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Crosstalk between histone modifications indicates that inhibition of arginine methyltransferase CARM1 activity reverses HIV latency

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ABSTRACT

In eukaryotic cells, the gene expression status is strictly controlled by epigenetic modifications on chromatin. The repressive status of chromatin largely contributes to HIV latency. Studies have shown that modification of histone H3K27 acts as a key molecular switch for activation or suppression of many cellular genes. In this study, we found that K27-acetylated histone H3 specifically recruited Super Elongation Complex (SEC), the transcriptional elongation complex essential for HIV-1 long terminal repeat (LTR)-mediated and general cellular transcription. Interestingly, H3K27 acetylation further stimulates H3R26 methylation, which subsequently abrogates the recruitment of SEC, forming a negative feedback regulatory loop. Importantly, by inhibiting methyltransferase activity of CARM1, the enzyme responsible for H3R26 methylation, HIV-1 transcription is reactivated in several HIV latency cell models, including a primary resting CD4+ T cell model. When combined with other latency disrupting compounds such as JQ1 or vorinostat/SAHA, the CARM1 inhibitor achieved synergistic effects on HIV-1 activation. This study suggests that coordinated and dynamic modifications at histone H3K27 and H3R26 orchestrate HIV-1 LTR-mediated transcription, and potentially opens a new avenue to disrupt latent HIV-1 infection by targeting specific epigenetic enzymes.

INTRODUCTION

Human immunodeficiency virus (HIV) is a retrovirus that causes acquired immunodeficiency syndrome. HIV infection of CD4+ helper T lymphocytes is a multistep process, involving fusion and entry through interaction with CD4 receptor and CCR5 co-receptor, reverse transcription of HIV viral RNA into double-stranded viral DNA, and integration of viral DNA into the host cell genome. Combination Antiretroviral Therapy (cART), also known as highly active antiretroviral therapy (HAART), employs the use of three or more antiretroviral drugs to inhibit the key steps of HIV infection, and has been very effective in HIV-infected patients by suppressing HIV replication and maintaining the virus at undetectable levels. However, although continuous cART is life-prolonging, it never eradicates HIV infection or cures this disease. Interruption of treatment quickly results in virus replication and recurrence of the disease (1). Therefore, HIV-infected patients must receive lifelong cART treatment, which presents economic and therapeutic burdens.

The principal impediment to eradication of HIV infection is the latent reservoir of HIV provirus. HIV provirus persists in multiple tissues and cells, particularly the long-lasting, resting CD4+ T cells. Reversing proviral latency is believed to be a major challenge for strategies to eradicate HIV from an individual and achieve a functional cure. To this end, recently discussed and tested approaches to target latency involve 'shock and kill' strategies, which propose to reactivate ('shock') latent HIV in the presence of cART and apply immune-based therapy to purge ('kill') these reservoirs (2,3). Mechanistically, epigenetic transcriptional processes drive HIV latency in T cells, as the repressive status of chromatin largely contributes to HIV latency (4). In order to reactivate the latent virus, it is essential to

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'open' the repressive structure of chromatin that harbors the provirus. For instance, it is generally accepted that histone deacetylases (HDACs) are involved in maintenance of repressive chromatin status on the HIV-1 promoter, and restoration of histone acetylation by an HDAC inhibitor treatment loosens the repressive chromatin structure and reactivates latent HIV (5–7). However, the molecular mechanism of how site-specific histone acetylation contributes to transcriptional reactivation on HIV proviral regulatory sequences remains elusive.

Lysine 27 (K27) on the histone H3 tail is subject to two covalent modifications: methylation and acetylation. Trimethylation of H3K27 (H3K27me3) is a transcriptional repression 'mark', and its function has been well documented (8–10). The responsible methyltransferase EZH2 (Enhancer of Zeste 2) is an essential component of the PRC2 (Polycomb repressive complex 2), and is involved in silencing many important genes during development and cell differentiation (11–13). Interestingly, EZH2 was found to participate in silencing HIV-1 by methylation of histones located at the HIV-1 LTR region (14). A histone lysine methyltransferase (HKMT) inhibitor that targets EZH2, 3-deazaneplanocin A, can reactivate silenced proviruses (15). Similarly, GSK343, a specific EZH2/EZH1 inhibitor, has been reported to sensitize the latent HIV to reactivation effect of the HDAC inhibitor vorinostat (16).

In contrast to H3K27 methylation, acetylation at H3K27 (H3K27ac) is a key epigenetic mark that correlates with gene transcriptional activation (17,18). Because methylation and acetylation occur on the same residue and have opposite functions, it is anticipated that there is an antagonistic switch between methylation and acetylation on H3K27. Indeed, knockout of EZH2 or Suz12 (another integral component in PRC2) abrogates H3K27me3, and meanwhile greatly enhances the level of H3K27ac in mouse ES cells (19). Similarly, histone deacetylase inhibitors LBH589 and LAQ824 increase histone acetylation levels, and simultaneously decrease H3K27 methylation levels in primary human acute leukemia cells (20). Therefore, it is expected that enhancing H3K27ac levels could contribute to re-activation of silenced HIV provirus.

In this study, we investigated how H3K27 acetylation regulates HIV-1 transcription. We found that H3K27ac is a preferable substrate for H3R26 methylation catalyzed by a transcriptional coregulator protein termed CARM1 (Coactivator-associated arginine methyltransferase 1).

CARM1 belongs to the protein arginine methyltransferase (PRMT) family, and it catalyzes the transfer of methyl groups from S-adenosyl-L-methionine to the guanidine nitrogens of arginine, producing asymmetric dimethylated arginine (Rme2a) as its final product (21,22). As a transcriptional co-regulator, CARM1 methylates multiple nuclear proteins involved in gene transcription, including histone H3, transcriptional coactivators CBP/p300 and SRCs, mediator Med12, RNA pol II, RNA-binding proteins PABP1, HuR, and HuD, as well as the splicing factors CA150, SAP49, and SmB (23–30). CARM1 methylates histone H3 mainly on two sites, R17 and R26, with an additional methylation site R42 that was recently identified (31,32).

In this study, the Super Elongation Complex (SEC) was identified as an H3K27ac-associated complex based on a peptide pull-down/mass spectrum analysis. Interestingly, the consequent H3R26 methylation event abrogated SEC recruitment, suggesting that H3K27ac is a positive regulator of HIV transcription, whereas H3R26me is a negative regulator. Consistent with this observation, a CARM1-specific small molecule inhibitor functions as a potent latency reversing agent (LRA) in both cell line and primary CD4+ T cell models of HIV latency. When combined with other latency disrupting compounds, the CARM1 inhibitor achieved synergistic effects on HIV-1 reactivation.

MATERIALS AND METHODS

In vitro histone methylation assays

The *in vitro* methylation assays were performed as described previously (25). Briefly, 100 ng of histone methyltransferase enzymes were incubated with substrates (recombinant histones or histone peptides) in the presence of 20 mM Tris–HCl (pH 8.0), 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 1 μl of [^{3}H]AdoMet (13.3 Ci/mM; PerkinElmer Life Sciences). The reactions were incubated at 30°C for 1 h before the proteins were separated in a 4–15% SDS-polyacrylamide gel (or 10–20% Tris-Tricine gel for methylation of histone peptides). After fixation in a mixture containing 50% methanol and 10% acetic acid for 20 min, the gels were treated with autoradiography Amplify reagent (Amersham Biosciences) for 20 min, dried, and exposed to X-ray films. Recombinant EZH2 protein complex, wildtype, K27-acetylated, and K27-trimethylated histone H3 proteins were purchased from Active Motif. Recombinant CARM1 protein was obtained from Millipore, and p300 protein was generated from the baculoviral system as previously described (33).

