation in its coordination with the Arg triad, we performed 100-ns molecular dynamics (MD) simulations of energy-minimized structures for both the phosphorylated and mutated form of Thr186 using the CDK9/CycT1 3BLQ.pdb X-ray structure as template (42). Results from MD simulations revealed that the T186A mutation destabilizes the triad network (Arg65, Arg-148, and Arg-172), evidenced by its increase in radius of gyration ($R_g$), compared to the phosphorylated form (Fig. 1B). The greater fluctuation of $R_g$ observed in the T186A simulation further showed that the Arg triad experienced increased structural flexibility due to the loss of the phosphate at Thr186.

Although T186A did not appear to significantly affect dynamic interactions at the dimer interface between CDK9 Arg65 and CycT1 Glu96, it clearly caused an increased perturbation of the intermolecular distance between CDK9 Arg-172 and CycT1 Glu96 (Suppl. Fig. 1). Thus, these simulation results suggest that phosphorylation of CDK9 at Thr186 is critical in coordinating and stabilizing the Arg network to enable proper dimer interactions with CycT1.

To confirm that pThr186 functions to promote the assembly of P-TEFb by positioning Arg65 and Arg-172 to favorably interact with CycT1, mutagenesis experiments were performed. Cells were infected with retroviral vectors expressing FLAG-CDK9. Complexes containing the FLAG-CDK9 were purified by immunoprecipitation and analyzed by Western blots using antibodies to CDK9, pThr186 CDK9, CycT1; the 7SK RNP complex components HEXIM1 and LARP7; and Hsp90 and Cdc37 (Fig. 2).

Mutation of CDK9 at either Arg65, Arg-148, or Arg-172 to Ala disrupted intracellular P-TEFb assembly resulting in the loss of association with CycT1, HEXIM1, and LARP7. These phenotypes were equivalent to the T186A mutant (Figs. 2A-2C and Suppl. Figs. 2A and 2C). Interestingly, in addition to disrupting heterodimer assembly and 7SK snRNP association, the R65A and R172A point mutations also caused a loss of the phosphate at Thr186 but, importantly, retained CDK9 interactions with the Hsp90/Cdc37 chaperone (Figs. 2B, 2C and Suppl. Fig. 2C). It is also noteworthy that 7SK snRNP dissociation to
free up P-TEFb can be triggered by the transient dephosphorylation of Thr186 (43,44).

A modest, but statistically significant, enhancement of CDK9 association with Hsp90/Cdc37 was observed with the T186A mutant (Fig. 2C). CDK9 mutants that did not stably interact with CycT1 and were not phosphorylated at Thr186 (R65A, R172A, and T186A) were found to be catalytically inactive (Suppl. Fig. 3).

Mutation of CycT1 at Glu96 to Ala had a modest disruptive effect on P-TEFb assembly and interactions with 7SK snRNP, but P-TEFb assembly and interactions with 7SK snRNP were abolished upon mutation of Glu96 to Lys (Suppl. Fig. 2B).

Similarly, mutation of CDK9 at Lys164, located right near the start of the activation loop and that was identified by tandem mass spectrometry to be subject to acetylation (Suppl. Fig. 4), resulted in the loss of P-TEFb assembly coupled with the dephosphorylation of Thr186 (Suppl. Figs. 5A and 5B).

None of these CDK9 mutations had an adverse effect on the extent of proviral reactivation in Jurkat 2D10 T cells (Suppl. Fig. 6). It seems likely that because these mutants fail to interact with CycT1 they are unable to disrupt formation of functional P-TEFb with the endogenous CDK9, which remains at sufficient levels to support proviral transcription.

**Hsp90/Cdc37 interacts with a CDK9 monomer that is unmodified at Thr186.**

The mutagenesis studies described above confirm that the coordination of an intramolecular CDK9 Arg hydrogen bonding network by pThr186, which extends to Glu96 of CycT1, is critical for stable heterodimer interactions. We therefore hypothesized that in the absence of P-TEFb assembly, CDK9 becomes bound to Hsp90/Cdc37 and that the phosphorylation of CDK9 at Thr186 is required for the de novo assembly of catalytically competent P-TEFb.

Unstimulated memory CD4+ T cells, are highly restricted for CycT1 expression (Fig. 3) (28,35,36). In these cells CDK9 is found to be primarily in the cytoplasm where it is likely to be present in an immature and catalytically inactive state. Stimulation of these primary T cells by T-cell receptor activation results in the nuclear import of CDK9 that is coupled with an exclusively nuclear elevation of both pSer175 CDK9 and CycT1 (Fig. 3).

We therefore tested the hypothesis that cytoplasmic CDK9 found in resting memory T cells was associated with the kinase-specific Hsp90/Cdc37 chaperone, which has been reported to bind CDK9 in HeLa and Jurkat T cells (2,36,37). Immunoprecipitation experiments demonstrated that a CDK9-Hsp90/Cdc37 complex can be isolated from unstimulated memory CD4+ T cells (Fig. 4A).

In order to conclusively demonstrate that Hsp90-bound CDK9 is not assembled with CycT1 and that CDK9 in these complexes is not phosphorylated at Thr186, a sequential immunoprecipitation of CDK9 and Hsp90 was performed using Jurkat T cells stably expressing FLAG CDK9 wildtype. Whole cell extracts from these cells were subjected to immunoprecipitation with anti-FLAG resin to pull down CDK9 complexes. After eluting these complexes with FLAG peptide, the eluates were further subjected to anti-Hsp90 immunoprecipitation. Both the FLAG peptide eluates and anti-Hsp90 precipitation of the eluates were analyzed by Western blotting for the presence of CycT1 and pThr186 CDK9. Fig. 4B shows that while both CycT1 and pThr186 could be detected in the FLAG-CDK9 eluates, they were absent from the anti-Hsp90 immunoprecipitation of the eluates. These results indicate that CDK9 that is not assembled with CycT1, is associated with Hsp90 and is very likely to be unphosphorylated at Thr186.

**Inhibition of Hsp90 disrupts P-TEFb activation and suppresses proviral reactivation.**

Blockade of the ATP-dependent chaperone activity of Hsp90 with ansamycins or their derivatives, which competitively inhibit ATP binding, has been shown to disrupt the maturation of already bound target client proteins by preventing their proper folding and release from the chaperone (45). Inhibition of Hsp90 can either ultimately lead to conformational arrest of immature client proteins or the proteasomal degradation of some of these clients (45-49). Recently, it was reported that Hsp90 inhibitors,
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some of which are currently under phase II clinical trials to treat cancer, can potently suppress the reactivation of latent HIV in response to T-cell activation signals, but this phenotype was attributed to impact of the drugs on NF-κB activation (50,51).

Since the latent reactivation of HIV in resting memory T cells is primarily responsive to NFAT, rather than NF-κB (52-54), we tested the hypothesis that Hsp90 inhibition leads to a block in P-TEFb assembly, and consequently, blocks HIV reactivation. Treatment of resting primary Th17 cells with the geldamamycin derivative 17-AAG at 1 μM during 18 h or 48 h TCR activation led to a substantial inhibition of P-TEFb activation as assessed by the induction of CycT1 and pSer175 CDK9 (Fig. 4C and Suppl. Fig. 7A). This inhibitor treatment also resulted in 2.5- and 2.6-fold reductions in TCR-induced pThr186 CDK9 levels at 18 h and 48 h, respectively (Fig. 4C and Suppl. Fig. 7A). However 17-AAG did not significantly reduce total CDK9 protein expression (Fig. 4C and Suppl. Fig. 7A). Overall, similar results were also obtained using Concanavalin A-stimulated primary memory resting CD4+ T cells (Suppl. Fig. 7A). Treatment of latently infected resting primary Th17 cells (Suppl Fig. 7B) with 17-AAG during 18 h or 48 h TCR activation also led to a 1.65 to 2-fold decrease in induced HIV Nef expression (Fig. 4D and Suppl. Fig. 7C).

Thus, inhibition of Hsp90 chaperone activity blocks P-TEFb biogenesis and thereby inhibits the eventual reactivation of latent HIV in primary T cells.

Rapid turnover of pSer175 CDK9 in Jurkat T cells.

We next examined the biological role of pSer175, a nearby signal-dependent T-loop modification that we previously showed was induced in response to T-cell receptor activation or treatment with the potent PKC agonist PMA (36). To assess whether both of these CDK9 T-loop modifications (pSer175 and pThr186) are catalyzed by the same or distinct kinase activities, Jurkat T cells were pre-treated with the pan-PKC inhibitor Ro-31-8220 at 100 nM prior to T-cell activation with phorbol ester. Not surprisingly, the drug potently blocked the rapid induction of pSer175 without affecting the pre-existing levels of pThr186 (Fig. 5A). Using this inhibitor, we were then able to conduct a pSer175 CDK9 chase experiment to measure the half-life of this modification. Jurkat T cells were stimulated with PMA for 2 h followed by treatment with Ro-31-8220 over a 6-hour time course. The chase experiment revealed that pSer175 is a short-lived modification that turns over with a half-life of ~ 3 h (Suppl. Fig. 8). It is important to note that the levels of pThr186 did not appear to substantially diminish with time of PKC inhibitor treatment (Suppl. Fig. 8).

