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Genome-wide analysis of histone modifications in latently HIV-1 infected T cells

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Objectives: The transcriptional silencing of HIV type 1 (HIV-1) provirus in latently infected cells is a major hurdle on the pathway to HIV-1 elimination. The epigenetic mechanisms established by histone modifications may affect the transcriptional silencing of HIV-1 and viral latency. A systematic epigenome profiling could be applicable to develop new epigenetic diagnostic markers for detecting HIV-1 latency.

Design: The HIV-1 latency cell lines (NCHA1, NCHA2 and ACH2] were compared with $CD4^+$ T-cell line (A3.01).

Methods: The histone modification profiles obtained from chromatin immunoprecipiation followed by sequencing (ChIP-Seq) for histone H3K4me3 and H3K9ac were systematically examined and differential gene expression patterns along with levels of histone modifications were used for network analysis.

Results: The HIV-1 latency gave rise to downregulation of histone H3K4me3 and H3K9ac levels in 387 and 493 regions and upregulation in 451 and 962 sites, respectively. By network analysis, five gene clusters were associated with downregulated histone modifications and six gene clusters came up with upregulated histone modifications. Integration of gene expression with epigenetic information revealed that the cell cycle regulatory genes such as *CDKN1A* (p21) and cyclin D2 (*CCND2*) identified by differentially modified histones might play an important role in maintaining the HIV-1 latency.

Conclusion: The transcriptional regulation by epigenetic memory should play a key role in the evolution and maintenance of HIV-1 latency accompanied by modulation of signalling molecules in the host cells.

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Introduction

HIV-1 can infect $CD4^+$ T cells preferentially and eventually the viral genome is permanently integrated in a host cell chromosome. The integrated viral genome, provirus, is replication-competent but dormant for years by forming stable reservoir of latently HIV-1 infected cells [1].

The epigenetic information stored in chromatin structure, which can be altered by DNA methylation, histone modifications and nucleosome positioning, affects the transcriptional competence by providing protein binding sites and modulating accessibility of transcription machinery [2–8]. Dynamic posttranslational modifications of histones have been well studied such that hyperacetylation of histones is related with transcriptional

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activation and histone methylations are associated with both gene activation and transcriptional silencing depending on the position and degree of modifications [2–4,9,10]. Although great interest in epigenetics of HIV-1 latency has been raised, epigenetic regulation in HIV-1 latency cells was not studied extensively as one of molecular mechanisms of HIV-1 latency [11]. Histone deacetylases (HDACs) in a complex with transcription factors such as transcription factor Yin Yang 1 (YY1), late SV40 factor (LSF) and CCAAT-box binding transcription factor 1 (CBF1) are recruited to the promoters and repress the long terminal repeat (LTR)-driven transcription [12–14]. c-MYC and SP1 were reported to be involved in HIV-1 latency by induction of HDAC1 binding to the HIV-1 promoter [15]. The viral protein Tat, transactivating the HIV-1 promoter associated with the displacement of HDAC1 and increase of histone acetylation, can block the establishment of HIV-1 latency [16].

Previously, we explored the genome-wide histone modification profiles in the human primary T cells extensively and sought the functional significance of specific domains with histone modification islands [3,4,17,18]. As a continuation of understanding of T-cell epigenetics, we established a series of HIV-1 latency T-cell lines called novel chronic HIV-1 infected cells derived from A3.01 Novel Chronic HIV-1 infected cells derived from A3.01 (NCHA) [19,20]. Here, a genome-wide histone modification analysis and further network analysis on HIV-l latency induced by epigenetic changes were performed in three types of latently HIV-1 infected cells and A3.01 uninfected cells.

Materials and methods

Cell lines and cell culture

A3.01, ACH2 and NCHA cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS, 5% penicillin–streptomycin and 2 mmol/l glutamine [20].

ChIP-Seq

The ChIP-Seq procedure was performed following our previous study [21]. Briefly, the fragmented chromatin was immunoprecipitated using antibodies specific to histones H3K4me3 (ab8580; Abcam, Cambridge, UK) and H3K9ac (ab4441; Abcam). The isolated DNA was sequenced by the Genome Analyzer IIx (Illumina, San Diego, California, USA).

Quantitative ChIP-PCR

To quantify the enrichment of immunoprecipitated DNA, ChIP-PCR was performed using StepOne Real Time PCR System (Applied Biosystems, Foster City, California, USA). The relative enrichment of histone modifications was calculated by ddCt method [fold change of enrichment = 2 delta (Ct-ChIP) – (Ct- input)]. The PCR primer sequences used for the validation of CD4 gene loci will be provided upon request.

Identification of differentially enriched histone islands

Differentially enriched histone islands were identified using SICER 1.1 program [22] with the following options; window size = 200, gap size = 400, E-value = 0.01 and FDR = 0.05. In addition, the significant fold changes of normalized tag counts were set to over 1.5-fold. The overlapped histone islands were identified by HOMER program with a parameter for maximum distance to merge: 'given'.

Gene expression data analysis

The gene expression data were obtained using Human whole genome 4 x 44K microarray (Agilent, Santa Clara, California, USA). To compare with ChIP-Seq data, mRNA expression level of each gene was calculated by averaging signals from all probes of a gene. The *Z*-score is calculated by subtracting the population mean from an individual expression value and then dividing the difference by the population standard deviation.