Plasmid DNA

Histone H3 K27M mutant and its corresponding wildtype H3 were constructed in a pET16b expression vector. The K27M mutation was generated by PCR-based site-directed mutagenesis. The sequences of the PCR primers are: 5′GCTACAAAAGCCGCTCGCATGAGTGCGCCC TCTACTGG-3′ (forward); 5′-CCAGTAGAGGGCGC ACTCATGCGAGCGGCTTTTGTAGC-3′ (reverse). Similarly, the pInducer20 lentiviral vector was used to generate expression vectors for wildtype H3 and H3K27M (34). pCMV-Flag-Tat was previously generated from Dr Andrew Rice's laboratory (35).

Histone peptides, pull-down and western blot analysis

Biotinylated histone H3(23-42) peptides (unmodified, K27ac, R26me2a, and R26me2a+K27ac) were purchased from JPT Peptides Inc. Biotinylated histone H3(18-35) peptides (unmodified, K27M mutated, and K27M mutation with R26me2a modification) were synthesized at Peptide 2.0 Inc. For each pull-down analysis, 0.25 μg of peptide was immobilized onto 25 μl Dynabeads 280 streptavidin (Life Technology, Inc), followed by addition of 1 mg HeLa nuclear extract and incubated at 4°C for

2 h. After binding, the beads were washed three times with wash buffer BC150 (20 mM Tris–HCl pH 7.9, 10% glycerol, 0.2 mM EDTA, 150 mM KCl) before addition of 2× SDS sample buffer and loading for SDS-PAGE and western blot analysis. The antibodies used for western blot were: H3R26me2a antibody from Millipore; H3R17me2a antibody from Active Motif; ELL, ENL, AF9, CARM1, AFF1 and AFF4 antibodies from Bethyl laboratories; p300, Cyclin T1 and CDK9 antibodies from Santa Cruz biotechnology, Inc; and β-actin antibody from Sigma.

Mass spectrometry sample preparation and analysis

Following the peptide pull-down assay, streptavidin beads were washed and then boiled in 30 ul of 1× NUPAGE® LDS sample buffer (Invitrogen) and subjected to SDS-PAGE (NuPAGE 10% Bis–Tris Gel, Invitrogen) and visualized with Coomassie Brilliant blue-stain. The SDS-PAGE gel was excised, destained and subject to in-gel digestion using 100 ng of trypsin (GenDepot T9600). The tryptic peptides were resuspended in 10 ul of 0.1% formic acid and subjected to nanoHPLC–MS/MS system, which consists of a nano-LC 1000 system (Thermo Scientific) and a LTQ Orbitrap Elite™ (Thermo Scientific) mass spectrometer. The peptides were loaded onto a Reprosil-Pur Basic C18 (1.9 μm, Dr Maisch GmbH, Germany) pre-column with a size of 2 cm × 100 μm. The pre-column was switched in-line with an in-housed 50 mm × 150 um analytical column packed with Reprosil-Pur Basic C18 equilibrated in 0.1% formic acid. The peptides were eluted using a 75-min discontinuous gradient of 4–26% acetonitrile/0.1% formic acid at a flow rate of 600 nl/min. The eluted peptides were directly electro-sprayed into mass spectrometer operated in the data-dependent acquisition mode acquiring fragmentation spectra of the top 50 strongest ions. Obtained MS/MS spectra were parsed against a target-decoy human refseq database in Proteome Discoverer 1.4 (Thermo Fisher) with the Mascot algorithm (Mascot 2.4, Matrix Science). The precursor mass tolerance was confined within 20 ppm with a fragment mass tolerance of 0.5 Da and a maximum of four missed cleavage allowed. Dynamic modification of oxidation (methionine), protein N-terminal acetylation and De-Streak (cysteine) was allowed. The peptides identified from the Mascot result file were validated with 5% false discovery rate (FDR). For relative quantification, the data was then grouped into gene products and assigned homology and identification quality groups using an in-house developed algorithm (36). All protein gene products were required to have at least one identification where a spectral match passing <1% FDR and >20 ion score or <5% FDR and >30 ion score thresholds were present in order to be chosen for follow-up in this study. Each gene product amount was estimated using a label-free intensity-based absolute quantification (iBAQ) approach (as the sum of peptide areas normalized to the theoretical tryptic peptide potential) and then reported as a fraction of total protein iBAQ amount per experiment (in 10–5 units for visual comprehension).

Cell lines and nuclear extract (NE) preparation

TZM-bl cells were initially obtained from the NIH AIDS Reagent Program, and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml). The 2D10 cell line is a gift from Dr Jonathan Karn (Case Western Reserve University), and the cells were maintained in RPMI 1640 medium (without phenol red) supplemented with 10% heat-inactivated FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml) and 25 mM HEPES at 37°C in 5% CO_2. For NE preparation, HeLa S3 cell pellets were obtained from the National Cell Culture Center (Minneapolis, MN), and NE was prepared following the standard Dignam protocol (37).

Transient transfection (plasmid and siRNA knockdown) and luciferase analysis

TransIT LT1 reagent (Mirus Bio LLC) was used to transfect plasmid DNA to TMZ-bl cells. Lipofectamine RNAiMax transfection reagent (Thermo Fisher Scientific) was used to transfect siRNA/plasmid DNA to TMZ-bl cells. CARM1 and control SMARTpool siRNA (Dharmacon ON-TARGETplus) were purchased from GE Healthcare. Luciferase activity was determined using the Promega luciferase assay kit following the manufacturer's instructions (Promega Corp., Madison, WI, USA).

Flow cytometry

Single cell suspensions were achieved by passage through a 22-gauge syringe before plating at a density of 5×10^4 on 24-well plates and treatment with pharmacological compounds for 24 h. GFP-expressing cells were quantified using a BD FACSCANTO II and the FACSDIVA software suite in the Cytometry and Cell Sorting Core at Baylor College of Medicine.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using the ChIP-IT Express kit (Active Motif) following the manufacturer's protocol except for the DNA purification step. We sheared the chromatin to an average size of 300–400 bp by sonication. The sheared chromatin was incubated with indicated antibodies overnight at 4°C. The QIAquick PCR purification kit was used for DNA purification after reverse-crosslinking of immunoprecipitated DNA. Purified DNA was eluted with 50 μl of elution buffer and 3 μl was used for quantitative PCR analysis using the SensiFAST SYBR Hi-ROX kit from Bioline. The primers for amplification of Nucleosome 0 locus (Nuc-0) of LTR are: 5′-TGGATCTACCACACACAAGG-3′ (forward); 5′-GTACTAACTTGAAGCACCATCC-3′ (reverse). The primer sequences for amplification of Nucleosome 1 locus (Nuc-1) of LTR are: 5′-GAGCCTGGGAGCTCTCTG-3′ (forward); 5′-GCTAGAGATTTTCCACACTG-3′ (reverse). And the primer sequences for amplification of DNase I hypersensitive sequence site (DHS) are: 5′-TTACACCCTATGAGCCAGCATG-3′ (forward); 5′-GCTCTCGGGCCATGTGAC-3′ (reverse) (38). The additional antibodies used in ChIP assays are: histone H3 antibody from Novus; H3K27ac, H3K36me3 and pol II pSer2 antibodies from Abcam. The H3R26me2aK27ac antibody was customized at Pocono Rabbit Farm &

Laboratory, Inc. and affinity purified by immobilized antigen.