CDK7 is required for CDK9 Ser175 phosphorylation.

Although signaling by PKC may be required to maintain high levels of pSer175 in Jurkat T cells, the preceding experiments did not demonstrate that pSer175 is directly modified by PKC itself. The CDK7 subunit of both CDK-activating kinase complex (CAK) and TFIH, was revealed by chemical genetics experiments to be the long-elusive kinase that switches CDK9 to a catalytically active state by phosphorylating the critical T-loop residue Thr186 (55). These experiments employed transgenic cells in which the wildtype CDK9 gene had been replaced by a F103G CDK9 mutant that could utilize bulky ATP analogs as substrate.

We therefore tested whether CDK7 could also mediate Ser175 phosphorylation of CDK9. For these experiments, we employed a more recent, well-characterized and highly selective inhibitor of CDK7, THZ1. This inhibitor fits into the ATP-binding pocket of CDK7 and covalently crosslinks to Cys312 at its C-terminal tail (56).

Since pThr186 CDK9 is observed to be a constitutive modification in actively replicating T cells (36), we first examined how THZ1 influenced its turnover rate in the presence of phorbol ester (PMA) treatment. We, and others, have shown previously that in addition to inducing pSer175, PMA treatment results in a partial dissociation of P-TEFb from 7SK snRNP (57,58).

Pretreatment of Jurkat T cells with 50 nM THZ1 for 15 min followed by stimulation with PMA for 2 h or up to 6 h in the presence of inhibitor did not elicit a significant change in the levels of pThr186 (Figs. 5B, 5C and Suppl. Fig. 9A). By contrast, phosphorylation of CDK9 at
Ser175 induced by PMA was effectively blocked by THZ1 (Figs. 5B, 5C and Suppl. Fig. 9A).

In a reciprocal design, Jurkat T cells were stimulated with PMA for 1 h prior to a 6-hour time course treatment with THZ1. This led to a noticeable, but transient, decay of pThr186 CDK9 (Suppl. Fig. 10). By contrast, pSer175 CDK9, whose expression is sustained following PMA induction (as described above), turned over with a half-life of ~4.5 h after addition of THZ1 (Suppl. Fig. 10). The turnover rate was comparable to that observed using the pan-PKC inhibitor (Suppl. Fig. 8). Thus, CDK7 is the primary kinase required for pSer175 CDK9 formation.

As reported previously (59), the selective CDK9 kinase inhibitor flavopiridol (FVP) induced a rapid and near total release of P-TEFb from 7SK snRNP as measured by its dissociation from HEXIM1 and LARP7 (Suppl. Figs. 9B and 10). In contrast to THZ1, FVP did not substantially block the induction of pSer175 by PMA at an inhibitor concentration of 100 nM (Suppl. Fig. 9B). Moreover, FVP appeared to enhance the levels of pSer175 CDK9 over the 6 h time course of inhibitor treatment in PMA-stimulated cells (Suppl. Fig. 10). Since pSer175 is found on CDK9 that is dissociated from 7SK snRNP, the observed modest enhancement of its levels by FVP in PMA-treated cells (Suppl. Fig. 10) could be a consequence of P-TEFb dissociation from the 7SK snRNP complex.

Flow cytometry experiments were performed to study the effects of CDK7 inhibition on P-TEFb assembly in resting primary T cells. Consistent with the above observations, inhibition of CDK7 by THZ1 in TCR-activated primary Th17 cells resulted in a 2.2-fold and 1.75-fold reduction of pSer175 CDK9 and pThr186 CDK9, respectively, without causing a significant reduction in the total CDK9 levels (Figs. 6A-D and Suppl. Fig. 11).

In summary, the inhibitor studies in both Jurkat T cells and primary T cells suggest that the CDK7 subunit of CAK is the major kinase that is directly responsible for the phosphorylation of both Ser175 and Thr186. However, since THZ1 stably inhibits CDK7 through a covalent linkage, the re-phosphorylation of Thr186 suggests that there could be a complementary kinase that restored this modification. One possible mechanism is autophosphorylation by P-TEFb since THZ1 is known to be a poor inhibitor of CDK9 (60).

**In vitro phosphorylation of CDK9 at Ser175 by CDK7.**

To provide definitive evidence that CDK7 can phosphorylate CDK9 at Ser175, *in vitro* kinase assays were performed using either commercially obtained recombinant CDK7-cyclin H-MAT1 (rCDK7) or CDK7 prepared by immunoprecipitation from Jurkat T cells. The trimeric CDK7/cyclin H/MAT1 complex (CDK-activating kinase, CAK) can be found both on its own where it functions in cell cycle regulation, or as a module of the general transcription factor TFIIH, which phosphorylates the RNAP II CTD during initiation (61). The immunoprecipitated fraction is expected to contain both forms of the enzyme.

As a control for the enzymatic activity of both these enzyme sources, a radioactive *in vitro* kinase assay using a His-tagged RNA polymerase II CTD as substrate was performed. Fig. 7A compares the activities of rCDK7 to that of CDK7 immunoprecipitates in the kinase assay performed with or without pretreatment of the enzyme sources with THZ1 for 15 min. The results demonstrate that both enzyme sources had CDK7 activity, which could be inhibited by THZ1. The immunoprecipitated CDK7 had higher activity than the recombinant enzyme, possibly because it retained a native configuration and a full complement of accessory factors.

We next performed radioactive *in vitro* kinase reactions assessing the phosphorylation of a CDK9 Ser175 peptide. As shown in Fig. 7B, both rCDK7 and the Jurkat-derived CDK7 immunoprecipitates could phosphorylate the Ser175 peptide. Phosphorylation of the Ser175 peptide by either enzyme source could be blocked by THZ1.

In complementary experiments, we performed a non-radioactive *in vitro* kinase assay using affinity purified R65A FLAG-CDK9 as the substrate and Jurkat-derived CDK7 immunoprecipitates as source of CDK7 kinase. Formation of pSer175 CDK9 was then detected by Western blotting analysis. Reactions were carried out with or without pretreatment of the enzyme immunoprecipitates with THZ1 for 15 min.
Incubation of CDK7 immunoprecipitates with R65A FLAG-CDK9 substrate for 1 h in the absence of inhibitor yielded pSer175 CDK9 (Fig. 7C). The in vitro phosphorylation of R65A CDK9 could clearly be blocked by THZ1 and the modification did not occur in the absence of CDK7 immunoprecipitates.

In conclusion, the in vitro kinase assays support the intracellular experiments described above, and confirm that the CDK7 subunit of CAK is the kinase that is responsible for phosphorylating CDK9 at Ser175.

**S175K CDK9 suppresses proviral reactivation and disrupts P-TEFb binding to BRD4.**

Previously, we found that CDK9 mutation of Ser175 to Ala enhanced basal proviral reactivation by 10-fold. The reactivation phenotype was associated with the complete abrogation of BRD4 binding to P-TEFb and a 1.7-fold reduction in Tat binding (36). Phosphomimetic mutation of Ser175 to Asp resulted in a 3-fold reactivation of latent HIV and a modest enhancement in Tat binding to P-TEFb (36).

To provide further evidence that pSer175 enhances Tat binding to P-TEFb relative to BRD4, and thereby promotes proviral gene expression, we studied the phenotype of a new mutation, S175K CDK9. We first tested the hypothesis that pSer175 can strengthen CDK9 – Tat intermolecular interactions by forming a stronger hydrogen bond with Lys12 of Tat.

MD simulations based on the 3MIA structure confirmed that pSer175 ought to favor a more stable interaction with Lys12 of Tat (Fig. 8A). This is also supported by examination of the MD structures before and after the 100 ns simulations (Suppl. Figs. 12A and 12B). By contrast, the more dynamic trajectory observed between non-phosphorylated Ser175 and Tat Glu9 suggested that although this intermolecular interaction may form, it appears to be less stable (Fig. 8B).

MD simulations of the S175K P-TEFb – Tat structure suggested that S175K should disfavor CDK9-Tat interactions by clashing with Lys12 of Tat (Figs. 8C and 8E). In the MD analysis of the distance between S175K and Tat Glu9, there was evidence of a stabilized structure (Figs. 8D and 8F), however, analysis of these structures at the end of the 100-ns simulations revealed that the intermolecular distance between S175K and Tat Glu9 is too large (6.1 Å) to form a hydrogen bond (Suppl. Figs. 12C and 12D). We concluded that S175K should disrupt intermolecular interactions between the CDK9 T-loop and Tat by abolishing the hydrogen bond between pSer175 (or non-phosphorylated Ser175) and Tat Lys12.

