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A Proteomic Glimpse into the Initial Global Epigenetic Changes During HIV Infection

Laura-Mae P. Britton¹, Pavel Sova^{2,3}, Sarah Belisle^{2,3}, Shichong Liu⁴, Eric Y. Chan¹, Michael G. Katze^{2,3,*}, and Benjamin A. Garcia^{4,*}

¹Department of Molecular Biology, Princeton University, Princeton, NJ 08544

²Department of Microbiology, University of Washington, Seattle, Washington, USA

³Washington National Primate Research Center, Seattle, Washington, USA

⁴Epigenetics Program, Department of Biochemistry and Biophysics, Smilow Center for Translational Research, Perelman School of Medicine, University of Pennsylvania, 3400 Civic Center Blvd, Bldg 421, Philadelphia, PA 19104-5157

Abstract

HIV-1 replication requires the insertion of viral DNA into the host genome, which is catalyzed by HIV-1 integrase. This integration event can lead to vast changes in the chromatin landscape and gene transcription. In this study, we sought to correlate the extensive changes of histone post-translation modification (PTM) abundances with the equally dynamic shifts in host transcriptional activity. To fully capture the changes that were occurring during the course of HIV-infection, we performed time-courses in which we extracted both histones and mRNA from HIV-infected, UV-inactivated HIV-infected and mock-infected SUP-T1 cells. We then analyzed the alterations to histone PTM profiles using nanoLC-MS/MS, as well as the expression of chromatin-associated enzymes, such as histone deacetylases, acetyltransferases, demethylases, methyltransferases and histone chaperone proteins. As expected, we observed major changes in histone PTM abundances, which we linked to massive fluctuations in mRNA expression of associated chromatin enzymes. However, we find few differences between HIV and HIV_{UV} (UV-inactivated) infection, which suggests that initial histone PTM changes during HIV infection are from the host in response to the infection, and not due to the HIV virus manipulating the transcriptional machinery. We believe that these preliminary experiments can provide a basis for future forays into targeted manipulations of histone PTM-regulated aspects of HIV progression through its replication cycle.

Keywords

HIV; provirus; deacetylases; acetyltransferases; demethylases; methyltransferases; histone chaperones

HIV-1 exists as a two-copy single-stranded RNA virus, whose genome is reverse transcribed and integrated into human chromatin upon infection of CD4+ T cells¹. The death of these

*To whom correspondence should be addressed: bgarci@mail.med.upenn.edu, Phone: 1-215-573-9423. honey@u.washington.edu, Phone: 1-206-732-6135.

immune cells is the most definitive symptom of HIV progression to AIDS^{2, 3}. In the past decade, highly active antiretroviral therapy (HAART) has proven to be a vastly effective treatment regime for clearing active virus from circulation, often to below detectable thresholds, thereby preventing or delaying the progression to immunodeficiency in infected persons. However the main obstacle to HAART serving as a final cure for HIV is the persistence and stability of the HIV-1 provirus, which latently infects resting CD4+ T cells early in primary infection and is protected within the host chromatin⁴. This Achilles heel of HAART raises the need for development of novel therapeutics, which can potentially clear the cells of the virus during active infection and thereby prevent integration and development of the proviral pool. In response, the unraveling of epigenetic mechanisms which control the state of chromatin with specific emphasis on the retroviral landscape, has opened a new space for therapeutics directly targeting the integration, latency and reactivation of the HIV virus.

One such portal into this relatively new realm of possibilities has been through the analysis of histone post translational modifications (PTMs) surrounding the sites of viral integration. These studies have revealed that the long terminal repeats (LTRs) of the proviruses are complexed with nucleosomes that contain highly methylated and hypoacetylated histones^{5, 6}. The CpG islands near the HIV-1 promoter are also hypermethylated⁷. In combination, these 'epigenetic' marks are believed to suppress HIV-1 gene expression by maintaining a region of 'silenced' heterochromatin and by blocking access to transcription factors⁸. The HIV-1 transactivator, Tat, has been shown to recruit the histone acetyltransferases (HATs), p300, CBP-associated factor (PCAF), and hGCN5 to the HIV-1 LTR, which acetylate histones near the HIV-1 promoter to facilitate transcription of HIV-1 mRNA⁹. Recent independent studies have shown that inhibitors of Enhancer of Zeste 2 (EZH2), and G9a, both histone lysine methyltransferases, were sufficient to reactivate the latent HIV provirus, comparable to similar investigations using histone deacetylase inhibitors, pabinostat, vorinostat and valproic acid^{8, 10}; some of which are a part of ongoing clinical trials (<http://clinicaltrials.gov>).