Primary T cell model of CCL19-induced HIV latency

The CCL19 latency model initially developed in Dr. Sharon Lewin's laboratory has been described previously (39–41). Briefly, primary CD4+ T-cells were isolated from healthy donors using a RosetteSep human CD4+ T cell enrichment kit (StemCell) following the manufacturer's instructions. The resting CD4+ T cells were then negatively selected using CD30 MicroBeads by MACS separation (Miltenyi Biotec). These cells were treated with CCL19 (R&D Biosystems) for 2 days before SF162 virus infection. Five days after infection, cells were stimulated by CARM1 inhibitor 7g (Millipore) in combination with SAHA or JQ1. Cells were harvested a day after compound treatment, followed by RNA extract using Tri-reagent RT (Molecular Research Center, Inc) and DNase treatment using Direct-zol RNA purification kit (Zymo Research). The expression of the *gag* RNA level was measured by RT-qPCR analysis using a sensiFAST SYBR Hi-ROX One-step kit (Bioline). The sequences of the primers are: 5′-GGGACCCAGCCATAAAGC-3′ (forward); 5′-GCTGAATTTGTTACTTGGCTCA-3′ (reverse). These experiments were performed at the Baylor-UT Houston Center for AIDS Research (CFAR) laboratory.

RESULTS

Crosstalk between H3K27ac and H3R26me2a

Because histone H3K27 acetylation is a key epigenetic mark for active gene transcription, we investigated if this modification has any functional crosstalk with histone modifications on other sites. We first examined the residues in immediate proximity to H3K27. Interestingly, we found that H3R26 methylation, catalyzed by CARM1, was greatly enhanced when H3K27 was acetylated. This appears to be specific for R26 site, as R17 methylation was not altered (Figure 1A and B).

Interestingly, when we aligned the known amino acid sequences of CARM1 methylation sites, only H3R26 and H3R17 contain a lysine residue at the '+1' position. All other substrates have a neutral or hydrophobic residue (Figure 1C). A previous study on H3R17 methylation reveals that the positive charge of lysine side chain at '+1' position can slow down the process of arginine methylation, and acetylation of this lysine neutralizes the charge and therefore accelerates the reaction (42). In order to confirm whether this occurs on H3R26, we mutated the H3K27 residue to methionine and generated the recombinant H3 protein. The H3K27M mutation mimics the natural methionine residue at '+1' position in SRC-3, CBP and p300 coactivators. Interestingly, H3K27M somatic mutation has been found in up to 70% of diffuse intrinsic pontine glioma (DIPG) patients and is a driver mutation for tumorigenesis (43–45). With an *in vitro* methylation assay, we showed that the histone H3K27M mutant is preferentially methylated by CARM1 on R26, but not on R17 (Figure 1D and E). We also generated stable cell lines that ectopically express wildtype or K27M mutated histone H3 proteins, and as shown in Figure 1F, the histone H3 extracted from cells with H3K27M mutation contains much higher level of H3R26me2a, suggesting that the crosstalk between H3K27ac and H3R26me2a exists at the cellular level. Furthermore, we compared the methylation activity on H3 recombinant proteins with different modifications, including unmodified, K27-acetylated or K27-tri-methylated histone H3. H3K27ac appears to be the most preferable substrate, whereas H3K27me3 is the least favorable (Figure 1G). Based on these results, we conclude that K27-acetylated histone H3 is a better substrate for CARM1 methylation, and acetylation on H3K27 could be a priming event for H3R26 methylation (Figure 1H).

H3K27 acetylation and H3R26 methylation control the recruitment of Super Elongation Complex (SEC)

Histone modifications exert functional consequences on epigenetic gene regulation through recruiting 'reader' proteins that specifically recognize the modified histone marks. The reader proteins subsequently recruit additional transcriptional machinery to the target gene promoter/enhancer to modulate transcription (46). Although H3K27ac is consistently correlated with gene activation, no specific reader has been identified. In addition, the role of methylation of H3R26, the residue immediately adjacent to H3K27, is poorly understood. Therefore we performed peptide pull-down assays followed by mass spectrometry analysis using K27-acetylated or R26-methylated H3 peptides as bait to identify specific binding partners, *i.e.* 'readers'. These biotinylated peptides were immobilized on streptavidin beads and incubated with HeLa nuclear extract. Proteins bound to the peptides were precipitated and identified by mass spectrometry. A complete list of proteins enriched by each peptide is shown in Table 1. Overall, 117 proteins were enriched on H3K27ac peptide relative to unmodified H3. In contrast, only 31 proteins showed enrichment with H3R26me2a peptide. Interestingly, when we searched for protein complexes that are enriched in H3K27ac peptide pull-down, we found multiple key subunits of the SEC. But these subunits were significantly decreased in H3R26me2a peptide pull-down (Figure 2A). The mass spectrum result was confirmed by a separate peptide pull-down followed by Western blot analysis (Figure 2B and C), and was further validated by a pull-down assay using full-length histone H3 proteins as bait (Figure 2D). These results indicate that H3K27ac directly recruits SEC subunits, whereas H3R26 methylation precludes this recruitment.

CARM1 methyltransferase activity is repressive for HIV-1 LTR-mediated transcription

Positive transcription elongation complex (P-TEFb), a major component of the SEC, is composed of kinase CDK9 and one of the cyclin subunits, cyclin T1and T2. The CDK9 and cyclin T1 subunits of P-TEFb are essential for HIV-1 transcription. P-TEFb directly interacts with Tat protein and viral TAR RNA to stimulate HIV-1 transcriptional elongation (47,48). Therefore, our results suggest that H3K27ac may enhance HIV-1 transcription by recruiting