To assess the effect of this mutation on proviral gene expression, latently infected Jurkat 2D10 cells were stably transduced with MSCV retroviral constructs containing FLAG-CDK9 forms of the wildtype, S175A, S175D, S175K, or the kinase-dead mutant D167N. The effects of these mutations on proviral gene expression at baseline and following treatment with SAHA, TNF-α, anti-CD3/anti-CD28 antibodies, or PMA were then examined by Western blotting for Tat and flow cytometry analysis of the EGFP reporter.

As shown in Fig. 9A, the S175K mutation does not affect in vitro CDK9 kinase activity. As expected, it also potently suppressed both the basal and stimulus-induced LTR-driven expression of Tat and EGFP (Figs. 9B and 9C). Immunoprecipitation experiments revealed that S175K disrupted P-TEFb binding to BRD4 by an average of 3-fold (Figs. 10A and 10C). The phenotype of S175K is therefore consistent with a mutation that is unable to bind to either Tat or BRD4.

D167N CDK9 also repressed reactivation of proviral HIV. This mutant is able to effectively assemble with CycT1 (Figs. 10B and Suppl. Fig. 13A) and is phosphorylated at Thr186 (Fig. 10B). We assume that this allows D167N to displace endogenous CDK9, forming a defective P-TEFb that cannot phosphorylate RNAP II or the negative elongation factors.

We unexpectedly found that the catalytically defective D167N mutation, showed little to no phosphorylation of Ser175 (Figs. 10B and 10D). In cellular fractionation experiments, we found that D167N CDK9 is retained in the chromatin fraction to a greater extent than the wildtype (Fig. 11 and Suppl. Fig. 14). Additionally, D167N CDK9 is associated with 7SK snRNP components, HEXIM1 and LARP7, to a lesser extent compared to the wildtype (Fig. 10B; Suppl. Figs. 13B, 13C and 14). Consistent with its increased chromatin association and
reduced incorporation into 7SK snRNP, D167N enhanced P-TEFb interaction with BRD4 by an average of 3.1-fold (Fig. 10C). The inability for D167N to be phosphorylated at Ser175 is therefore likely to enhance its association with chromatin-bound BRD4 and block reassembly of the 7SK RNP complex.

In sharp contrast to D167N, CDK9 mutants that were unable to assemble with CycT1 and incorporate into 7SK snRNP (ie. T186A, R65A and R172A), remained bound to Hsp90/Cdc37 in the low salt fraction (Suppl. Fig. 14). The observed presence of a smaller amount of these mutants in the nuclear chromatin fraction might be attributed to poor solubility as a consequence of their ectopic overexpression. As described above, these overall results explain why T186A, R65A, and R172A did not substantially suppress proviral reactivation (Suppl. Fig. 6) unlike the D167N mutant (Figs 9B and 9C).

In summary, we have identified a CDK9 mutation of Ser175 (S175K) that strongly suppresses proviral reactivation while also significantly disrupting P-TEFb interactions with Tat’s competitor, host BRD4. In addition, we unexpectedly noted that D167N, which also suppresses proviral reactivation for reasons described above, promotes P-TEFb association with BRD4 while abrogating inducible T-loop phosphorylation at Ser175. Overall, these observations strengthen the argument that although Ser175 is required for P-TEFb binding to BRD4, pSer175 may interfere with BRD4 binding and, instead, serves to facilitate CDK9 T-loop interactions with Tat.

DISCUSSION

Biogenesis and activation of P-TEFb are regulated by post-translational modifications.

The central role of P-TEFb in HIV transcription, and the necessity to assemble functional P-TEFb in resting memory T cells make studies of this transcription factor central to the HIV Cure agenda. The studies presented here represent the first detailed analyses of P-TEFb assembly in resting memory T cells and provide a structural framework for understanding the key phosphorylation steps regulating this HIV Tat co-factor.

Figure 12 outlines the complex series of molecular events that are coordinated by post-translational modifications to regulate the assembly of P-TEFb, during the activation of resting memory T cells and the exchange of P-TEFb from 7SK snRNP to proviral and cellular promoters. As highlighted in Figure 12, major new findings presented here include: (i) phosphorylation of CDK9 at Thr186 serves to hold P-TEFb together, is unlikely to occur on CDK9 not bound to CycT1, and is also partly dependent on CDK7 activity; (ii) that dephosphorylated CDK9 that is not assembled with CycT1 is found in a complex with Hsp90 in T cells; (iii) the inducible phosphorylation of CDK9 at Ser175 is highly dependent on CDK7 kinase activity; and (iv) pSer175 is essential for mediating proviral reactivation by Tat, and is absent when P-TEFb is bound to BRD4. Our studies also emphasize for the first time the importance of nuclear and cytoplasmic exchange in the biogenesis of P-TEFb in T cells.

Formation of the heterodimer interface between CDK9 and CycT1 is nucleated by pThr186.

CDK9 phosphorylation at the conserved T-loop residue Thr186 is inducible in quiescent CD4+ T lymphocytes, but is found to be constitutive in actively replicating T cell lines and expanding, fully activated, T cells (34-36,62,63). Consistent with molecular dynamics simulations, cell-based mutagenesis experiments demonstrated that the assembly of P-TEFb in T cells is critically dependent on the stabilization of the CDK9/CycT1 interface by phosphorylation of CDK9 at Thr186. This modification nucleates an intramolecular hydrogen bond network with three arginine residues (Arg65, Arg-148, and Arg-172). At the dimer interface, Glu96 on the CycT1 subunit was identified in the MD simulations and mutagenesis analysis to also serve as a crucial link to the Arg/pThr186 bonding network.

Mutation of these Arg residues not only blocked P-TEFb assembly but also resulted in a loss of pThr186. In the first P-TEFb X-ray crystal structure (3BLQ.pdb), Baumli et al. observed that while pThr186 could form hydrogen bonding interactions with Arg-148 and Arg-172, Arg65 was not close enough (3.9 Å) to form a hydrogen bond (42). However, our findings are in agreement
with the observation that in the analogous CDK2/cyclin A structure phospho-Thr160 serves to nucleate an intramolecular hydrogen bonding network with an arginine triad (Arg50, Arg126 and Arg150) that is critical for the catalytic activation of CDK2 and its heterodimerization with the cyclin subunit (41). It is important to note that in the 3BLQ.pdb structure, Glu96 of CycT1 had been replaced with a glycine. Since Arg65 of CDK9 is observed to form a salt bridge interaction with Glu96 based on more recent P-TEFb structures (38,64) and our MD simulations, it is quite likely that the glycine substitute may have caused Arg65 to shift away from both pThr186 and CycT1. Consistent with this idea we observed that, although the E96A CycT1 mutation caused a modest dissociation of P-TEFb, the E96K CycT1 mutant abrogated dimer assembly.

**Hsp90 stabilizes CDK9 in the cytoplasm of resting memory T cells.**

While the disruption of CDK9/CycT1 assembly by the T186A, R65A and R172A mutations destroyed CDK9 kinase activity, it did not negatively affect CDK9 interactions with the Hsp90/Cdc37 chaperone complex. These observations support our model that inactive unassembled CDK9 is bound to Hsp90/Cdc37 in the cytoplasm of T cells.

Hsp90 is an ATP-dependent molecular chaperone that is constitutively resident in the cytoplasm and functions as a homodimer to ensure the proper folding and stability of its client proteins which include kinases, unliganded steroid receptors, and transcription factors (65,66). ATP hydrolysis by client-bound Hsp90 leads to loss of affinity for client proteins and their release from the chaperone in a properly folded conformation (65,67). Cdc37, which has been shown to bind both client protein kinases as well as Hsp90, is thought to inhibit the chaperone’s ATPase activity in order to stabilize the interactions between Hsp90 and the client kinase (68).

Treatment of cells with ansamycin-derived Hsp90 inhibitors has been shown to force dissociation of client proteins from Hsp90, leading to their subsequent degradation (37,45,46,49,69-72). In some cases, inhibition of Hsp90 may also lead to the conformational arrest of already bound client proteins prior to their subsequent degradation (49).

In the current study we provide evidence that these inhibitors could mediate their suppressive effects on HIV transcription in part by blocking the formation of P-TEFb. Inhibition of Hsp90 in primary T cells substantially diminished CycT1 protein expression without affecting total CDK9 levels. It is possible that a conformational arrest of immature CDK9 by inhibitor-bound Hsp90/Cdc37 could prevent P-TEFb assembly and therefore trigger the proteasomal degradation of CycT1, particularly considering that CycT1 possesses a C-terminal PEST sequence.