Generation of interaction networks

Interaction networks based upon direct (physical) and indirect (functional) interactions were defined by using the STRING 9.05 (http://string-db.org/) and the BioGRID 3.1.74 (http://thebiogrid.org/) databases. Interactions with a combined STRING score greater than 700 are only used. Genes with differential histone modification islands were used for network analysis. The graphic view of interactions was generated using Cytoscape (http://www.cytoscape.org) platform. Among these interactions, highly interconnected significant gene clusters were calculated using a molecular complex detection algorithm (MCODE) with Fluff parameter [23]. Functional annotations of clusters were confirmed using BiNGO program [24].

Results

Genomic distribution of histone modifications in latent HIV-1 infection

Two active histone modifications such as H3K4me3 and H3K9ac were examined in A3.01 cells as uninfected $CD4^+$ T cells and three latently HIV-1 infected cells, NCHA1, NCHA2 and ACH2 cells using ChIP-Seq (Fig. 1a). The overall profile of H3K4me3 and H3K9ac did not show big differences between uninfected and HIV-1 latently infected cells (Fig. 1b and Supplementary Fig. S1, http://links.lww.com/QAD/A523). In good agreement with the previous results, both H3K4me3 and H3K9ac were highly enriched at the promoter regions and positively correlated with gene expression levels. Both H3K4me3 and H3K9ac levels of specific loci such as

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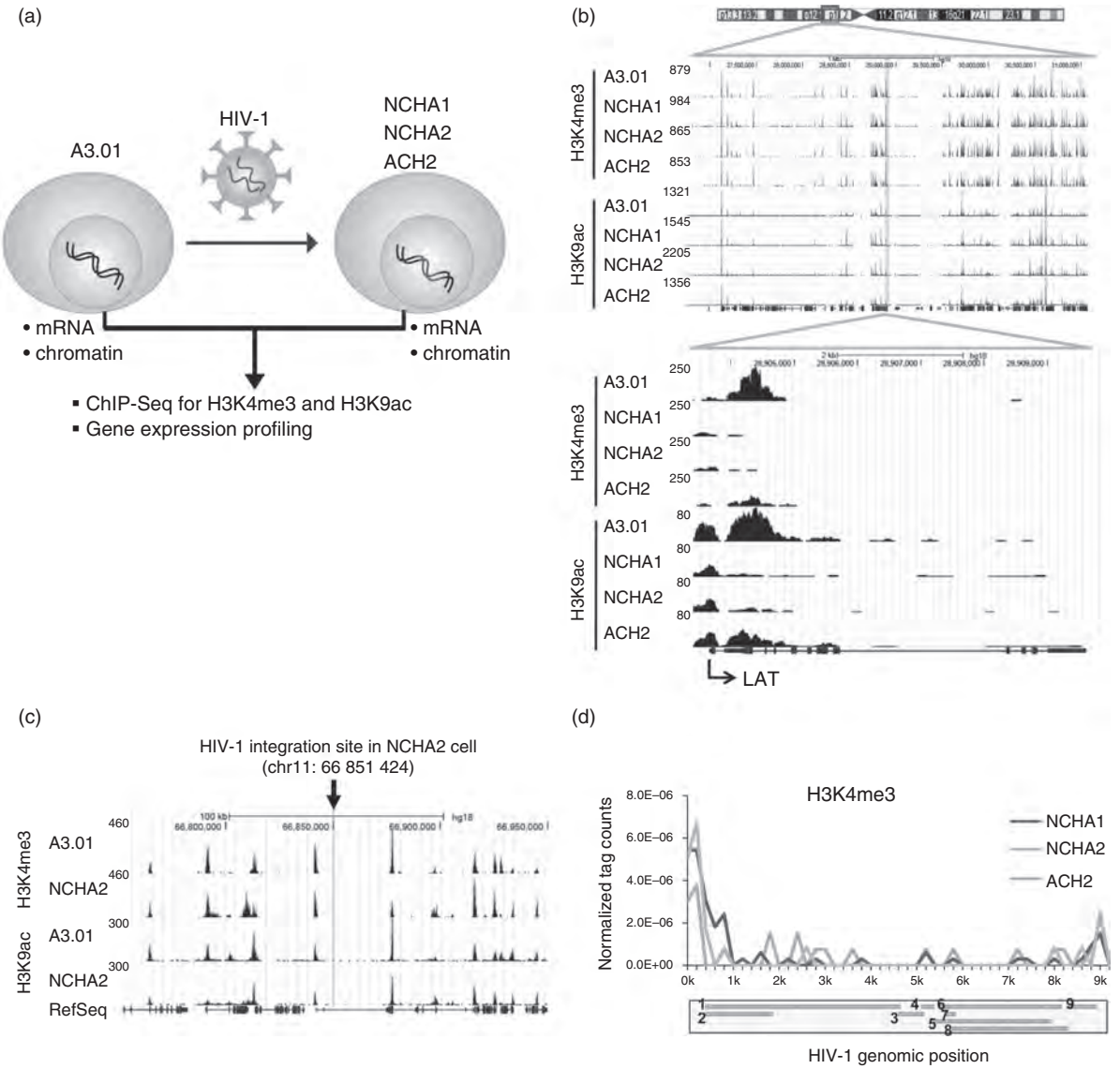

Fig. 1. Genome-wide distribution of histone modifications and mRNA profiles in a control and HIV-1 latently infected cells. (a) Overall experimental design of this study. (b) The genome-wide enrichments of histone modifications, H3K4me3 and H3K9ac, of chromosome 16 and LAT (linker for activation of T cells) locus were displayed on the UCSC genome browser as an example. (c) The HIV-1 integration site in NCHA2 cells was shown in the above panel. (d) The integrated HIV-1 genome itself was also subjected to enrichment analysis of histone H3K4me3. 1: Gag-Pol (336–4642), 2: Gag (336–1838), 3: Vif (4587–5165), 4: Vpr (5105–5396), 5: Tat (5377–7970), 6: Rev (5516–8199), 7: Vpu (5608–5856), 8: Env (5771–8341), 9: Nef (8343–8963).