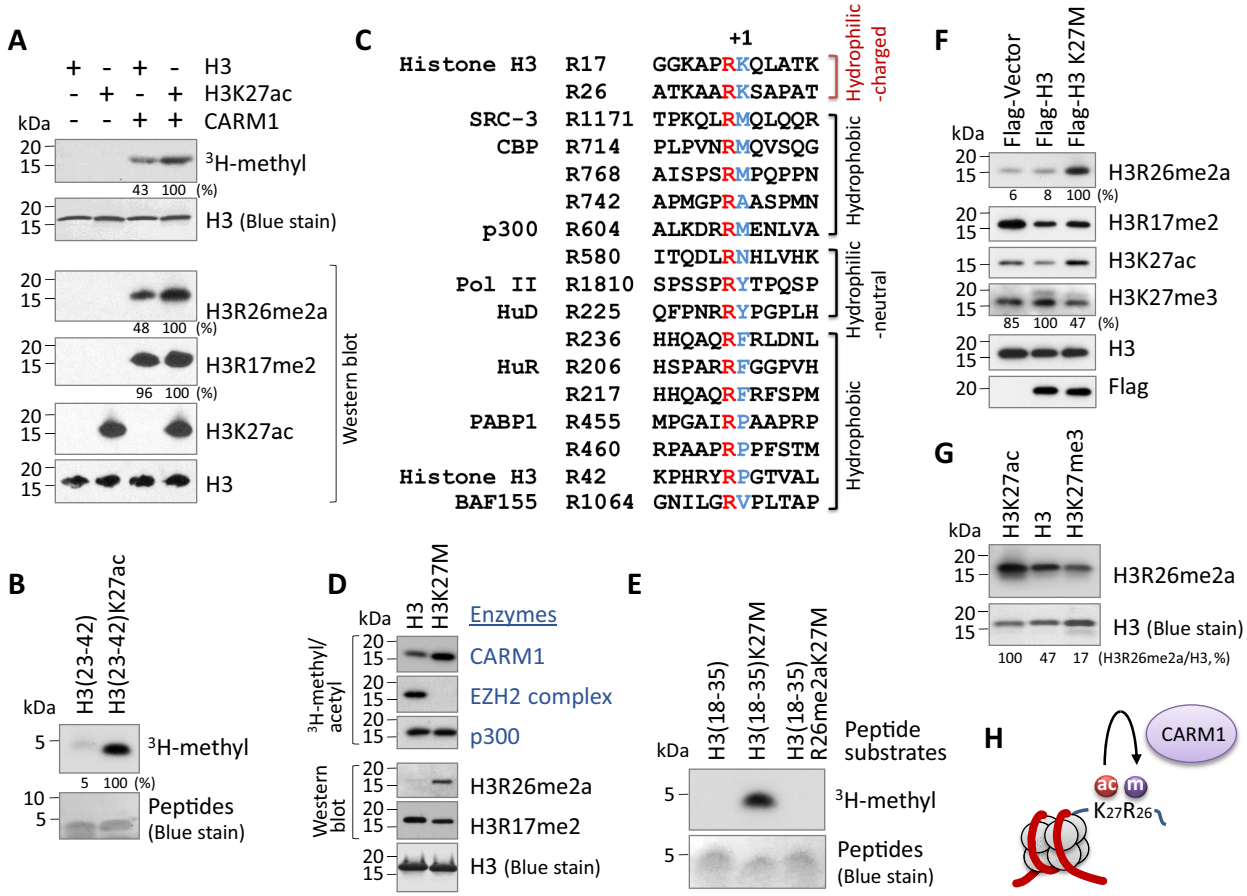


Figure 1. Histone H3 with K27 acetylation is a better substrate for CARM1 methylation. (**A**) *In vitro* histone methylation assay catalyzed by CARM1. Unmodified and K27-acetylated recombinant histone H3 proteins were used as substrates, and the methylation activity was determined either by tritium-labeled assay (top panel), or western blot analysis (bottom panel). The relative intensity of signals was determined by ImageJ. (**B**) Unmodified and K27-acetylated histone peptides (amino acids 23–42) were used as substrates for tritium-labeled *in vitro* methylation assay catalyzed by CARM1. (**C**) A sequence alignment of known CARM1 substrates. The 'R' residues in red are CARM1 methylation sites. (**D**) *In vitro* methylation assay using wildtype histone H3 or histone H3 with a K27M mutation. The names of the recombinant methyltransferases/acetyltransferase were shown in blue. (**E**) Unmodified and R26-methylated histone H3 peptides (amino acids 18–35) harboring the K27M mutation were used in a methylation assay catalyzed by CARM1 *in vitro*. (**F**) Western blot analysis of histones extracted from 293T cells stably expressing vector, wildtype histone H3, or K27M mutated histone H3. The relative intensity of signals was determined by ImageJ. (**G**) Histone methylation assay catalyzed by CARM1. Unmodified, K27-acetylated, and K27-trimethylated histone H3 proteins were used as substrates. The total histone proteins were shown by Blue stain reagent, and H3R26me2a level was shown by Western blot analysis. The relative intensity of H3R26me2a was normalized by H3 and shown at the bottom. (**H**) A cartoon model of H3K27ac-regulated H3R26 methylation.

and stabilizing the SEC, Tat and TAR multi-complexes. In contrast, the subsequent H3R26 methylation catalyzed by CARM1 may dissociate the SEC and attenuate HIV-1 transcription.

We performed Chromatin-IP analysis in 2D10 cells, a latency model with a single proviral insertion of HIV-d2EGFP in the genome of Jurkat CD4+ T cells (49). When we treated the 2D10 cells with TNFα to activate the latent provirus, we observed a significant increase of H3K27ac levels on Nuc-0 and Nuc-1 regions of HIV-1 5′ LTR. The level of increase is correlated with H3K4me3, another epigenetic mark for active transcription, and enrichment of RNA polymerase II (Figure 3A). This result suggests that H3K27 is acetylated when HIV-1 viral transcription is activated.

Based on the mass spectrometry data, we hypothesized that H3R26 methylation is a repressive mark for HIV-1 LTR-mediated transcription through dissociation of SEC. We tested this hypothesis in TZM-bl cells, a HeLa cell HIV-1 latency model that contains Tat-inducible HIV LTR-Luc and HIV LTR-β-galactosidase reporter genes. As shown in Figure 3B, overexpression of wild-type CARM1 significantly reduced Tat-mediated transcriptional activity, whereas methyltransferase mutants of CARM1 did not. Our results indicate that the intrinsic methyltransferase activity of CARM1 indeed suppresses HIV-1 transcription. In addition, when CARM1 was depleted in TZM-bl cells by siRNA, Tat-mediated transcription was moderately increased (Figure 3C). Transcriptional activity was further increased by the addition of JQ1, suggesting that inhibition of CARM1 can work with other LRAs to activate HIV-1 proviral transcription.

Table 1. A complete list of proteins enriched/reduced on H3K27ac or H3R26me2a peptides

Proteins enriched on H3K27ac peptide							
ACACA	DNAJA2	FASN	KRT3	NKRF	ROR2	RSL1D1	TAF6L
AFF1	DOT1L	FYTTD1	MAML1	NOP2	RPL10A	SCAF11	TBK1
AFF4	DSC1	GEMIN5	MAU2	NR4A1	RPL11	SDC4	TMOD3
ATAD5	DYNC1H1	GPATCH8	MCM3AP	NRDE2	RPL13	SEC24B	TOP3A
ATP6V1B2	EEF2	GPC1	MED13	PDE12	RPL14	SH3GL1	TWISTNB
BRCA1	EHMT2	GTPBP4	MED27	PGAM5	RPL15	SLFN5	USP39
CCNT1	EIF2S1	HIST1H1C	MEN1	PKP2	RPL18	SLX4	VPRBP
CDC42EP1	EIF3B	HNRNPLL	MLH1	PRKDC	RPL21	SMARCA2	VWA9
CDT1	EIF5B	HOXA10	MLLT1	PRPF38A	RPL27	SMCHD1	WTAP
CNOT3	ELAVL1	HPS3	MOV10	PRRC2A	RPL27A	SNAPC4	YLPM1
CSTF1	ELL2	IRF2BP2	MYBBP1A	RAD18	RPL30	SPAG5	ZMYM4
CWF19L2	EPRS	KAT7	MYBL2	RBM34	RPL7A	STAT3	ZNF318
DDX24	ERBB2IP	KHDRBS1	NAT10	RERE	RPS19	SUGP2	
DDX50	ESRP2	KIAA1429	NCBP1	RFX1	RPS2	SYDE1	
DNAJA1	FANCI	KPNA1	NDE1	RNF169	RPS25	SYNCRIP	
Protein enriched on H3R26me2 peptide							
ACTR8	CTSH	IRF2BP2	PDIA3	S100A9	VWA9		
APOD	DDX20	KRT79	RAD18	SH3GL1			
ARFGAP3	DYNC1H1	MAML1	RFX1	SLX4			
AZGP1	ELL2	NAT10	RPS25	STAT3			
CALML3	FAM83D	NR4A1	RUNX2	TMOD3			
CSTA	HMGB1	PDE12	S100A7	UBN1			

CARM1 small-molecule inhibitor 7g is a potential latency-reversing agent for HIV-1