Supplementary Fig. 15 shows the MS/MS sequence coverages of trypsin-cleaved Hsp90 and Cdc37 co-purified with FLAG-CDK9 from Jurkat T cells. For the captured tryptic precursor peptides, Supplementary Table 1 lists the post-translational modifications found on Hsp90 and Cdc37 that are bound to CDK9. We were able to detect N-terminal phosphorylation of Cdc37 at Ser-13, a modification that is attributed to the activity of casein kinase 2 and has been demonstrated to be essential for the efficient binding of the co-chaperone to client protein kinases that include Akt, Cdk4, MOK, and Raf1 (73,74).

The mass spectometry analyses also identified multiple acetyl-lysine modifications on both Hsp90 and Cdc37, N-terminal-end acetylation of Cdc37, phosphorylation of Hsp90-β on Ser255 and phosphorylation of both Hsp90 isoforms on a tyrosine (Y284 on Hsp90-α and Y276 on Hsp90-β) adjacent to an acetylated lysine residue. Identification of this wide range of post-translational modifications on the subsets of Hsp90 and Cdc37 that are specifically bound to CDK9, will enable the future characterization of chaperone modifications which are essential for mediating interactions with CDK9 and, therefore, regulating P-TEFb biogenesis.

**Transport of newly assembled P-TEFb to the nucleus.**

As clearly illustrated by the immunofluorescence micrographs shown in Fig. 3 the restriction of CycT1 expression in unstimulated (ie. resting) memory CD4+ T cells causes unassembled CDK9 to have a
predominantly cytoplasmic distribution. It is therefore likely that in resting T cells, Hsp90 functions to hold the unphosphorylated CDK9 in the cytoplasm in order to prevent its nuclear import prior to its assembly with the cyclin T subunits.

The cellular mechanisms that trigger pThr186 on CDK9 are still poorly understood. Baumli et al. have proposed that pThr186 is the result of an auto-modification that is triggered by CDK9-CycT1 assembly (42). However, chemical genetics studies conducted by Bob Fisher and colleagues have identified the CDK7 subunit of CAK and TFIIH to be the kinase that switches CDK9 to a catalytically active state by phosphorylating Thr186 (55).

We propose that Thr186 phosphorylation is separately regulated in memory T cells and occurs in different cellular compartments for functionally different reasons. Specifically, we propose that Thr186 phosphorylation, due either to autophosphorylation or phosphorylation by a cytoplasmic form of CDK7, is a prerequisite for its assembly with CycT1 in the cytoplasm. Thr186 phosphorylation by CDK7 could also occur in the nucleus and be part of a process for regenerating P-TEFb pools during transcription (Fig. 12).

Several phosphatases, namely, PP1, PPM1A and PPM1G, have been reported to target pThr186 (43,44,62,75,76). However, the data on their role in HIV transcription has been somewhat contradictory. Rice and colleagues (62) found that the siRNA knockdown of PPM1A expression in HIV-infected resting CD4+ T cells increased the level of CDK9 Thr186 phosphorylation in the absence of T-cell stimulation, and this was coupled with enhanced HIV gene expression. By contrast, Ammosova et al. (76) reported that the stable expression of a peptide-based inhibitor of PP1, cdNIPP1, promoted CDK9 Thr186 phosphorylation. Surprisingly, they also found that cdNIPP1 inhibited HIV replication in 293T cells and also increased the association of CDK9 with 7SK RNA. We think it is likely that this discrepancy arose because of an additional requirement for PP1 during HIV transcription rather than a direct effect on P-TEFb.

Since dephosphorylation of Thr186 disrupts the P-TEFb heterodimer, we believe that dephosphorylation provides mechanism to regenerate P-TEFb pools and that it is not part of the delivery of P-TEFb to genes (43,44,77). We suggest that dephosphorylated CDK9 reassociates with Hsp90/Cdc37 following its export to the cytoplasm, where reassembly with CycT1 takes place (Fig. 12).

Phosphomimetic substitution of two adjacent tyrosine residues (Tyr271 and Tyr274) on HEXIM1, which we recently identified by tandem mass spectrometry to be subject to phosphorylation, disrupts the incorporation of P-TEFb into 7SK snRNP and causes a redistribution of CDK9 into the cytoplasm (31). Based on these findings, we have proposed that the dissociation of 7SK snRNP induced by post-translational modifications is essential for generating transcriptionally active P-TEFb. The 7SK snRNP complex therefore serves to retain newly assembled P-TEFb in the nucleus in order to provide an exchangeable pool of the enzyme that can be readily accessed to stimulate transcription.

**CDK9 Ser175 phosphorylation by CDK7 regulates the interaction of P-TEFb with Tat and BRD4.**

The kinase responsible for intracellular phosphorylation of the CDK9 T-loop at Ser175 has not been previously identified. Our original work showed that pSer175 becomes elevated in response to protein kinase C (PKC) agonists or T-cell activation (36). We demonstrate here that pSer175 is a dynamic modification (t1/2 ~ 3 h) that was dependent on activation of PKC in Jurkat T cells. However, PKC is unlikely to directly phosphorylate Ser175 because this modification is highly dependent on CDK7 kinase activity in both primary and transformed T cells. Moreover, we were able to demonstrate in *in vitro* kinase assays that CDK7 can directly phosphorylate CDK9 at Ser175. It will be important to examine whether a signaling or mechanistic relationship exists between the activation of PKC and the phosphorylation of Ser175 by CDK7.

The phenotypes of S175A and S175D CDK9 are consistent with a model that pSer175 enhances P-TEFb interactions with HIV Tat. Here we provide additional evidence that pSer175 mediates binding of Tat to P-TEFb from our analysis of the S175K mutant. S175K is predicted to block interactions with Tat from MD simulations, and it substantially suppressed the
LTR-driven expression of proviral HIV. For this mutant, the inhibition of proviral reactivation is unlikely to be due to a competition effect because this mutation also disrupted P-TEFb binding to BRD4 by 3-fold.

Our findings that Ser175 phosphorylation of CDK9 by CDK7 promotes proviral gene expression contrasts with those of Ammosova et al. (78) who reported that Ser175 is an automodification site whose dephosphorylation by PP1 activates CDK9 activity and facilitates HIV transcription in 293T cells. We think autophosphorylation of Ser175 is highly unlikely since the potent CDK9 inhibitor flavopiridol did not significantly block its formation in both Jurkat and primary T cells. Moreover, several CDK9 mutants that are catalytically defective (i.e. T186A and R65A) are still subject to inducible Ser175 phosphorylation in vivo.

The catalytically dead D167N mutant is able to effectively assemble with CycT1, unlike the CDK9 Arginine triad mutants. Surprisingly, this mutant is also unable to become phosphorylated at Ser175. However, D167N CDK9 exhibited enhanced chromatin association, most likely because of its increased binding to BRD4. Although these observations might suggest superficially that autophosphorylation is responsible for pSer175 formation, we think it is much more likely that the enhanced association of P-TEFb with BRD4 is incompatible with the formation of pSer175.

In summary, the results presented here clearly identify novel roles for Hsp90 and CDK7 in P-TEFb biogenesis. This new information suggests that these factors can be targeted to either activate or repress HIV replication in latently infected cells. The remaining gaps in our knowledge of P-TEFb biogenesis include identification of the signaling pathways in primary T cells that initiate P-TEFb biogenesis and Ser175 phosphorylation of CDK9; defining how, where and when CDK7 phosphorylates pSer175, and identifying the phosphatases that negatively regulate this modification in T cells.

EXPERIMENTAL PROCEDURES
Reagents and constructs

RPMI 1640 medium and fetal bovine serum were purchased from Hyclone. CDK7 inhibitor THZ1 was from EMD Millipore. Flavopiridol and the geldanamycin derivative tanespimycin (17-AAG) were purchased from Sigma-Aldrich. CDK9 (H-169 and D-7), CycT1 (H-245), CDK7 (C-19 and FL-346), Hsp90 (4F10) and Cdc37 (H-271) antibodies were from Santa Cruz Biotechnology. Antibody towards pThr186 CDK9 (#2549) was purchased from Cell Signaling Technology. HIV Nef antibody (3D12) was from Abcam. PMA and LARP7 antibody (SAB2108536) were purchased from Sigma. HIV Tat expression was detected using the NT3.2D1.1 monoclonal antibody (79). HEXIM1 and BRD4 antibodies were custom synthesized and affinity purified by Covance Research Products. Human T-cell activator α-CD3 and α-CD28 antibody cocktail were obtained from ThermoFisher Scientific. SAHA was purchased from Cayman Chemical. Recombinant TNF-α was purchased from Invitrogen. Generation and purification of the pSer175 CDK9 antibody has been described previously (36). The MSCV retroviral expression system (Clontech) was used for the creation of stable Jurkat T-cell lines expressing FLAG-CDK9 wildtype or mutants.