linker for activation of T cells (LAT) gene locus showed dramatic decrease in NCHA1, NCHA2 and ACH2 cells (Fig. 1b). Apparently, there was no characteristic histone modification pattern at the integration sites of HIV-1 genome in NCAH1, NCAH2 and ACH2 cells as shown in Fig. 1c and Supplementary Fig. S2, http://links.lww.com/QAD/A523. The LTR regions near the 5'-end and 3'-end of the HIV-1 genome were marked by relatively high levels of H3K4me3 (Fig. 1d). Minor H3K4me3-enriched regions were detected but not associated specifically with promoters of HIV-1 genes. Similarly, the H3K9ac patterns also showed high level of enrichment in ACH2 but in NCHA1 and NCHA2 cells, not as high as in ACH2 (Supplementary Fig. S2c, http://links.lww.com/QAD/A523).

Identification of differentially enriched histone islands in HIV-1 latently infected cells

The Pearson correlation coefficients for H3K4me3 and H3K9ac between two cell lines were calculated by counting sequence reads within consecutive 200-bp genomic segments (Fig. 2a). When compared with A3.01 cells, the correlation coefficients of H3K4me3 were 0.76, 0.74 and 0.93 for NCHA1, NCHA2, and ACH2 cells, respectively. The coefficients between two latently infected cells were 0.96 for NCHA1 and NCHA2,

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0.77 for NCHA1 and ACH2, and 0.75 for NCHA2 and ACH2. The H3K9ac also gave the analogous range of correlation coefficients from 0.98 to 0.76.

The differential histone modification islands that were domains with dominantly enriched or depleted histone modification in the HIV-1 latency cells were defined by SICER 1.1 program. Among increased H3K4me3 islands in NCHA1, NCHA2 and ACH2, 451 of H3K4me3 islands were commonly upregulated in all HIV-1 latency cells. From decreased H3K4me3 islands, 387 of H3K4me3 islands were consistently downregulated. For H3K9ac islands, 962 islands were upregulated and 493 islands downregulated in all HIV-1 latency cells (Fig. 2b). The genomic distribution of differential histone islands was also analysed and most of islands were located in the promoter regions where the percentage of downregulated histone modification islands was greater than that of the upregulated ones (Fig. 2c). The percentages of upregulated islands are 49, 63 and 64% but the percentages of downregulated islands are increased to 77, 78 and 66% for NCHA1, NCHA2 and ACH2, respectively. The commonly upregulated H3K4me3 and H3K9ac islands occupied a higher percentage than differential islands detected in each of the HIV-1 latency cells (72% for H3K4me3, 61% for H3K9ac). Total of 1171 upregulated islands from 451 H3K4me3 islands and 962 H3K9ac islands common in all HIV-1 latency cells and 785 downregulated islands from 387 H3K4me3 and 493 H3K9ac islands were selected for further analysis to identify the transcriptional signalling pathway essential to HIV-1 latency (Fig. 2d).

Differential enrichment of histone modifications and changes of gene expression associated with CD4 signalling pathway

The genes possessing differential histone modification islands were sorted by their expression levels (Fig. 3a). Specifically, CD4 signalling pathway related genes were further examined regarding histone modification status and gene expression (Fig. 3b). The number of sequenced reads for two histone modifications around ± 1 kb regions of transcription start site (TSS) was counted within 200-bp bin. As consistent with our previous result [20], the gene expression levels, involved in CD4 signalling pathway such as CCR5 (chemokine receptor 5), CXCR4 [chemokine (C-X-C motif) receptor 4], LCK (lymphocyte-specific protein tyrosine kinase) and ZAP70 [Zeta-chain (TCR) associated protein kinase 70 kDa], were decreased in the HIV-1 latently infected cells, especially in NCHA1 and NCHA2. About half of 15 genes showed lower histone enrichment of H3K4me3 and H3K9ac in the HIV-1 latency cells (red boxes in Fig. 3b), which implied that these active chromatin markers were not deposited in the promoter regions, and consequently led to transcriptional downregulations. The histone modification profiles of CD4 gene loci obtained from ChIP-Seq experiment were illustrated (Fig. 3c). The ChIP-Seq peak patterns of both H3K4me3 and H3K9ac at the immediate downstream of CD4 TSS were also reproduced in the PCR result (Fig. 3d).