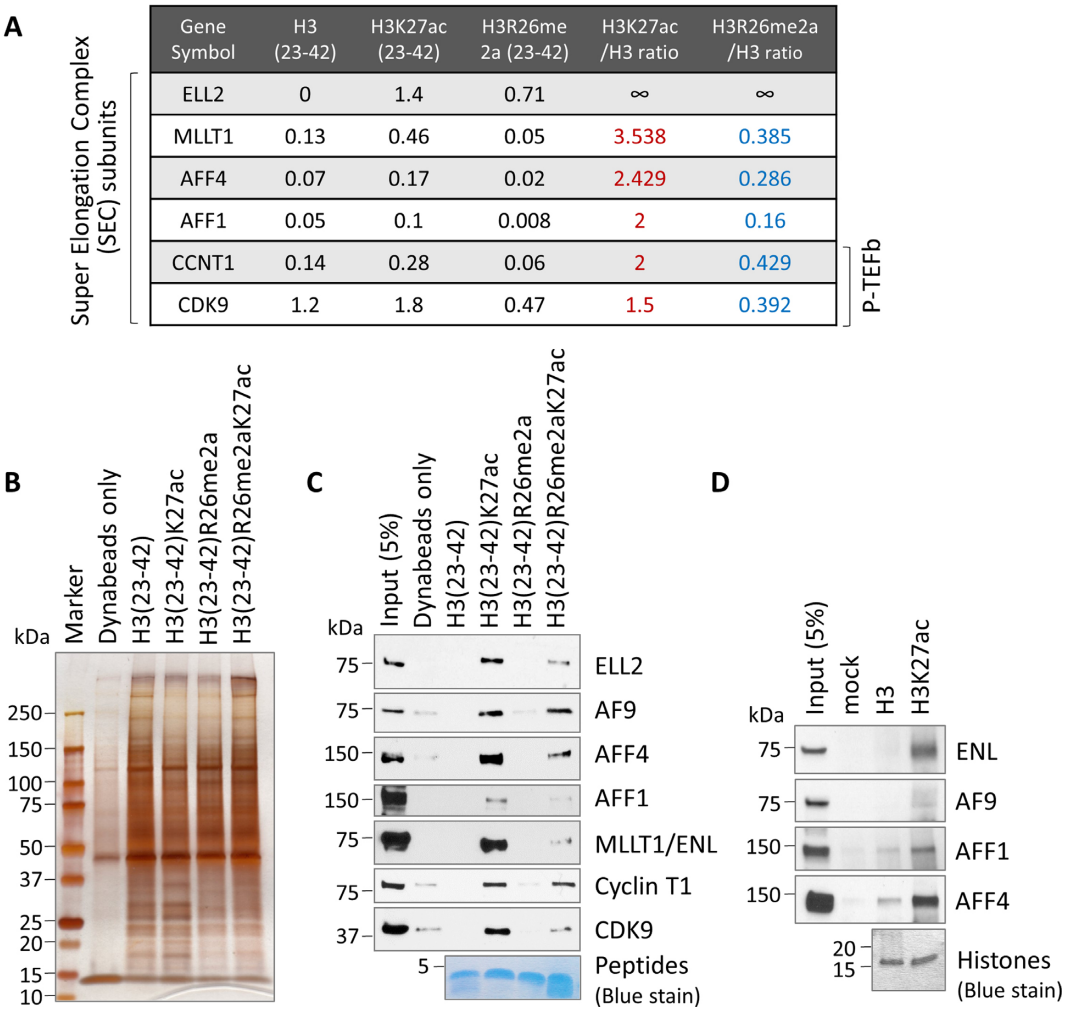
Given that the repressive function of CARM1 depends on its methyltransferase activity, we predicted that a CARM1 inhibitor could activate HIV-1 proviral transcription. We tested the commercially available CARM1-specific inhibitor 1-Benzyl-3,5-*bis*-(3-bromo-4-hydroxybenzylidene) piperidin-4-one (termed 7g) in HIV latency models (Figure 4A) (50). At the cellular level, 7g potently inhibits H3R26 methylation (Figure 4B). Treatment of TZM-bl cells with 7g strongly induced HIV-1 transcriptional output as indicated by luciferase activity. Moreover, the co-treatment of 7g with bromodomain inhibitor JQ1 synergistically increased the transcriptional activity (Figure 4C). Similar results were observed in 2D10 cells. Treatment of increasing doses of 7g activates the latent provirus in 2D10 cells in a dose-dependent manner (Figure 4D), and 7g exhibits a synergistic effect with the HDAC inhibitor SAHA and JQ1 (Figure 4E and F), suggesting that both acetylation and methylation are key regulators of HIV latency. Additionally, the geometric means of GFP-positive cells were calculated and exhibited a positive correlation with the doses of 7g, indicating that 7g not only increases the number of cells expressing HIV, but also increases the level of expression at the single cell level.

Because both TZM-bl and 2D10 latency models are based on cancer cell lines, we next sought to determine the effect of 7g in a primary resting T cell model of HIV latency. First, we determined the toxicity of 7g in PBMCs by measuring the cell viability and apoptosis after 7g treatment. As shown in Figure 4G and H, 7g exhibits little toxicity when being used at 6.3 μM. However, it affects cell viability and induces apoptosis at higher concentrations such as 12.5 μM. Therefore to avoid toxicity, we chose to use 2 μM of 7g when combined with other LRAs in primary cells.

To examine the efficacy of 7g during latency reversal in combination with SAHA and JQ1, we used a model system that establishes latent HIV-1 infection in resting CD4+ T cells treated with the cytokine CCL19 [26]. As shown in Figure 4I, when 7g was used alone, *gag* mRNA was produced in a dose-dependent manner. And when 2 μM of 7g was used in combination with SAHA or JQ1, a synergistic effect was observed, in agreement with the result observed in 2D10 cells (Figure 4J). Taken together, our data indicate that the small-molecule inhibitor of CARM1 7g can induce activation of latent HIV and therefore has potential as a novel therapeutic agent for shock-and-kill strategies.

CARM1 inhibitor 7g activates expression of latent HIV-1 through promoting transcriptional elongation

Because of the critical role of the SEC in HIV transcriptional elongation, we sought to examine the roles of histone modifications and the recruitment of the SEC on the HIV-1 LTR in Jurkat 2D10 cells. Due to the lack of ChIP-grade commercial antibodies for H3R26me2a, we generated and purified our own rabbit polyclonal antibodies recognizing the dual modification H3R26me2aK27ac. The specificity of the antibody was confirmed by the histone H3 peptide-based dot-blot experiment shown in Figure 5A, and further validated by western blot analysis using full-length histone H3 and H3K27ac recombinant proteins. Both proteins were methylated by CARM1 protein *in vitro*, but only the methylated H3K27ac protein can be recognized by H3R26me2aK27ac antibody (Figure 5B). Because HDAC inhibitors have been reported as LRAs for HIV-1 in many studies, we used SAHA in our ChIP analysis as a positive control. As shown in Figure 5C, treatment of 2D10 cells with SAHA or 7g did not alter the total protein levels of the SEC subunits including Cyclin T1, CDK9, ENL, and AFF4, or the transcriptional coregulator



Gene Symbol	H3 (23-42)	H3K27ac (23-42)	H3R26me 2a (23-42)	H3K27ac /H3 ratio	H3R26me2a /H3 ratio
ELL2	0	1.4	0.71	∞	∞
MLLT1	0.13	0.46	0.05	3.538	0.385
AFF4	0.07	0.17	0.02	2.429	0.286
AFF1	0.05	0.1	0.008	2	0.16
CCNT1	0.14	0.28	0.06	2	0.429
CDK9	1.2	1.8	0.47	1.5	0.392

Figure 2. Super Elongation Complex (SEC) are H3K27ac 'readers'. (**A**) Key components of SEC were highly enriched on H3K27ac peptide by pull-down analysis. The fold changes over unmodified histone H3 peptide are shown as red (enriched) or blue (reduced). A complete list of all proteins identified by mass spectrometry is shown in Table 1. (**B**) Peptide pull-down followed by silver stain. (**C**) Peptide pull-down followed by Western blot analysis to confirm the mass spectrum results in (A). The peptides shown on the bottom were in proportion to the amount of peptides used in the pull-down. (**D**) Immunoprecipitation (IP) of SEC subunits from HeLa nuclear extract by recombinant full-length histone H3 proteins (unmodified or K27-acetylated). Histone H3 antibody and protein G beads were added to the IP samples following 2 h of incubation. The mock sample contains all the reagents except for recombinant histone H3. The amount of histones used in the IP was shown by Blue stain.

proteins p300 and CARM1. The ChIP assay showed that H3K27ac was significantly increased on HIV-1 LTR when the cells were treated with SAHA, despite the fact that the levels of total histones were reduced (Figure 5D). Consistent with our biochemical observations that H3K27 acetylation functions as a priming event for H3R26 methylation, we observed that the levels of H3R26me2aK27ac increased in SAHA treated samples by ChIP, but not in 7g-treated cells, suggesting that endogenous H3R26 methylation on HIV-1 LTR depends on CARM1 methyltransferase activity. Consistently, the recruitment of CylcinT1 and AFF4 was greatly enhanced in both SAHA and 7g-treated cells, and consequently promoted the transcriptional elongation exhibited by the increase of Ser2-phosphorylated RNA polymerase II (Figure 5D). We also observed that 7g treatment enriched H3K36me3, a histone modification mark for transcriptional elongation, further supporting our conclusion that 7g activates HIV-1 LTR mediated transcription by promoting transcriptional elongation. It is worth noting that the recruitment of p300 was elevated in 7g-treated cells, in agreement with our previous observation that CARM1 methyltransferase activity is critical to dissociate the p300/SRC-3/CARM1 coactivator complex (25).