T-cell culture and latent HIV reactivation studies

The Jurkat 2D10 T-cell line model of HIV latency suitable for the study of proviral transcription has been extensively characterized (80). The proviral gene in this model is derived from the NL4-3 isolate and contains the entire HIV 5' LTR. It is modified to contain an EGFP reporter gene in place of Nef to allow for measurement of latent HIV reactivation via fluorescence detection of proviral gene expression by microscopy and flow cytometry. Jurkat 2D10 cell lines, including those that were engineered to stably express N-terminally FLAG-tagged versions of CDK9 wildtype or the mutants S175K, D167N, T186A, R65A, R172A, K164A and K178A were cultured at 37°C in complete media made up of RPMI 1640 medium (Hyclone), 10% fetal bovine serum, 100 I.U/ml penicillin, 100 μg/ml streptomycin, and 25 mM Hepes, pH 7.2. Reactivation studies were initiated by treating cells for 18 h with TNF-α, SAHA, PMA, or a combination of anti-CD3/anti-CD28 antibodies. Thereafter, LTR-driven EGFP expression was
measured by flow cytometry analysis on a LSR Fortessa instrument (BD Biosciences).

Immunoprecipitation and Western blotting analysis

Cells expressing wildtype or mutant versions of FLAG-CDK9 or HA-CycT1 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 25 mM Hepes, pH 7.2. After washing the cells with ice-cold 1X phosphate-buffered saline (PBS), whole cell extracts (WCE) were prepared in microcentrifuge tubes using cell lysis buffer A (150 mM NaCl, 10 mM KCl, 1.5 mM MgCl₂, 0.5% NP-40, 1 mM DTT, 10 mM Hepes, [pH 8.0]) containing an EDTA-free protease and phosphatase inhibitor cocktail (Roche). WCE were cleared by centrifugation at 5000 rpm for 5 min and 2 h incubation with washed protein A sepharose beads (25 μl packed beads per ml of WCE) at 4°C. This was followed by an overnight incubation at 4°C of the cleared WCE with anti-FLAG M2 affinity resin (Sigma) or anti-HA agarose beads (Thermo Scientific) on a microcentrifuge tube rotator (30 μl packed beads per ml of WCE). Immunoprecipitates were washed extensively with lysis buffer A and then boiled in 1X LDS sample loading buffer containing 50 mM DTT. 1-D SDS-PAGE and nitrocellulose transfer of the immunoprecipitates and WCE were performed followed by Western blotting using primary antibodies against CDK9, CycT1, HEXIM1, LARP7, Hsp90, Cdc37, pSer175, and pThr186. Except where indicated, SDS-PAGE of the immunoprecipitates and whole cell extracts were done on 4-12% Bis-Tris gels in MOPS buffer at 150V for 60-70 min.

pSer175 CDK9 chase experiments

1.0 X 10⁸ 2D10 Jurkat T cells stably expressing FLAG-CDK9 wildtype were stimulated or not with 50 ng/ml PMA to induce phosphorylation of CDK9 at Ser175. Thereafter, cells were treated with either 1 μM Ro-31-8220, 50 nM THZ1, or 100 nM flavopiridol for 1 h, 2 h, 4 h or 6 h. Preparation of whole cell extracts and immunoprecipitation of FLAG-CDK9 were then performed as described above. Immunoprecipitates collected at the different times after inhibitor addition, were subjected to 1D SDS-PAGE, nitrocellulose transfer and immunoblotting with antibodies against pSer175 CDK9, pThr186 CDK9 and total CDK9. Remaining levels of these proteins following inhibitor treatment was quantified using Quantity One software (Bio-Rad).

CDK9 subcellular fractionation analysis

Low salt and chromatin nuclear fractions were prepared as previously described but with some modifications to the protocol (57). Briefly, 1 X 10⁸ 2D10 T cells stably expressing FLAG-CDK9 wildtype or mutants were collected and washed with ice-cold 1X PBS. Cells were then resuspended in low salt cytosolic extract (CE) lysis buffer (10 mM KCl, 10 mM MgCl₂, 0.5% NP-40, 1 mM DTT, and 10 mM Hepes [pH 7.8]) containing an EDTA-free protease and phosphatase inhibitor cocktail (Roche) and placed on ice for 30 min. Thereafter, nuclei were gently pelleted by centrifugation at 4000 rpm for 5 min and washed with CE buffer. The nuclear pellets were placed in cell lysis buffer A and lysed in multiple quick freeze-thaw cycles using isopropanol/dry ice and 37°C water bath. This was followed by sonication using a microtip to mechanically shear DNA and solubilize chromatin-bound proteins. Nuclear extracts were then centrifuged at 4000 rpm for 3 min to remove insoluble debris. Protein concentration in both extracts was measured at 280 nm using a Nanodrop spectrophotometer (Thermo Scientific). Western blotting was performed on the low salt and chromatin nuclear fractions and the anti-FLAG immunoprecipitates prepared from these fractions.

In vitro CDK9 kinase assay

Cell lysis buffer A whole cell extracts were prepared from 1 X 10⁸ Jurkat 2D10 T cells belonging to uninfected controls, FLAG-CDK9 wildtype, or the FLAG-CDK9 mutants S175K, D167N, T186A, R65A, and R172A. After clearance of these extracts by centrifugation as described above, CDK9 complexes were immunoprecipitated by overnight incubation at 4°C with anti-FLAG M2 affinity resin (Sigma). To assess CDK9 kinase activity, 50 μl in vitro kinase reactions were set up as previously described (36).
using 20 µl of the anti-FLAG protein immune complex with or without the addition of 250 ng His-tagged full length human RNAP II CTD substrate (Abcam). The kinase assay was performed at 30°C for 1 h. Reactions were terminated by boiling samples in 1X LDS sample loading buffer containing 50 mM DTT, subjected to SDS-PAGE and the dried gel was analyzed by autoradiography.

**In vitro kinase assay to assess CDK9 Ser175 phosphorylation**

Recombinant CDK7 (Abcam; ab64303) and CDK7 immunoprecipitates prepared from Jurkat T cells were used as CDK7 enzyme sources for in vitro kinase assays to assess the phosphorylation of CDK9 at Ser175. CDK7 immunoprecipitates were prepared from whole cell extracts belonging to approximately 1 X 10⁹ Jurkat T cells. Briefly, whole cell extracts cleared by centrifugation and 2 h incubation with washed protein A sepharose beads, were incubated overnight at 4°C with a 1:50 dilution of an equal mixture of two CDK7 antibodies (C-19 and FL-346). This was followed by an incubation with protein A sepharose beads at a 1:1 antibody to bead ratio for 1 h at room temperature. Bead-immune complexes were washed extensively with cell lysis buffer A and successful immunoprecipitation of CDK7 was confirmed by Western blotting.

CDK7 activity from Jurkat T-cell immunoprecipitates or recombinant CDK7 (rCDK7) was measured in radioactive in vitro kinase assays reactions using a His-tagged RNA polymerase II CTD as substrate with or without 15 min pretreatment with 50 nM THZ1. To assess for CDK9 Ser175 phosphorylation by CDK7, non-radioactive in vitro kinase reactions were set up using CDK7 immunoprecipitates as source of CDK7 kinase and affinity purified R65A FLAG-CDK9 as substrate. Reactions were carried out with or without pretreating CDK7 immunoprecipitates for 15 min with 50 nM THZ1. Reactions were terminated as described above, resolved by SDS-PAGE and subjected to Western blotting analysis for formation of pSer175 CDK9 using a well validated antibody (36), that specifically detects this modification.

**Immunoprecipitation of CDK9-Hsp90 complexes from resting memory T cells**

Approximately 2.5 X 10⁷ memory CD4⁺ T cells were isolated from a healthy donor leukopak by negative selection using the EasySep™ Human Memory CD4⁺ T Cell Enrichment Kit (STEMCELL Technologies). After preparing whole cell extracts using Cell Lysis Buffer A and setting aside a tenth of the total volume for Western blotting, half of the remainder of the extract was mixed with an anti-CDK9 antibody at a 1:25 dilution while the other half was mixed with a non-specific IgG antibody at the same dilution. Extracts were incubated with either antibody overnight at 4°C prior to addition of washed protein A sepharose beads for another 4 h. Antibody-bound beads were washed extensively with Cell Lysis Buffer A and boiled in 1X LDS sample loading buffer containing 50 mM DTT. Boiled samples were resolved by SDS-PAGE prior to Western blotting analysis using antibodies against Hsp90, Cdc37 and CDK9.