Identification of functional clusters implicated in genes downregulated by HIV-1 latency

Genes selected from Fig. 3a were subjected to identification of HIV-1 latency-related gene group. Using the STRING and the BioGRID databases, 558 genes with downregulated histone levels of H3K4me3 or H3K9ac in the HIV-1 latency cells than those in A3.01 cells were assembled into five significant gene clusters (Fig. 4a). The prominent gene ontology terms were cell surface receptor linked signalling pathway, response to chemical stimulus, cell death, protein import into nucleus, T cell activation, response to organic substance and regulation of molecular function (Supplementary Table S1, http://links.lww.com/QAD/A523). Focused on LAT, LCK, ITK (intracellular tyrosine kinase), SH3BP2 (SH3-domain binding protein 2), DGKζ (diglyceride kinase zeta) and SLA (Src-like adapter) in cluster 1, which showed greater than 1.5-fold down-regulation in the level of histone modifications, the interacting neighbouring genes were identified (Fig. 4b–4d). The individual gene expression levels were not always proportional to the enrichment of two active histone modifications, implying that other epigenetic factors should be involved in controlling the expression programme. Consistent with downregulation of H3K4me3 and H3K9ac levels in three HIV-1 latency cells, the expression of LCK and ITK was decreased. However, the expression level of LAT and SLA was decreased only in two types of cells and slightly increased in another cells. The functional significance of identified genes such as ZAP70 and LAT was confirmed in our previous experiments [20,25]. When the expression of ZAP70 was knock-downed by siRNA, the HIV-1 P24 viral antigen production was decreased. Reactivation of HIV-1 with treatment of phobol 12-myristic 13-acetate (PMA) in the HIV-1 latency cells induced LAT expression levels. These results support that identified essential genes are truly involved in the HIV-1 latency.

Identification of functional clusters implicated in genes upregulated by HIV-1 latency

By the STRING and the BioGRID database, 899 genes identified by upregulated H3K4me3 or H3K9ac islands in the HIV-1 latency cells gave rise to six clusters and were involved in regulation of cell cycle, regulation of cell proliferation, second-messenger mediated signalling and metabolic process (Fig. 5a and Supplementary Table S2, http://links.lww.com/QAD/A523). The centre of cluster 1 was occupied by CCND1, CCND2 (cyclin D1 and D2), CDKN1A, CDKN2A (cyclin-dependent kinase inhibitor 1A and 2A) and NPDC1 (neural proliferation, differentiation and control 1). Their interacting neighbor genes such as HDAC1, HDAC2, SMARCA4 (SWI/SNF-related, matrix-associated,

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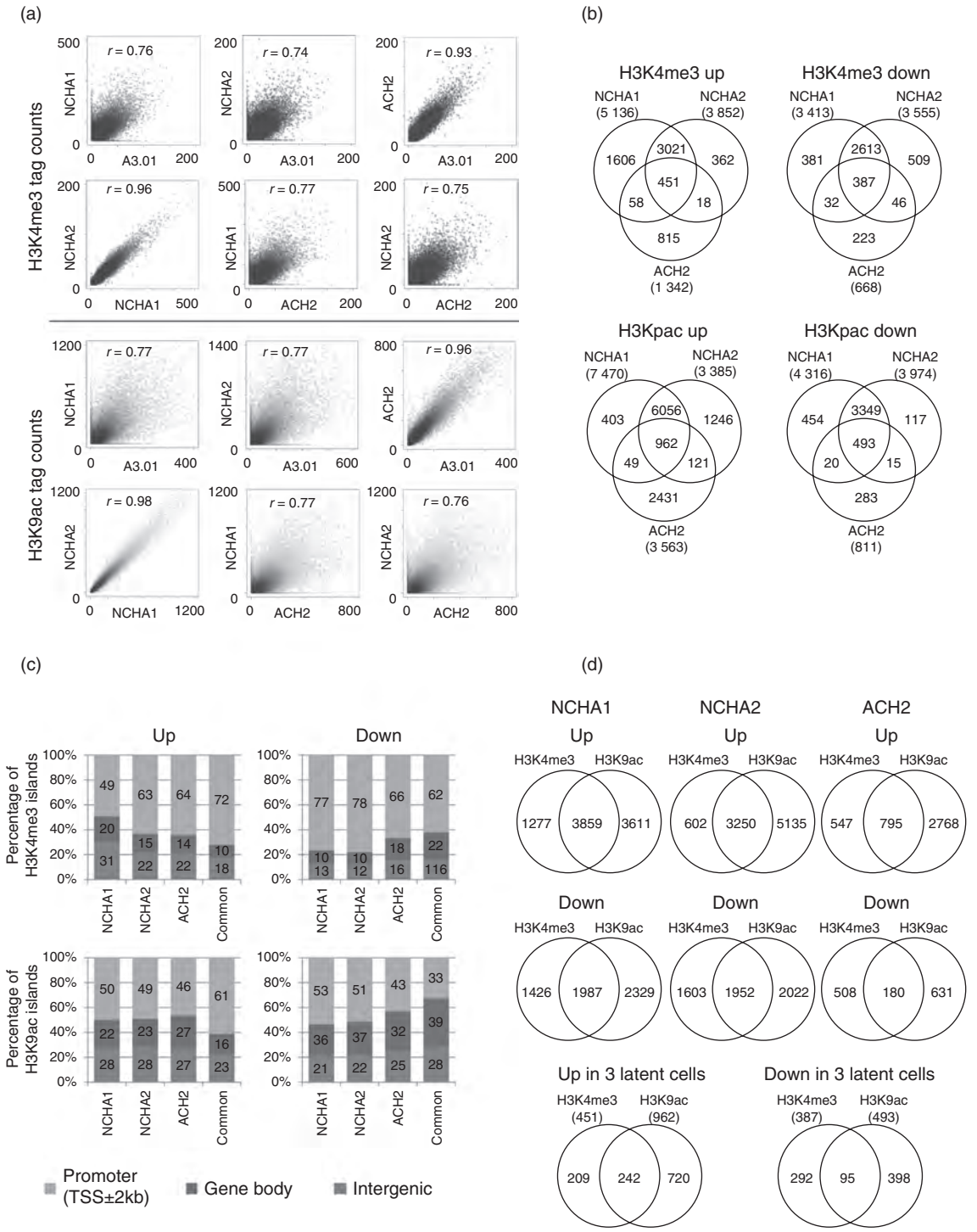

Fig. 2. Identification of differentially enriched histone modification islands in HIV-1 latently infected cells. (a) The genomic loci were divided into 200-bp windows and their histone modification levels were compared. The pair-wise correlation coefficients (*r*-values calculated by Pearson correlation coefficients) were shown in each graph. (b) Differentially enriched histone modification islands in HIV-1 latently infected cells were identified by SICER program with at least 1.5-fold difference. (c) The differential histone modification islands were distributed in intergenic, gene body and promoter (TSS ±2kb) regions. (d) The differential histone H3K4me3 and H3K9ac islands were compared with each other. At the bottom panel, the occurrence of two modification islands was examined.