DISCUSSION

Gene transcription in eukaryotic cells is a complex process involving recruitment of multiple transcriptional machineries. These enzymatic activities/complexes are recruited to the chromatin in a sequential order to modify chromatin structure and facilitate transcriptional initiation and elongation. However, how these processes are coordinated is largely unknown. In our current study, we demonstrate that crosstalk occurs between histone H3K27 acetylation and H3R26 methylation. Importantly, the change of histone modifications on K27 and R26 determines the recruit-

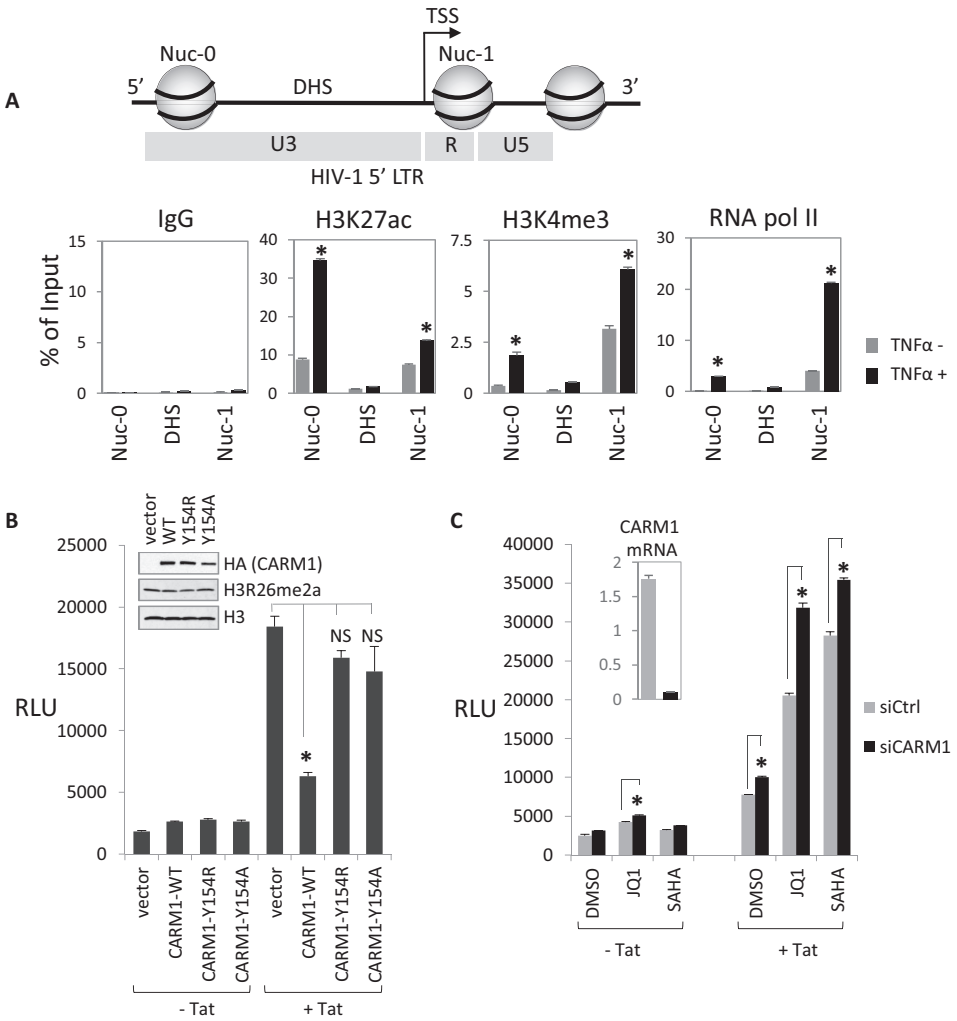


Figure 3. CARM1 methyltransferase activity is repressive for HIV-1 LTR-mediated transcription. (**A**) Chromatin IP on HIV-1 LTR in 2D10 cells treated with or without TNFα for 2 h. The experiment has been repeated two times and the most representative results were shown. *$P < 0.05$ determined by two-tailed *t*-test with duplicated ChIP samples per treatment. The error bars were shown as standard error of mean (SEM). DHS: DNase I hypersensitive site. (**B**) Wildtype or CARM1 methyltransferase-dead mutants Y154R and Y154A were transfected in TZM-bl cells in the absence or presence of Tat, and the relative luciferase activity was measured two days after transfection. Western blot analysis showed the expression levels of CARM1 proteins and H3R26me2a. *$P < 0.05$ determined by two-tailed *t*-test. NS, not significant. All transfections were in quadruplicates, and the error bars were shown as SEM. (**C**) TZM-bl cells were transfected with Tat expressing vector and siRNA for 24 h, followed by treatment of DMSO, JQ1 (0.5 μM), or SAHA (0.5 μM) for another 24 hours before harvesting cells for luciferase assay. RT-qPCR analysis showed the knockdown efficiency of CARM1 siRNA in TZM-bl cells. *$P < 0.05$ determined by two-tailed *t*-test. The experiment was performed in duplicates. The error bars were shown as SEM.

ment and dissociation of the SEC protein complex on the HIV-1 LTR. Based on our findings, we propose a working hypothesis as shown in Figure 6. H3K27ac is an active mark for HIV-1 transcription. SEC interacts and is stabilized by H3K27ac on HIV-1 LTR, and the recruitment of SEC is essential for HIV-1 transcriptional elongation. Subsequently, H3K27ac induces CARM1-dependent H3R26 methylation, resulting in dissociation of SEC and attenuation of HIV-1 transcription. An interesting question that arises here is which component of the SEC is the 'reader' for H3K27ac. AF9 and ENL, the YEATS domain containing proteins, have been previously reported as H3K9ac readers. They can also recognize H3K27ac and H3K18ac but with lower affinity (51). It remains to be determined whether AF9/ENL or other component recognizes H3K27ac and bring the SEC to the HIV-1 promoter.