Maintenance of the HIV-1 provirus and transcriptional activation has been attributed to a number of detailed molecular mechanisms, which have been extensively reviewed elsewhere¹¹. More relevant to this work are several studies, which have revealed a preference for viral integration into actively transcribed genes¹², although how this preference is maintained or targeted is not completely understood. Here, we seek to unravel the histone PTM conditions, which may provide an advantage to the virus during its cycle of active infection. In light of the continually emerging roles of individual PTMs and their enzymes during viral infection, we will focus our study on the role of global histone modifications during acute infection of highly proliferating cells. We compare the gene expression data for HIV-infected cells with their accompanying changes in histone post-translational modifications, in hopes of understanding the interplay between active HIV infection and chromatin state.

SUP-T1 cells were obtained from American Type Culture Collection (CRL-1942) and propagated in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (HyClone), penicillin (100 U/ml), streptomycin (100 µg/ml), and GlutaMAX-I. HIV-1 LAI

strain (catalog no. 2522) was obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD) and propagated in SUP-T1 cells. U373-MAGI-CXCR4_{CEM} cells were obtained from M. Emerman through the NIH AIDS Research and Reference Reagent Program, and the virus titer in these cells was measured by the protocol of Vodicka *et al*¹³. Typical titers reached 10⁷ infectious units per ml. Infections were carried out at a multiplicity of infection (MOI) of 2.5 and performed in triplicate, with triplicate UV-inactivated virus infections carried out in parallel. UV-inactivation of the virus was performed as previously described¹⁴. Briefly, HIV_{UV} was generated by irradiating HIV preparations for 5 min, and confirmed as replication-incompetent in SUP-T1 cells by viral mRNA load by Taq-Man qPCR¹⁵. Mock-infected samples received SUP-T1 cell conditioned medium and were performed in duplicate. The infectious dose was optimized to achieve 100% infected cells at 24 hpi with over 50% cell viability as measured by trypan blue exclusion assay. Infected cells were visualized by immunofluorescence assay with rabbit HIV-1_{SF2} p24 antiserum kindly provided by BioMolecular Technologies through the NIH AIDS Research and Reference Reagent Program as a quality control step.

At 4 and 20 hours post infection, 1×10⁷ cells were harvested. The cells were pelleted and washed thrice with cold Dulbecco's phosphate-buffered saline (DPBS); twice with 15 ml and once with 1 ml. The pellets were flash frozen in liquid nitrogen and stored overnight at -80°C. The pellets were thawed on ice and nuclei were obtained from cells by incubating pellets briefly with NP-40 in nuclei isolation buffer with 5 µmol/l microcystin, 0.3 mmol/l 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and 10 mmol/l sodium butyrate. From the nuclei, the histone proteins were extracted with 0.4 N H₂SO₄, precipitated with trichloroacetic acid, washed in acetone, dried overnight and resuspended in ammonium bicarbonate (pH 8). The purified histones were trypsin-digested in solution, following chemical derivatization with propionic anhydride, as previously described¹⁶. Briefly, propionic anhydride reagent (3:1 isopropanol:propionic) was added to the sample, and the solution vortexed and pulse-centrifuged for several seconds. Ammonium hydroxide was added dropwise to ensure that a pH 8 value was maintained. This reaction was incubated at 37°C for 15 min and dried down to approximately 5 µl in a SpeedVac concentrator. After a second round of propionylation, the histones were digested with trypsin in a 20:1 (wt/wt) protein:trypsin ratio and incubated at 37°C for 5 hours. Two additional rounds of propionic anhydride derivatization were performed on the digested peptides. The reconstituted samples were desalted using homemade STAGE tips, and prepared for MS analysis, as previously described¹⁷.