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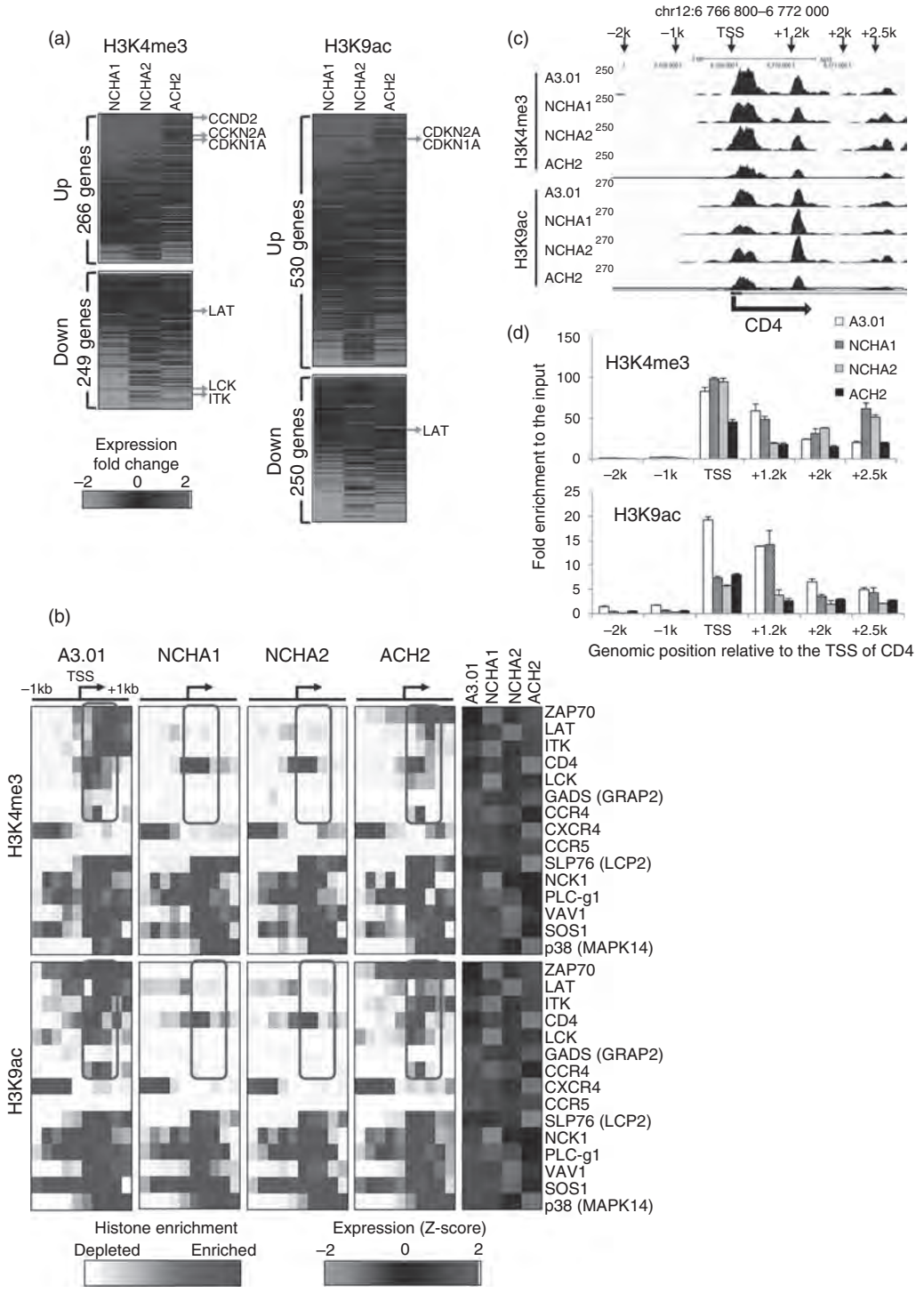

Fig. 3. Differential enrichment of histone modifications and changes of gene expression associated with CD4 signalling pathway. (a) Genes with differential enrichment of histone modifications were sorted according to their expression levels. These genes were selected only when they are occupied by differential histone islands in promoter and gene body regions. (b) Histones H3K4me3 and H3K9ac enrichment patterns of genes related with CD4 signalling pathway were analysed within ±1 kb regions from transcription start site (TSS). The genes in CD4 signalling pathway were selected from the KEGG pathway database. (c) The ChIP-Seq reads were counted at CD4 promoter region. (d) The histone modification enrichment was confirmed by quantitative PCR using PCR primer sets at designated positions. Genomic regions are shown on the x-axis and enrichment compared with total input DNA is indicated on the y-axis. The coordinates of loci are presented on the top. Data are graphed as mean ± SEM.