CARM1 has been shown to function as both a transcriptional activator and an attenuator in a context-dependent manner. CARM1 methylates diverse substrates including histone H3, RNA binding proteins, transcription factors, and coactivators. H3R17 is a major CARM1 methylation site on histone, and this modification promotes transcriptional elongation through recruiting TDRD3 and TOP3B to prevent R loop accumulation and/or recruiting PAF1c (52–54). On the other hand, we and others have shown that CARM1 can methylate the SRC family of coactivators and subsequently attenuate gene transcription by dissociation of coactivator complex and degradation of SRC-3 protein (25,27). Our current study reveals an additional mech-

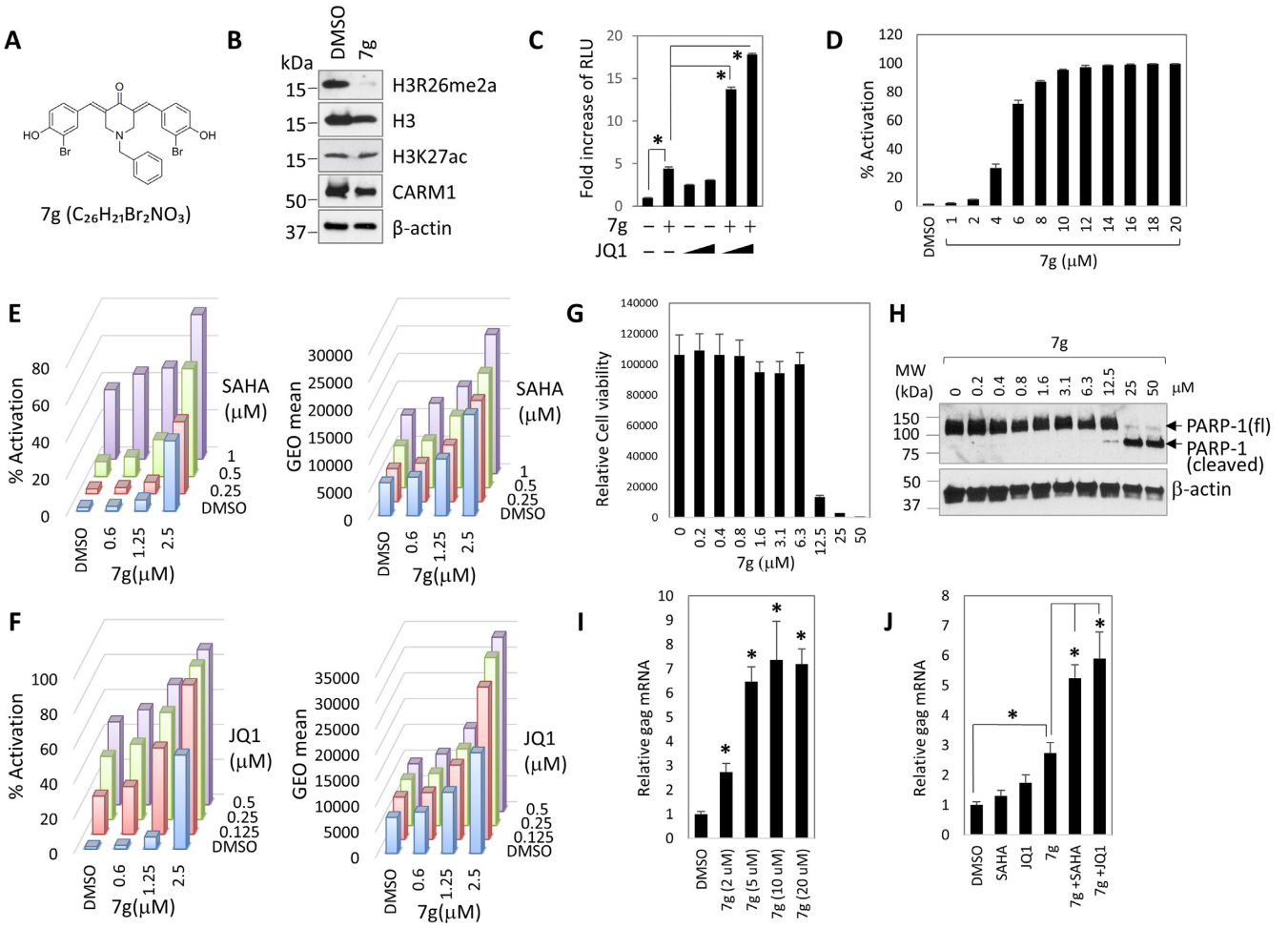


Figure 4. The CARM1 inhibitor 7g strongly induces HIV-1 transcription in latent cell models. (**A**) Chemical structure of 1-Benzyl-3,5-bis-(3-bromo-4-hydroxybenzylidene) piperidin-4-one, the CARM1 inhibitor 7g. (**B**) 293T cells were treated with 7g for 24 h and Western blot analysis was performed. (**C**) TZM-bl cells were first transfected with a Tat expressing vector, and then treated with 7g (5 μM) with or without the bromodomain inhibitor JQ1 (0.1 or 1 μM). Luciferase assay was performed after 24 h of drug treatment. $*P < 0.05$ determined by two-tailed *t*-test. The experiment was performed in duplicates, and error bars were shown as standard deviation (SD). (**D**) 2D10 cells were treated with increasing doses of 7g for 16 h and GFP level was determined by FACS analysis. Each treatment was duplicated and averages were shown. Error bars were shown as SD. (**E**) 2D10 cells were treated with different doses of 7g and SAHA for 16 h followed by FACS analysis to measure the GFP level. The percentage of activated cells and their geometric mean (GEO) were both shown. The GEO Mean was calculated specifically for GFP+ cells, and was done by gating GFP+ cells and displaying the geometric mean from that gate. (**F**) 2D10 cells were treated with different doses of 7g and JQ1 for 16 h followed by FACS analysis to measure the GFP level. (**G**) PBMCs were treated with various concentrations of 7g for 3 days, and the cell viability was determined by CellTiter-Glo luminescent assay. The experiment was performed in triplicates, and error bars were shown as SD. (**H**) PBMCs were treated with various concentrations of 7g for 24 h, and the levels of full-length (fl) PARP-1 and cleaved form of PARP-1 were determined by Western blot analysis. (**I**) CCL19-treated primary resting CD4+ T cells were infected by SF162 virus. Five days after infection, cells with latent HIV-1 were stimulated by increasing doses of 7g. The mRNA level of *gag* was measured by RT-qPCR to determine the activity of 7g on HIV-1 activation at the transcriptional level. The assay was performed in triplicates and SEM was shown. $*P < 0.05$ determined by two-tailed *t*-test in comparison with DMSO-treated sample. (**J**) Evaluation of 7g with a combinatory treatment of SAHA and JQ1 using CCL19 primary T cell model. 7g, 2 μM; SAHA, 0.5 μM; JQ1, 0.5 μM. The assay was performed in triplicates and SEM was shown. $*P < 0.05$.

anism by which CARM1 suppresses transcription through H3R26 methylation. It is postulated that at the early stage of gene transcription, CARM1 preferentially methylates those substrates that are important for transcriptional activation; whereas after transcription is completed, CARM1 changes substrates and plays repressive role on transcription, facilitating the turn-off stage of transcriptional cycle. It remains to be determined how the substrate selectivity of CARM1 is precisely regulated during transcription. It has been reported that the full-length of CARM1 protein (aa 1–585) is required for H3R26 methylation, whereas the minimal methyltransferase domain (aa 147–490) is sufficient to methylate H3R17, suggesting a putative mechanism of regulating the CARM1 substrate selectivity (42).