MS sequencing analysis of sample histone peptides was performed as previously described¹⁶; samples were loaded via an Eksigent autosampler (Eksigent Technologies Inc., CA, USA) onto a 75 µm C18 reverse phase capillary column packed with Magic C18 reversed-phase 5µm particles and constructed with an integrated electrospray ionization tip. Samples were resolved using an 110-minute 1–100% buffer B gradient (buffer A = 0.1 mol/L acetic acid, Buffer B = 70% acetonitrile in 0.1 mol/L acetic acid at 0.070 µl/min on an Agilent HPLC system (Agilent, Santa Rosa, CA, USA). The peptides were directly electrosprayed into and analyzed by an LTQ-Orbitrap XL (ThermoFisher Scientific, San Jose, CA, USA). Full scan MS spectra were acquired from over an m/z range 200–1500 at

30,000 resolution in the Orbitrap after accumulation of approximately 500,000 ions, followed by 5 to 7 data-dependent MS/MS spectra collected in the ion trap (CAD energy 40%) after accumulation of approximately 10 000 ions. Peak abundance for each modified peptide was calculated by manual chromatographic peak integration of full MS scans using Qual Browser software (ThermoFisher). Precursor ions of low-level modifications were selected for targeted analysis to increase identification of these PTMs. To quantify the relative abundance of each modified peptide species, the area under the curve for each peak is expressed as a percentage of the total histone peptide (sum of all forms of a particular peptide) for 3 biological replicates. The MS data have been deposited to the Chorus Public MS cloud application (<https://chorusproject.org/pages/dashboard.html#/projects/public/365/experiments>). The statistical significance (P-values) of the relative abundance of each histone modification as compared to the relative abundance of other genomic histone PTMs was calculated using a two-tailed, unpaired Student's t-test.

Samples for microarray assays were generated in a separate experiment carried out under identical conditions. 1×10^6 cells were collected at 4, 8, 12, 16, 20, 24, 28, and 32 hours post infection and lysed immediately in TRIzol (Life Technologies). Total RNA was isolated was using RNeasy columns (Qiagen). cRNA probes were generated from each sample by the use of an Agilent one-color Low Input Quick Amp labeling kit (Agilent Technologies) and individual samples were hybridized to Agilent 4x44k Human WG microarrays (G4112F) according to the manufacturer's instructions. Slides were scanned with an Agilent DNA microarray scanner, and the resulting images were analyzed using Agilent Feature Extractor version 9.5.3.1. Microarray expression data were background corrected and quantile normalized in R. Average expression in the HIV infected samples at each time point was calculated relative to time matched mock samples. Primary microarray data has been submitted to Gene Expression Omnibus (GEO; GEO accession no. pending) in accordance with proposed Minimum Information About Microarray Experiment (MIAME) standards.

Our initial aim was to use the combination of these microarray and proteomics datasets to correlate expression trends for several candidate PTM enzymes over the course of HIV infection. Our histone PTM platform was based on sensitive nanoLC-MS/MS experiments which detected changes in the relative abundance for over 60 peptides representing histone H3 and H4 PTMs from histones collected at 4 and 20 hpi (HIV and HIV_{UV}) compared to a mock infection (Figure 1). We sought to differentiate between changes that can be attributed to integration events (HIV), as opposed to 'non-specific' host responses triggered by viral entry of replication-incompetent viral particles (HIV_{UV}). It should be noted that the HIV_{UV} viral particles are ideal candidates for comparing host response versus viral entry response and integration, as the UV-inactivated virion will be enveloped into the cell and so may contribute to the host response, although it cannot replicate. In our histone H3 analysis, we find several peptides whose abundances change when comparing the HIV infected SUP-T1 cells to mock infected cells. The most dramatic change initially observed for HIV-infected cells was a nine-fold increase in the relative abundance of the histone H3 lysine 4 dimethyl (H3K4me2) peptide at 20 hpi, when normalized to mock-infected cells (no virus). A significant decrease in KDM1A; a K4 and K9 specific demethylase, is similarly observed from 20–32 hpi (Figure 1). In contrast, several H3 9–17 peptide species K9acK14un, K9acK14ac, K27acK36un, K27me2K36me2 decreased significantly early in infection-