**Assessing P-TEFb activation in primary T cells**

Primary resting CD4⁺ Th17 cells were assayed for quiescence by immunostaining for cyclin D3, Ki67, and pSer175 CDK9 with or without T-cell receptor activation for 18 h. In order to analyze the effect of THZ1, flavopiridol, and the Hsp90 inhibitor 17-allylamino-geldanamycin (17-AAG) on phosphorylation of CDK9 and activation of P-TEFb, cells were pretreated with these inhibitors for 30 min followed by T-cell receptor stimulation in the presence of inhibitor. Cells were washed once with 1X PBS and fixed in 4% formaldehyde.
for 15 min at room temperature. This was followed by permeabilization with a sequential treatment with 0.2% Triton X-100 for 10 min and 1X BD Perm/Wash buffer (BD Biosciences) for 15 min. After blocking with a non-specific IgG for 15 min, cells were separately immunostained with fluorophore-conjugated antibodies against total CDK9, cyclin T1, pSer175 CDK9, and pThr186 CDK9 for 30 min in the dark. Thereafter, these cells were rinsed twice with 1X BD Perm/Wash buffer and subjected to flow cytometry analysis using the LSR Fortessa instrument equipped with the appropriate laser and filters.

Immunofluorescence microscopy of memory CD4\(^+\) T cells

Memory CD4\(^+\) T cells isolated from a healthy donor leukopak were stimulated or not with a T-cell receptor activator cocktail for 18 h prior to adhering the cells to plastic cover slips coated with poly-L-lysine. The cover slips were washed once with 1X PBS and bound cells were fixed in 4% formaldehyde for 15 min at room temperature. Fixed cells were permeabilized by sequential treatments with 0.2% Triton X-100 for 10 min followed by 1X BD Perm/Wash buffer for 30 min. After blocking with 10% Normal Donkey Serum (The Jackson Laboratory) for 15 min, cells were immunostained for 1 h at room temperature with primary antibodies against CDK9, pSer175 CDK9 and cyclin T1. Thereafter, the cells were washed three times with 1X BD Perm/Wash buffer and then incubated with a 1:100 dilution of AF488-conjugated anti-Rabbit IgG secondary antibody for 1 h. After washing the cover slips with 1X BD Perm/Wash buffer they were counterstained with 1 µg/ml DAPI, mounted onto glass slides with ProLong Antifade (Thermo Fisher Scientific), and viewed using a DeltaVision epifluorescent microscope (Applied Precision). Images were captured in z series, deconvolved, and processed using the Softworx analysis program (Applied Precision).

Molecular dynamics simulations of P-TEF\(\beta\) protein structures

Wild-type and mutants of the CDK9-cyclin T1 complex were modeled using the Amber ff99SB force field (81), the TIP3P water model, and the phosphorylated parameter was set for phospho-Threonine (82). The system was simulated in the presence of ATP and Mg, with their initial atomistic coordinates taken from its corresponding structure (3BLQ.pdb) (42) where Gly96 of cyclin T1 was mutated back to Glu prior to running the simulations. Using a standard simulation protocol, an initial equilibration was performed via brute-force 2 ns NPT simulations at 300 K and 1 atm followed by a total of 100 ns production simulations using the Amber 12 package (83). All simulations using the 3MIA.pdb structure as a template that are presented in Fig. 8 and Suppl. Fig. 12 were performed using Amber 16. Residue-to-residue distances were calculated based on the center-of-mass position of residue side chains with the exception of pSer175, where the distance was calculated from the phosphorus atom.

Purification and preparation of CDK9 complexes for mass spectrometry analysis

Samples for mass spectrometry analysis were purified and prepared as previously described (36). Briefly, whole cell extracts (WCE) were prepared from 5.0 X 10\(^8\) Jurkat T cells stably expressing FLAG-CDK9 using cell lysis buffer A. WCE were cleared by centrifugation at 2000 rpm for 5 min followed by incubation for 2 h with protein A sepharose beads. Cleared WCE were then incubated with anti-FLAG M2 affinity resin (Sigma) overnight at 4\(^\circ\)C on a gentle rocker (30 µl packed beads for 1 ml of cleared WCE). Immunoprecipitates were isolated by centrifugation at 2000 rpm for 2 min followed by five 30-min washes of the resin with 10 ml cell lysis buffer A. Bound CDK9 protein complexes were eluted off the beads with 200 µg/ml FLAG peptide in 2 ml cell lysis buffer A at 4\(^\circ\)C on a gentle rocker. FLAG peptide protein eluates were concentrated by methanol-chloroform precipitation and rehydrated with 1X LDS loading buffer containing 50 mM DTT before being resolved by 1D SDS-PAGE on a 4-12% Bis-Tris gel. SYPRO Ruby (Invitrogen) stained gel bands containing CDK9-associated proteins were subjected to in-gel digestion with sequencing grade modified trypsin (Promega, WI) as described previously (36). Briefly, excised gel pieces were first washed with 50% acetonitrile in 50 mM ammonium bicarbonate, and then
dehydrated with acetonitrile. Before an overnight proteolytic digestion, proteins were reduced with 20 mM DTT at room temperature for 1 h and alkylated with 55 mM iodoacetamide in 50 mM ammonium bicarbonate for 30 min in the dark. After proteolytic digestion, peptides were extracted from the gel with 5% formic acid in 50% acetonitrile and then resuspended in 0.1% formic acid after being dried completely under the Speed Vacuum.

**LC-MS/MS**

The digests were analyzed by LC-MS/MS using a LTQ Velos-Orbitrap mass spectrometer (Thermo-Finnigan, Bremen, Germany) equipped with a Waters nano Acquity UPLC system (Waters Inc, MA). The mass spectrometer was operated in a data-dependent MS to MS/MS switching mode, with the 10 most intense ions in each MS scan subjected to MS/MS analysis. The full scan at 60,000 resolution was obtained in the Orbitrap for eluted peptides in the range of 380–1800 amu followed by 10 MS/MS scans in ion trap detector. MS/MS spectra were generated by collision-induced dissociation of the peptide ions at normalized collision energy of 35% using dynamic exclusion with a repeat count of 2, repeat duration of 45 s, exclusion duration of 60 s, and exclusion size list of 500. The total analysis time was 90 min. Raw LC-MS/MS data were submitted to database search through Mascot search engine (version 2.2.0, Matrix Science) against human SwissProt database (20,249 sequences) with Cysteine carbamidomethylation as a fixed modification, Met oxidation, protein N-terminal and Lys acetylation, Ser, Thr, and Tyr phosphorylation as variable modifications. The mass tolerance was set as 10 ppm for precursor ion and 0.8 Da for product ion. The significance threshold setting is P < 0.05. Mascot ion score was set as no less than 20 for peptides identification. Candidate modification sites were further verified by manual examination of each tandem mass spectrum of the modified peptides.

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**AUTHOR CONTRIBUTIONS:** UM performed the cell-based biochemical experiments with cell lines and primary cells, analyzed and interpreted the data, and wrote most of the manuscript. SY performed the molecular dynamics simulations and analyzed the subsequent data. BW and GG carried out the mass spectrometry analysis of protein samples purified by UM. BW manually analyzed the mass spectrometry data, identified post-translational modifications, and prepared MS/MS fragmentation spectra. WS prepared the molecular models for molecular dynamics simulations and helped with the design of these experiments. JK and UM conceived the project and co-wrote most of the manuscript. All authors reviewed the results and edited and approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1. Role of CDK9 T-loop phosphorylation at Thr186. (A) Cartoons of the P-TEFb-Tat X-ray structure (3MIA.pdb) (38) showing how the hydrogen bonding coordination of pThr186 of CDK9 (Green) extends to Glu96 of cyclin T1 (Blue). Colored gray is Tat. The inset shows inter-atomic distances after 4-ns molecular dynamics (MD) simulations. (B) Graphical representation of MD simulations of CDK9/cyclin T1 wildtype and T186A CDK9/cyclin T1 conducted over a period of 100 ns. Shown are the radius of gyration (Rg) for Arg65, Arg-148, and Arg-172 of wildtype and mutant T186A.