Recently, several HDAC inhibitors have been tested as LRAs in clinical trials, but the results were not very encouraging. The size of the latent HIV-1 reservoir was not changed by these interventions despite an increase of HIV-1 transcription (55–58). This outcome suggests that more effective immune response is needed for eradiation of reactivated virus; and probably more likely, short-term treatment with a single LRA may not be potent enough to activate much latent provirus. Therefore, combination of two or more LRAs, especially LRAs with different targeting mech-

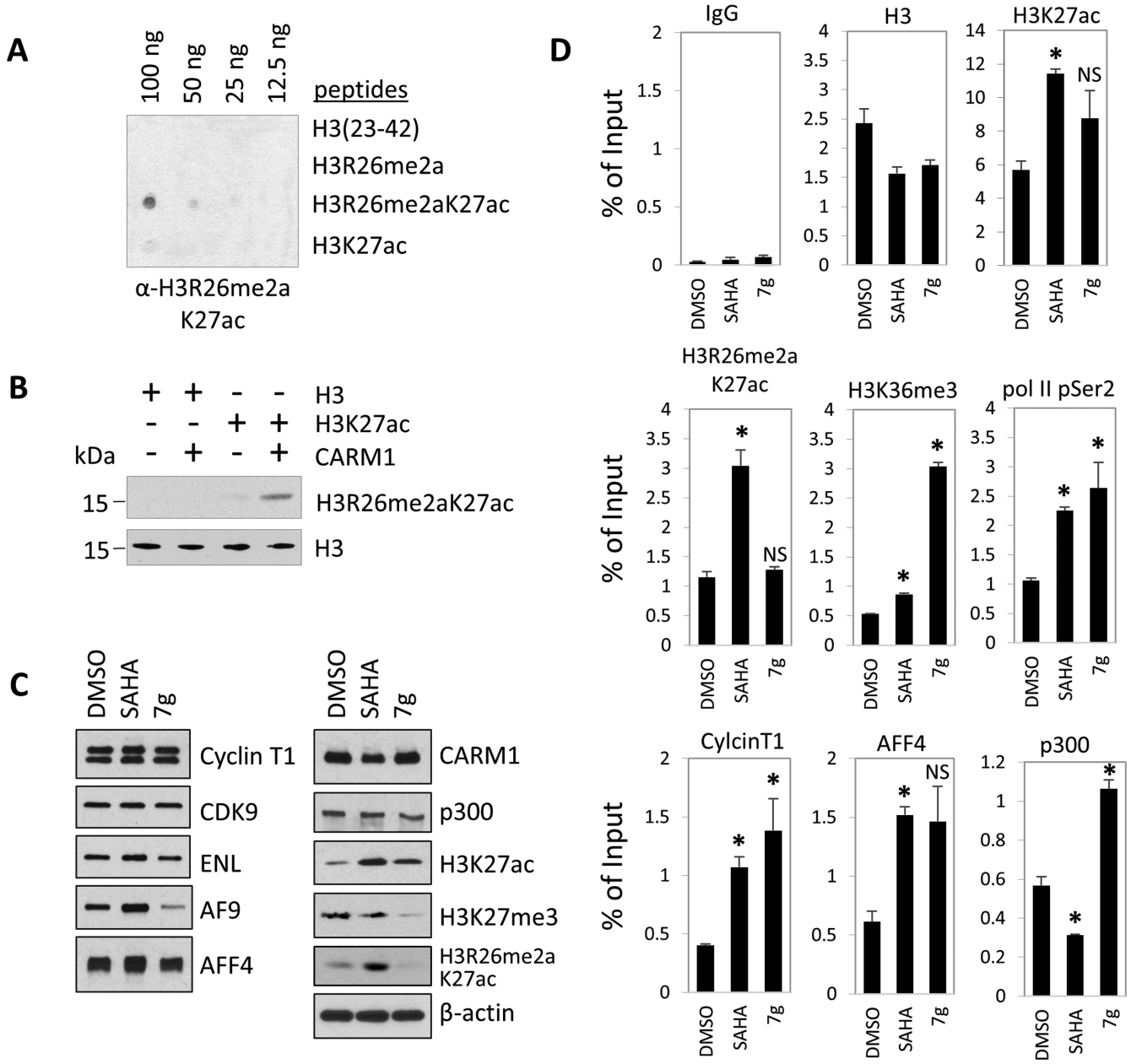


Figure 5. The CARM1 inhibitor 7g promotes transcriptional elongation by increasing the recruitment of SEC subunits on HIV-1 LTR. (**A**) Immuno-dot-blot assay to determine the specificity of the antibody. Various amounts of histone H3.1 (aa 23–42) peptides with/without methylated R26 or acetylated K27 were blotted on nitrocellulose membrane followed by western blot analysis. The H3R26me2aK27ac antibody was affinity-purified and used at 0.2 μg/ml. (**B**) Western blot assay to further determine the specificity of H3R26me2aK27ac antibody. Recombinant histone H3 and H3K27ac proteins were pre-methylated by CARM1 protein *in vitro* before being analyzed by Western blot analysis. PVDF membrane was used for protein transfer. (**C**) Western blot analysis to determine the protein levels of SEC subunits and histone modification enzymes in 2D10 cells treated with DMSO, 0.5 μM of SAHA, or 5 μM of 7g. Cells were treated for 24 h before harvest. (**D**) ChIP-qPCR analysis of histone modifications and the recruitment of transcriptional coregulators on HIV-1 LTR. The PCR primers amplified the DNA sequence on Nucleosome 1 (Nuc-1). 2D10 cells were treated with DMSO, 0.5 μM of SAHA, or 5 μM of 7g for 24 h before fixation for ChIP. The assay was performed in duplicates and SEM was shown. $*P < 0.05$ determined by two-tailed *t*-test in comparison with DMSO treated samples.

anisms, will likely be needed to achieve a better clinical response. Indeed, a previous study showed that using a combination of EZH2 inhibitors and HDAC inhibitors led to a synergistic activation of latent virus in comparison to single treatments (16). In this study, we aimed to identify additional epigenetic enzymes that control HIV gene transcription, with an anticipation that selectively targeting multiple suppressive epigenetic marks would synergistically activate HIV transcription.

Treatment with HDAC inhibitors increases total histone acetylation levels, which generally facilitate transcriptional initiation and elongation. The BET inhibitor JQ1 frees the limited amount of P-TEFb used by host transcription factor-mediated transcription by inhibition of

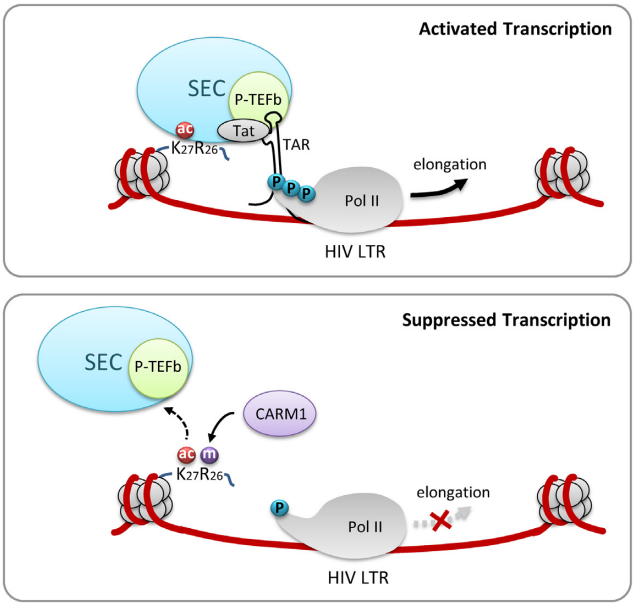


Figure 6. Working model. Histone H3 K27 acetylation (H3K27ac) is an epigenetic mark that recruits SEC to HIV-1 LTR. K27ac also promotes R26 methylation (H3R26me2a) catalyzed by CARM1, which subsequently causes dissociation of SEC and attenuation of transcription, resulting in a negative feedback regulatory loop.

BRD4, meanwhile relieving the direct suppression function of BRD2 and ultimately facilitating the elongation process (59–61). In contrast to these known LRAs, CARM1 inhibitor functions through a completely different mechanism. It limits histone methylation at H3R26, therefore increasing the enrichment of SEC for HIV-1 transcription. It was reported that the subunits of SEC, including ELL2, AFF1, or AFF4, facilitate the activation of latent HIV-1 in Jurkat/2D10 cells (62), indicating that up-regulation of transcriptional elongation is an efficient strategy to reverse HIV-1 latency, and that a combination of therapeutic approaches at multiple steps of HIV transcription will synergistically activate HIV-1 RNA production. Indeed, treatment with a CARM1 inhibitor synergizes with SAHA and JQ1 to increase HIV transcription. In summary, our results reveal a crosstalk between histone modifications, and suggest a novel epigenomic approach for activation of latent HIV-1.

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