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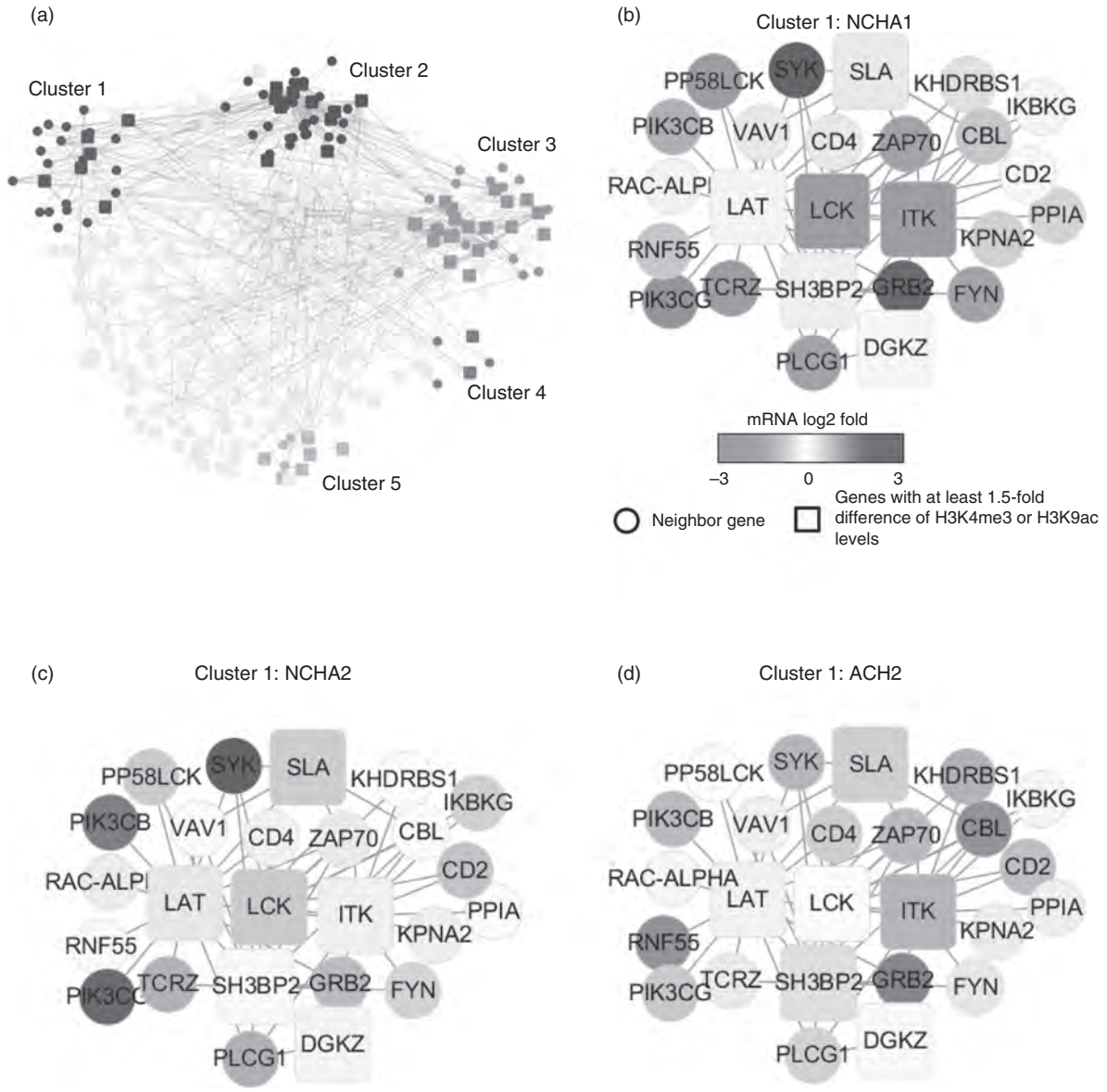

Fig. 4. Identification of functional clusters implicated in downregulated genes by HIV-1 latency. (a) Network analysis was conducted on genes with histone H3K4me3 and H3K9ac levels decreased in HIV-1 latency cells using two functional protein association databases (STRING and BioGRID). Node colours represent different clusters identified by MCODE. (b) The cluster 1 contained six genes with at least 1.5-fold decreased levels of H3K4me3 or H3K9ac and also their neighbour genes in NCHA1 cells. The gene expression levels were shown in light-dark grey. (c) The cluster 1 contained six genes with at least 1.5-fold decreased levels of H3K4me3 or H3K9ac and also their neighbour genes in NCHA2 cells. (d) The cluster 1 contained six genes with at least 1.5-fold decreased levels of H3K4me3 or H3K9ac and also their neighbour genes in ACH2 cells.

actin-dependent regulator of chromatin, subfamily a, member 4), SP1 (specificity protein 1), STAT3 (signal transducer and activator of transcription 3), RB1 (retinoblastoma 1), TP53 (tumour protein p53), c-MYC (v-myc avian myelocytomatosis viral oncogene) and c-JUN were identified and their expression levels were examined in NCHA1, NCHA2 and ACH2 (Fig. 5b–5d). The cell cycle regulatory proteins, CCND2, CDKN1A and CDKN2A, were overexpressed in NCHA1 and NCHA2. The neighbour genes' expression levels were not always positively related with the core cell cycle regulatory genes determined by differential histone modification islands. The discrepancies in examined histone modifications and gene expression levels could be caused by another regulatory effect of different chromatin structure between uninfected and HIV-1 latency cells.