K9me3K14un, K9acK14un, K9me2K14ac and K9me3K14ac all exhibit 1.5–2 fold changes at 4 hpi and none at 20 hpi (See Figure 1). Concurrently, potential candidate enzymes, KDM4B, a K9me3 demethylase, and SUV39H1, a K9me3 methyltransferase showed substantial increases and decreases, respectively (Figure 2). The overall surge in expression for the enzymes with lysine deacetylase activity, HDAC5, SIRT2, SIRT3 and SIRT4, may account for some of the reduction in acetylation on the H3 9–17 peptide (Figure 1 and 2), however these correlations would need to be tested more extensively with in vivo targeting of the enzymes through chemical inhibition and or knock down studies.

The K18me1K23un and K18unK23me1 abundances spiked nine- and ten-fold, respectively from 4 hpi to 20 hpi whereas K27acK36un increased approximately two-fold at 4 hpi but then decreased at 20 hpi (Figure 1). K27acK36un decreases ~3-fold at 20 hpi and K27me2K36me2 increase 4-fold at 4 hpi. K79me2 increased more than two-fold at 4 hpi but with no significant change at 20 hpi. Overall, the most acute changes were observed for several di-acetylated species of the H4 4–17 peptide; K5acK12ac, K5acK16ac and K12acK16ac, which all decreased steadily over the course of infection. We also observe an approximate two-fold increase at both 4- and 20 hpi for the K5acK8ac peptide species. The microarray data for comparative time points show drastic decreases in expression of two histoneH4-specific HATs, MYST1 and MYST2, which may account for these fluctuations, in combination with the largely decreasing expression of histone deacetylase enzymes (Figure 1 and 2). All combinations of the tri- and the tetra-acetylated peptide species increased significantly but only later in infection at 20 hpi. Expression of SETD8, an H4K20me1-specific methyltransferase decreased steadily over the course of infection, which correlated with an almost simultaneous decrease in H4K20me1.

The maintenance of an HIV reservoir is highly dependent on the viral genome remaining silenced within the cell's chromatin and so the histone PTMs and gene expression changes we've detected over the course of HIV infection may hint at a recruitment or inhibition of chromatin remodeling and/or histone PTM enzymes, towards maintaining a chromatin landscape conducive to infection. For instance, in this acute infection model in fast-growing cells, it's possible that the cell's defense mechanism leads to a silencing of specific chromatin regions. Conversely, the increases in activating enzymes and chromatin remodelers could suggest that HIV is able to manipulate chromatin modifying machinery to open up chromatin and activate overall transcription. We hope this work will be an index of data that will be useful for future work on the epigenetic mechanisms involved in HIV infection.

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Figure 1. Relative abundance of histone (a) H3 and (b) H4 PTMs at 4 and 20 hpi
Heat map was generated using fold change values (Log2 scale) for each highlighted peptide species, as quantified from the HIV-, HIV₁₁₉- and Mock-infected cells.

Figure 2. Gene expression data for histone PTM enzymes during infection

Microarray data for histone PTM enzyme mRNA at 4, 12, 20, 24 and 32 hpi (Log2 scale).

The enzyme classes for the above are as follows: **histone lysine methyltransferases** (EZH1, SETD3, SETD8, SUV39H1, SUV39H2), **histone demethylases** (JHDM1D, JMJD1C, JMJD4, KDM1A, KDM4B, KDM5A, KDM5D, KDM6A), **histone arginine methyltransferases** (PRMT1, PRMT3, PRMT5, PRMT6, PRMT7), **histone acetyltransferases** (MYST1, MYST2), and **histone deacetylases** (HDAC5, SIRT2, SIRT3, SIRT4). mRNAs from PTM enzymes and chromatin binder and remodelers accounted for more than 200 of the detected mRNA in the data. However, only those with significant changes (± 0.5) were included in this figure. The statistical significance of the relative abundance of each histone modification as compared with the relative abundance of other existing histone PTMs was calculated using a two-tailed, unpaired Student t-test, and the associated P values were generated using Microsoft Excel.