Figure 2. Point mutations of the arginine triad of CDK9 abrogates P-TEFb assembly and causes loss of Thr186 phosphorylation without disrupting CDK9 interactions with the Hsp90/Cdc37 chaperone. (A) Anti-FLAG immunoprecipitates (IP) prepared from whole cell extracts (WCE) belonging to control Jurkat T cells or those stably expressing FLAG-CDK9 wildtype, T186A, R65A, or R172A were analyzed by immunoblotting to assess the effect of these mutations on CDK9 binding to cyclin T1 (CycT1) or the Hsp90/Cdc37 chaperone. (B) Phosphorylation of CDK9 at Thr186 is critically dependent on P-TEFb assembly. Whole cell extracts prepared from Jurkat T cells stably expressing FLAG-CDK9 wildtype or the mutants shown were subjected to anti-FLAG IP followed by Western blotting for the indicated proteins. (C) Extent of CDK9 Thr186 phosphorylation and association with Hsp90/Cdc37, HEATM1, or LARP7 following mutation of Arg65, Arg-172 and Thr186. The graph shows fold changes in protein levels relative to those corresponding to CDK9 wildtype. Error bars denote +/- standard deviation of the mean from at least 3 experiments. Statistical significance (****p-value < 0.0005; *p-value < 0.05) was calculated relative to FLAG-CDK9 wildtype and is based on a two-tailed Student’s t-test.

Figure 3. Subcellular expression of CDK9, pSer175 CDK9 and cyclin T1 (CycT1) in memory CD4^+ T cells before and after T-cell receptor (TCR) activation. Healthy donor memory CD4^+ T cells were stimulated or not for 18 h with an anti-CD3/anti-CD28 antibody cocktail. Both cell populations were fixed in 4% formaldehyde, permeabilized and then immunofluorescently stained for total CDK9, pSer175 CDK9 or CycT1 and counterstained for DAPI. Images were obtained using a DeltaVision deconvolution microscope at 60X. Scale bar represents a length of 15 μm.

Figure 4. Inhibition of Hsp90 disrupts the biogenesis and activation of P-TEFb in primary T cells and suppresses proviral reactivation in latently infected Th17 cells. (A) Existence of a CDK9-Hsp90-Cdc37 complex in resting memory CD4^+ T cells. Endogenous CDK9 was immunoprecipitated from whole cell extracts prepared from memory CD4^+ T cells isolated from a healthy donor leukopak. CDK9 immunoprecipitates (IP) were then immunoblotted (WB) for Hsp90 and Cdc37 as shown. (B) Hsp90-bound CDK9 in T cells is neither associated with cyclin T1 nor phosphorylated at Thr186. Whole cell extracts (WCE) from Jurkat T cells stably expressing FLAG-CDK9 were subjected to immunoprecipitation with anti-FLAG antibody resin. FLAG peptide eluates were further subjected to anti-Hsp90 immunoprecipitation. Both the FLAG peptide eluates and anti-Hsp90 immunoprecipitates were then analyzed by Western blotting for the presence of CycT1 and pThr186 CDK9. (C) Immunofluorescence flow cytometry analysis of the expression of cyclin T1 (CycT1), pSer175 CDK9, pThr186 CDK9 and total CDK9 in resting primary Th17 cells following 18 h T-cell receptor (TCR) activation with anti-CD3/anti-CD28 antibody cocktail in the presence or absence of treatment with a Hsp90 inhibitor (17-AAG). Error bars denote +/- standard deviation of the mean from three experiments. Statistical significance (****p-value < 0.01; *p-value < 0.05) is based on a two-tailed Student’s t-test. (D) Latently infected Th17 cells were stimulated through the TCR for 18 h in the presence or absence of the Hsp90 inhibitor 17-AAG. Proviral reactivation was assessed by measuring the expression levels of HIV Nef. The graphed data, expressed as a fold change in Nef levels relative to the non-stimulated condition, are from six different experiments (color-coded). Statistical significance (p-value < 0.005) is calculated based on a two-tailed paired Student’s t-test.
Figure 5. Inducible phosphorylation of CDK9 at Ser175 is both PKC- and CDK7-dependent in Jurkat T cells. (A) Pre-treatment with a pan-PKC inhibitor Ro-31-8220 potently blocks inducible phosphorylation of CDK9 at Ser175. Jurkat T cells stably expressing FLAG-CDK9 were stimulated or not with 50 ng/ml PMA for 2 h in the presence of varying concentrations of Ro-31-8220. ‘Control’ denotes Jurkat T cells that do not express FLAG-CDK9. (B) THZ1, a highly selective and covalent CDK7 inhibitor, blocks inducible phosphorylation of CDK9 at Ser175. Following 15 min pre-treatment with 50 nM THZ1, Jurkat T cells stably expressing FLAG-CDK9 were subjected to a six-hour time course of PMA treatment in the presence of inhibitor. ‘No Inh’ denotes PMA treatment in the absence of THZ1. Whole cell extracts (WCE) and anti-FLAG immunoprecipitates (IP) were then analyzed by Western blotting for the indicated proteins. (C) Quantitation of the inhibition of pSer175 CDK9 induction by THZ1. The graph to the left shows the fold change in levels of pSer175 CDK9, pThr186 CDK9 and cyclin T1 (CycT1) present within immunoprecipitates of FLAG-CDK9 from Jurkat T cells before and after 2 h PMA stimulation. The graph to the right shows the fold change in levels of pSer175 CDK9, pThr186 CDK9 and CycT1 in CDK9 immunoprecipitates from Jurkat T cells treated with PMA for 2h in the presence or absence of 50 nM THZ1. In both graphs protein levels have been normalized to the corresponding levels of immunoprecipitated CDK9. Error bars denote +/- standard deviation of the mean from at least three experiments. Statistical significance (**p-value < 0.005; *p-value < 0.05) is calculated based on a two-tailed Student’s t-test.

Figure 6. THZ1 blocks Ser175 phosphorylation of CDK9 in primary resting T cells. The box plots in (A), (B), (C) and (D) show a quantitative immunofluorescence staining of intracellular pSer175 CDK9, pThr186 CDK9, cyclin T1, and total CDK9, respectively from at least three different experiments conducted using either resting primary Th17 cells (black) or memory CD4+ T cells (red). Statistical p-values were calculated using a two-tailed Student’s t-test. Cells were pre-treated with the indicated concentrations of THZ1 or flavopiridol for 30 min. prior to stimulation of the T-cell receptor (TCR) in the presence of either inhibitor for 18 h. Intracellular immunofluorescence staining and subsequent flow cytometry analysis were performed as described in the Experimental Procedures.

Figure 7. In vitro phosphorylation of CDK9 at Ser175 by CDK7. (A) Radioactive in vitro kinase assay using His-tagged RNA polymerase II C-terminal domain (RNAP II CTD) as substrate comparing the activities of commercially obtained recombinant CDK7-cyclin H-MAT1 (rCDK7) to that of CDK7 immunoprecipitates (CDK7 IP) prepared from Jurkat T cells. The kinase assay was performed with or without pretreatment of the enzyme sources with 50 nM THZ1 for 15 min. Reactions were terminated by boiling samples in 1X LDS sample loading buffer containing 50 mM DTT and resolved by SDS-PAGE using a 12% Bis-Tris gel in MOPS buffer. (B) Radioactive in vitro kinase assay assessing phosphorylation of a CDK9 Ser175-containing peptide by either Jurkat-derived CDK7 immunoprecipitates (CDK7 IP) or commercially obtained recombinant CDK7 (rCDK7). The kinase assay was performed with or without pretreatment of the kinase samples with 100 nM THZ1 for 15 min. Non-specific IgG bound to resin was used as a negative control for the enzymes used in the experiment. Reaction samples were denatured by boiling in 1X LDS sample loading buffer containing 50 mM DTT. Denatured samples were resolved by SDS-PAGE on a 12% Bis-Tris gel in MES buffer. ‘Non-specific’ denotes a band series for an unidentified phosphorylated species that is present in the CDK7 immunoprecipitates. Bottom image is of the same gel stained for protein with Sypro Ruby to show levels of CDK7 present in the kinase reactions. (C) Non-radioactive in vitro kinase assay (IVKA) using CDK7 immunoprecipitates (IP) as source of CDK7 kinase and affinity purified R65A FLAG-CDK9 as substrate. The reactions were carried out with or without pretreatment of the CDK7 IP with 50 nM THZ1 for 15 min. Reactions were terminated as described above, resolved by SDS-PAGE on a 4-12% Bis-Tris gel in MOPS buffer and subjected to Western blotting (WB) analysis for formation of pSer175 CDK9.

Figure 8. Effect of S175K on potential CDK9 interactions with the N-terminal region of Tat. Shown in A-F are 100-ns MD trajectories for the distances of Ser175, pSer175, or S175K to either Tat Lys12 or
Glu9. All simulations were conducted with energy-minimized models prepared using the 3MIA.pdb structure as a template and were performed using Amber 16. Residue-to-residue distances were calculated based on the center-of-mass position of residue side chains with the exception of pSer175, where the distance was calculated from the phosphorus atom.