Discussion

To understand the role of epigenetic regulation in establishment and maintenance of HIV-1 latency, the

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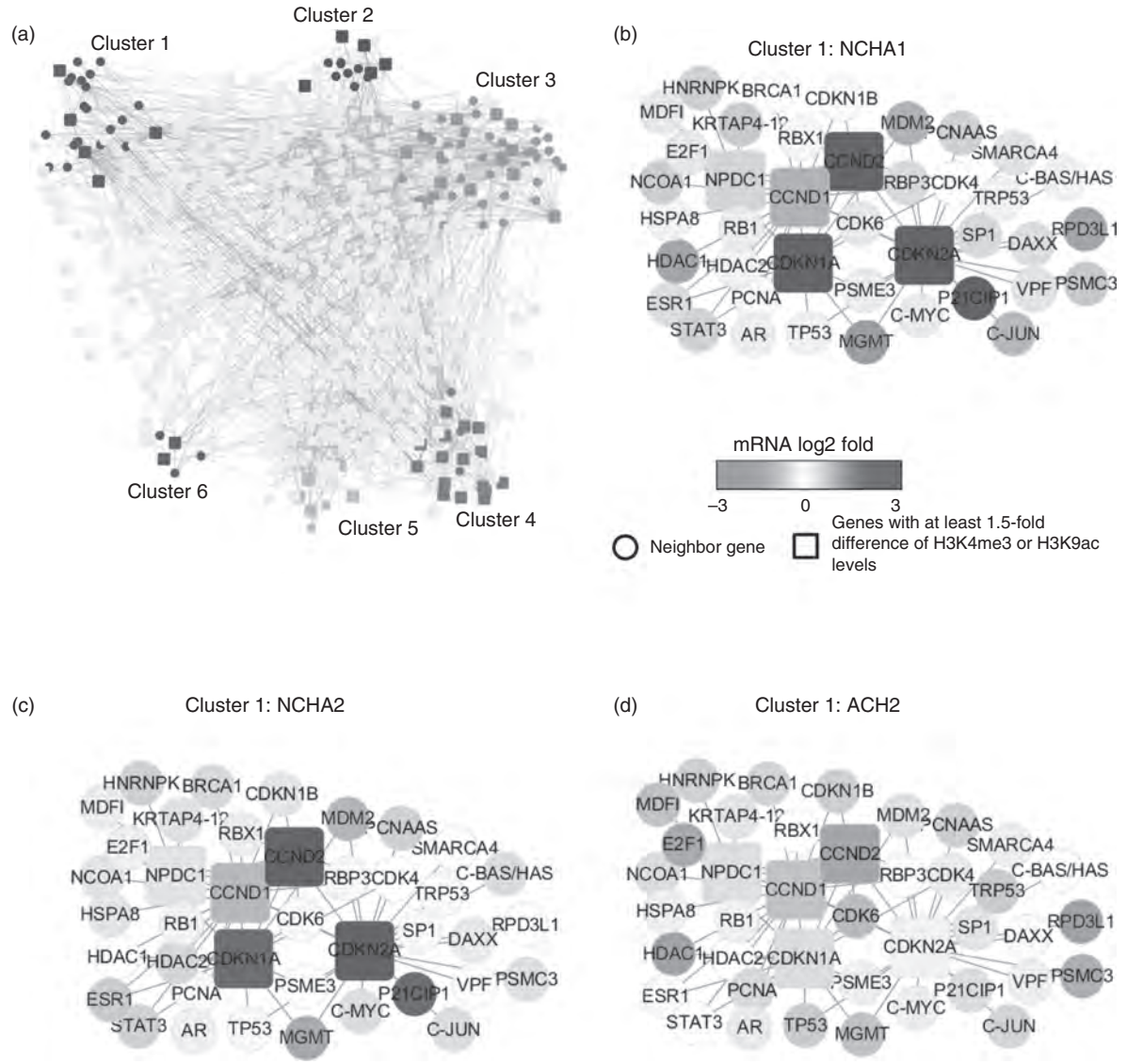

Fig. 5. Identification of functional clusters implicated in upregulated genes by HIV-1 latency. (a) Network analysis was conducted on genes with histone H3K4me3 and H3K9ac levels increased in HIV-1 latency cells using two functional protein association databases (STRING and BioGRID). Node colours represent different clusters identified by MCODE. (b) The cluster 1 contained five genes with at least 1.5-fold increased levels of H3K4me3 or H3K9ac and also their neighbour genes in NCHA1 cells. The gene expression levels were shown in light-dark grey. Grey colour nodes are not annotated genes in microarray data. (c) The cluster 1 contained five genes with at least 1.5-fold increased levels of H3K4me3 or H3K9ac and also their neighbour genes in NCHA2 cells. (d) The cluster 1 contained five genes with at least 1.5-fold increased levels of H3K4me3 or H3K9ac and also their neighbour genes in ACH2 cells.

genome-wide maps of histone modifications for H3K4me3 and H3K9ac were obtained in HIV-1 latently infected cell lines and uninfected A3.01 cells. The HIV-1 virus particles in our HIV-1 latency cell models were actively generated upon activation [25]. The overall histone modification patterns near HIV-1 integrated sites were not significantly different from those of control in this study. However, there were reports showing that the integration sites from activated T cells were highly associated with histone modification profiles of active genes, including H3 acetylation, H4 acetylation and H3K4 methylation [26,27]. The HIV-1 genome itself showed upregulation of H3K4me3 and H3K9ac levels near LTR regions. The integrated HIV-1 genome is known to be marked by H3K9me3, which is mostly accompanied by DNA methylation [19]. These observations suggest that both open chromatin markers such as H3K4me3 and H3K9ac, and repressive markers such as CpG methylation and H3K9me3 corporately maintain the HIV-1 latency state. In host cells, the overall enrichment of histone modification was not changed before and after HIV-1 integration. Some fraction of

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chromatin domains showing altered epigenetic features could be potentially important for establishment and maintenance of HIV-1 latency. The protein levels of four core histone proteins (H2A, H2B, H3 and H4) and HDAC Classes I and II were not changed in the latently HIV-1 infected cells [19]. Therefore, the target-specific changes of histone modifications in transcriptional regulatory elements or latency-related genes could either be mediated directly by low level of viral proteins produced by provirus or occur when the HIV-1 latent cells were established [28]. In both cases, the diversity of chromatin status in host cells induced by HIV-1 infection could play a crucial role in the viral latency. Apart from our observations of target-specific differences in the HIV-1 latency models, a couple of groups have showed that cell line dependent and model-specific discrepancy should exist and differentially affect the mechanism inducing HIV-1 latency [29,30]. The differentially enriched H3K4me3 and H3K9ac peaks were detected by SICER program and the genomic colocalization of peaks among NCHA1, NCHA2 and ACH2 cells was examined. The preference of histone modification enrichment to the promoter regions reflects that the HIV-1 latency-related genes might be coregulated by histone methylation and acetylation complementarily.

To see the epigenetic linkage to the gene activation, the levels of gene expression were integrated into the enrichment profiles of histone methylation and acetylation. Generally, the gene expression was positively correlated with the level of histone H3K4me3 and H3K9ac even though there were some minor discrepancies as expected. The CD4 receptor and its downstream genes possessed dramatically depleted levels of H3K4me3 and H3K9ac, suggesting that the HIV-1 latency is sustained by the epigenetic repression of $CD4^+$ downstream signalling molecules. Interestingly, the levels of H3K4me3 in the CD4 promoter maintained before and after HIV-1 infection led to latency, but the levels of H3K9ac were reduced in latently HIV-1 infected cells. But the protein level of CD4 gene examined was dramatically decreased in the HIV-1 latently infected cells, which implies that the protein amount is regulated by the translational control [20].

Using gene sets with differentially enriched islands of histone modifications, direct and indirect interactions among potential viral latency-related genes were examined. The epigenetically downregulated genes belonged mainly to signalling pathway, immune system process and cell death. It is noteworthy that HIV-1 latency induced epigenetic repression with which genes seemed to be involved in cellular signalling pathway to escape or evade immune response. The genes with increased histone modification levels in latency cells were linked to diverse cellular processes including cell cycle regulation and metabolic process. As potential candidates for viral latency markers, cell cycle regulating genes such as *CCND2*, *CDKN1A* and *CDKN2A* were of special interest. Their H3K4me3 and H3K9ac levels were upregulated and the expression levels were highly induced. The cell cycle might be modulated to be favourable for the maintenance of HIV-1 latency. Therefore, the host cells seem to be epigenetically adapted to harbour the viral genome and repress the viral gene expression at or below a threshold not detected by conventional test.

The host cellular transcription factors were also known to be important for the integrated HIV-1 activation, for example, nuclear factor of kappa-light-chain-enhancer of activated B cells (NF-κB), nuclear factor of activated T cells (NFAT) and specificity protein 1 (SP1) [31]. Among these transcriptional activators, a NFAT member, NFATC4, showed dramatically abolished levels of H3K4me3 and H3K9ac in NCHA1 and NCAH2 cells, which might explain the difference between NCHA and ACH2 cells even though both were derived from the same origin, A3.01 cells. The histone modifications of *NFATC2* gene were enriched at the different positions near the known TSS between NCHA and ACH2 cells, demonstrating that the transcription start might occur at different sites. This provides an evidence that the different epigenetic transitions could determine the cell type specificity.

Here, we performed the first genome-wide ChIP-Seq analysis using HIV-1 latency cells. The differential enrichment patterns helped us to define potential effector genes leading to the viral latency. In summary, first, there was no characteristic histone modification pattern at the HIV-1 genome integration site. Instead, the HIV-1 genome itself was associated with histone methylation and acetylation discretely. Second, apparently, the overall histone modification profiles of latency cells were similar to that of uninfected $CD4^+$ cells. However, the differential histone modification islands were detected at specific chromatin domains and genes colocalized with these islands could be regarded as potential candidates responsible for the maintenance of HIV-1 latency. Third, integrated with gene expression data, differentially histone modified genes were used for the generation of interaction networks, which defined several functional network clusters such as CD4 signalling, cell death, cell cycle and metabolic process. Therefore, our genomic profiling of epigenetic patterns and network analysis in HIV-1 latency cells could provide new insight into host cellular epigenetic mechanisms maintaining HIV-1 latency and are applicable to develop new diagnostic markers controlled epigenetically in HIV-1 latency cells. Furthermore, more accurate approaches to complete understanding of the mechanism inducing HIV-1 latency would be made if genome-wide epigenetic information such as repressive histone modifications and DNA methylation were collected for integrative interpretation.

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S.S.K., B.S.C. and T.Y.R. conceived and designed the experiments. J.P. and C.H.L. performed the experiments. J.P. and S.H. analysed the data. J.P., B.S.C. and T.Y.R. wrote the manuscript.

Conflicts of interest

There are no conflicts of interest.

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