**Figure 9. S175K CDK9 suppresses proviral reactivation.** (A) The S175K mutant is not defective for CDK9 catalytic activity. Radioactive *in vitro* kinase assay using anti-FLAG immunoprecipitates prepared from 2D10 Jurkat T cells stably expressing FLAG-CDK9 wildtype (WT), D167N or S175K. (B) (i) Western blotting analysis of proviral Tat expression in 2D10 Jurkat controls with or without 18 h PMA stimulation. (ii) Western blotting analysis of proviral Tat in 2D10 Jurkat T cells stably expressing wildtype, S175K or D167N CDK9 following 18 h challenge with PMA. (C) S175K CDK9 suppresses proviral gene induction to a similar extent as the kinase-dead mutation D167N. 2D10 Jurkat T cells, which carry stably integrated and latent proviral DNA containing an EGFP reporter, were engineered to stably express FLAG-CDK9 wildtype, S175K, or D167N. Cells were analyzed by flow cytometry to assess proviral EGFP expression with or without 18 h treatment with SAHA, TNF-α, anti-CD3/anti-CD28 antibody cocktail or PMA. Error bars denote +/- standard deviation of the mean from at least three experiments. Statistical significance (**p-value < 0.01; *p-value < 0.05) is calculated relative to CDK9 wildtype and is based on a two-tailed Student’s t-test.

**Figure 10. D167N CDK9 is defective for Ser175 phosphorylation but exhibits enhanced association with BRD4.** (A) S175K disrupts P-TEFb association with BRD4. Immunoblotting analysis of anti-FLAG-CDK9 immunoprecipitates conducted to assess effect of S175A, S175D, and S175K CDK9 mutations on binding of P-TEFb to BRD4. (B) D167N CDK9 is defective for Ser175 phosphorylation, exhibits enhanced BRD4 binding and shows reduced incorporation into 7SK snRNP. Whole cell extracts (WCE) prepared from unstimulated or 18 h PMA-treated Jurkat T cells belonging to FLAG-CDK9 wildtype, D167N or S175K were subjected to anti-FLAG IP followed by Western blotting for the indicated proteins. (C) Quantitation of the effect of D167N and S175K on the extent of CDK9 association with BRD4. The graphed data are from two different experiments. (D) Quantitation of the effect of D167N on the phosphorylation of CDK9 at Ser175. Graphed data are from three different experiments. Statistical significance (**p-value < 0.005) is calculated relative to CDK9 wildtype and is based on a two-tailed Student’s t-test.

**Figure 11. D167N CDK9 exhibits enhanced association with chromatin-bound BRD4.** Nuclear chromatin fractions belonging to Jurkat T cells stably expressing FLAG-CDK9 wildtype or the mutants shown and stimulated with PMA for 18 h, were subjected to anti-FLAG immunoprecipitation (IP). Both the anti-FLAG IP and chromatin fractions were then analyzed by Western blotting for indicated proteins.

**Figure 12. Proposed model for the assembly and activation of P-TEFb in HIV-infected primary CD4+ T cells.** Phosphorylation of the CDK9 T-loop at Thr186 regulates the initial assembly of P-TEFb and incorporation into 7SK snRNP. T-loop phosphorylation at Ser175 regulates the efficiency by which the Tat-containing Super Elongation Complex (SEC) is formed in HIV-infected CD4+ T cells. In resting CD4+ T cells, CDK9 is sequestered in the cytoplasm by Hsp90/Cdc37 due to restricted expression of cyclin T1 (CycT1) and limited T-loop phosphorylation at Thr186. T-cell receptor (TCR) activation of these cells allows for the assembly of P-TEFb following post-transcriptional expression of CycT1 which then triggers phosphorylation at Thr186. It remains unclear whether the assembly and T-loop phosphorylation of P-TEFb occur prior to nuclear import. T-loop phosphorylation at Thr186 further stabilizes the heterodimer interactions and is a prerequisite for the incorporation of assembled P-TEFb into 7SK snRNP. Posttranslational modifications such as the phosphorylation of HEXIM1 at Ser158 and Tyr271/274 promote the dissociation of 7SK snRNP as reported previously (31,58). Following release of P-TEFb from the 7SK complex, CDK9 becomes phosphorylated at Ser175 by CAK CDK7 in a manner dependent upon the sustained presence of TCR signals. Phosphorylation of CDK9 at Ser175 can then be
co-opted by Tat in HIV-infected CD4+ T cells to more efficiently compete with BRD4 for free P-TEFb. Dephosphorylation of CDK9 at Thr186 triggers the disassembly of 7SK snRNP, as reported previously (43), and may prompt the nuclear export of both CDK9 and CycT1. By contrast, the dephosphorylation of Ser175 may facilitate the reincorporation of P-TEFb into 7SK snRNP. Treatment of these cells with 17-AAG (17-allylamino-1,3,7-triazacyclononane) will disrupt the initial assembly of P-TEFb and perhaps lead to the degradation of CycT1. Flavopiridol (FVP), CDK9 inhibitor, should prevent the incorporation of assembled P-TEFb into 7SK snRNP and also induce dissociation of existing 7SK snRNP. The CDK7 inhibitor THZ1 blocks T-loop phosphorylation at Ser175 and may also compromise P-TEFb assembly by preventing phosphorylation at Thr186.
Figure 1

A. pThr186/Arg triad network

B. Molecular Dynamics

Rg for R65/R148/R172 (Å)

Simulation time (ns)
Figure 2

A. P-TEFb or Hsp90 assembly

B. Thr186 phosphorylation

C. Protein association with CDK9

Protein Association (Fold Change)

CDK9 WT  T186A  R65A  R172A

CycT1  HEXIM1  LARP7  Hsp90  Cdc37  pThr186 CDK9

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Figure 3

Post-translational control of P-TEFb and HIV reactivation
Figure 4

A. CDK9 binding to Hsp90/Cdc37 in resting T cells

B. Dephosphorylated CDK9 binding to Hsp90

C. Hsp90-dependent biogenesis of P-TEFb in primary T cells

D. Inhibition of HIV expression in Th17 cells

Post-translational control of P-TEFb and HIV reactivation

** p < 0.005
** p < 0.005

No stim 18 h TCR 18 h TCR + 1 μM AAG

Fold change in Nef levels

p < 0.005 p < 0.005
Figure 5

A. Induction of pSer175 CDK9 by PKC

B. THZ1 blocks pSer175 CDK9 induction

C. Inhibition of pSer175 CDK9 induction in Jurkat T cells by THZ1
Figure 6

A. pSer175 CDK9

- p = 0.135
- p = 0.0217
- p = 0.00022

B. pThr186 CDK9

- p = 0.241
- p = 0.158
- p = 0.0536

C. Cyclin T1

- p = 0.241
- p = 0.0581
- p = 0.00724

D. Total CDK9

- p = 0.113
- p = 0.0294
- p = 0.177
Figure 7

A. CDK7 activity (RNAP II CTD substrate)

B. CDK7 activity (Ser175 CDK9 peptide)

C. Immunodetection of pSer175 following CDK7 IVKA

Post-translational control of P-TEFb and HIV reactivation
Figure 8

A. CDK9 S175/pS175 to Tat K12

B. CDK9 S175 or pS175 to Tat E9

C. CDK9 pS175/S175K to Tat K12

D. CDK9 pS175/S175K to Tat E9

E. CDK9 S175/S175K to Tat K12

F. CDK9 S175/S175K to Tat E9
Figure 9

A. Kinase Assay with FLAG-CDK9 IP

B. Inhibition of Tat induction by S175K CDK9

C. Inhibition of proviral reactivation by S175K CDK9

Activated Cells (% EGFP+)

Unstim. SAHA (0.25 μM) SAHA (0.5 μM) SAHA (1 μM) TNF-α (0.5 ng/ml) TNF-α (2 ng/ml) TCR (α-CD3/CD28) PMA (5 ng/ml)

Control CDK9 WT D167N S175K

CDK9 WT

CDK9

His-RNAP II CTD

CDK9

CycT1

WB

Tat

Tat

Control CDK9 WT D167N S175K
Figure 10

**A. S175K CDK9**

**FLAG CDK9 IP**

**B. D167N CDK9**

**Unstimulated**

**PMA (18 hr)**

**C. D167N and S175K CDK9**

**D. D167N CDK9**

**BRD4 Binding (Fold Change)**

**pSer175 CDK9 (Fold Change)**
Figure 11

PMA (18 hr)

| Sample       | CDK9 WT | T186A | R65A | R172A | S175K | D167N |
|--------------|---------|-------|------|-------|-------|-------|
| Control      |         |       |      |       |       |       |
| CDK9         |         |       |      |       |       |       |
| CycT1        |         |       |      |       |       |       |
| pSer175      |         |       |      |       |       |       |
| BRD4         |         |       |      |       |       |       |
| CDK9         |         |       |      |       |       |       |
| CycT1        |         |       |      |       |       |       |
| BRD4         |         |       |      |       |       |       |
Figure 12

Post-translational control of P-TEFb and HIV reactivation
