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Epigenetic transcriptional repression of cellular genes by a viral SET protein

Shiraz Mujtaba¹, Karishma L. Manzur¹, James R. Gurnon², Ming Kang², James L. Van Etten², and Ming-Ming Zhou¹

¹ Department of Structural and Chemical Biology, Mount Sinai School of Medicine, New York University, 1425 Madison Avenue, Box 1677, New York, NY 10029, USA

² Department of Plant Pathology and Nebraska Center for Virology, University of Nebraska, Lincoln, Nebraska 68583, USA

Abstract

Viruses recruit host proteins to secure viral genome maintenance and replication. However, whether they modify host histones directly to interfere with chromatin-based transcription is unknown. Here we report that *Paramecium bursaria* chlorella virus 1 (PBCV-1) encodes a functional SET domain histone Lys methyltransferase (HKMTase) termed vSET, which is linked to rapid inhibition of host transcription after viral infection. We show that vSET is packaged in the PBCV-1 virion, and that it contains a nuclear localization signal and probably represses host transcription by methylating histone H3 at Lys 27 (H3K27), a modification known to trigger gene silencing in eukaryotes. We also show that vSET induces cell accumulation at the G2/M phase by recruiting the Polycomb repressive complex CBX8 to the methylated H3K27 site in a heterologous system. vSET-like proteins that have H3K27 methylation activity are conserved in chlorella viruses. Our findings suggest a viral mechanism to repress gene transcription by direct modification of chromatin by PBCV-1 vSET.

Introduction

Dynamic control of H3K27 methylation^{1, 2, 3} is central to gene silencing in development,^{4, 5, 6, 7} X-inactivation,⁸ stem-cell pluripotency,^{9, 10} cancer,^{11, 12} and inflammation.¹³ So far, only the mammalian EZH2 of the Polycomb repressive complex 2 (PRC2) is known to catalyze this important epigenetic histone modification.^{14, 15} H3K27 methylation as a viral strategy to regulate host transcription has not been reported. Viruses recruit chromatin-associated transcription proteins for maintenance of their genome and replication. For example, the papillomavirus E2 protein binds Brd4, tethering the viral genome to mitotic chromosomes to ensure persistence of viral episomes in infected cells.¹⁶ The adenovirus E1A protein interacts with the retinoblastoma protein p130 to silence E2F-responsive genes in quiescent cells.¹⁷ The HIV *trans*-activator Tat recruits histone acetyltransferases for chromatin acetylation and remodeling for transcriptional activation of the provirus.^{18, 19} Despite the ability of viruses to recruit cellular histone-modifying enzymes to facilitate viral transcription, no viral enzymes have been identified that directly modify host histones and modulate transcription. Of the large family of SET domain HKMTases,^{20, 21} a small subset of SET proteins is encoded by viruses

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Corresponding authors — Ming-Ming Zhou, ming-ming.zhou@mssm.edu; James L. Van Etten jvanetten@unlnotes.unl.edu.
Author contributions

S. M. characterized vSET function in mammalian cells; K. L. M. performed the *in vitro* histone Lys methylation study of vSET and vSET-like proteins; J. R. G. and M. K. conducted the study of PBCV-1 infection of *Chlorella* cells and *vset* gene probing of chlorella viruses; M.-M. Z. and J. L. V. E. directed the project and all authors contributed to the preparation of the manuscript.

and bacteria.^{22, 23} The cellular functions of these proteins are unknown but their presence in viruses raises questions about whether they are able to modify histones and, if so, how this would affect the host cells. To address these questions, we have characterized SET domain proteins from chlorella viruses.

We first focused on the function of vSET, a SET domain protein encoded by PBCV-1, which is a large double-stranded DNA virus that replicates in the unicellular, eukaryotic green algae *Chlorella* NC64A.²⁴ The 330-kb PBCV-1 genome has 366 non-overlapping protein-encoding genes and 11 tRNA genes.²⁴ Despite its large genome, PBCV-1 lacks an RNA polymerase gene, suggesting that it is dependent on the host transcription machinery. Notably, PBCV-1 infection causes a rapid inhibition of total RNA synthesis in *Chlorella* cells, achieving 60–80% inhibition by 20–40 min after infection (Figure 1a). PBCV-1 transcripts can be detected 5–10 min after infection, indicating that transition from host to virus transcription occurs rapidly.²⁴ These results led us to postulate that a factor(s) packaged in the virion may be responsible for this rapid inhibition of host RNA synthesis. vSET is a possible candidate as it methylates H3K27, which has been linked to PRC-mediated gene silencing in eukaryotes.^{1, 2, 3}

To investigate vSET function, we generated an anti-vSET antibody to immunoprecipitate vSET from PBCV-1 infected *Chlorella* cell extracts. vSET is present in mature virions and a small amount was detected as early as 10 min after infection of *Chlorella* cells, presumably, released from PBCV-1 (Figure 1b). Expression of vSET was markedly enhanced 120 min after infection. Pretreatment of isolated virus particles with trypsin, which removes any exogenous vSET contamination on the virus surface, had no effect on viral infectivity or the amount of vSET detected in PBCV-1 particles (Figure 1c). The presence of vSET in PBCV-1 virions was confirmed by Q-TOF mass spectrometry (data not shown). By comparing western blots from a known number of virus particles with blots containing different concentrations of vSET, we estimated that four vSET molecules are packaged per virion (data not shown). Furthermore, vSET in the disrupted virions possess H3K27 methylation activity (Figure 1d), which indicates that PBCV-1 virions contain active vSET.

To detect *vset* transcripts during PBCV-1 replication, RNA from infected *Chlorella* cells was hybridized with the *vset* gene. The probe hybridized to an RNA of about 1.8 kb, beginning approximately 60 min after infection (Figure 1e). This transcript is larger than expected for a 119-residue protein and may be a bicistronic transcript encoding the co-linear genes *a609l* (approximately 1.2 kb) and *a612l* (approximately 0.4 kb). The latter gene encodes vSET. This result is consistent with vSET expression occurring about 120 min after infection (Figure 1b).²⁴

vSET adopts a core β -barrel structure,^{25, 26} a fold that is conserved in eukaryotic SET domain HKMTases.²¹ To test whether vSET functions as a *bona fide* HKMTase in *Chlorella* cells, we cloned histone H3 from *Chlorella* NC64A and found that it has high sequence identity with human H3 (Figure 2a). We measured vSET methylation activity using both the nucleosome and individual core histones and confirmed its activity on full-length free H3 and H3 within the nucleosome, but not on other core histones (Figure 2b). To identify vSET methylation site (s) in H3, we expressed a series of GST–H3 peptides (residues 1–57) with Arg substitutions at Lys methylation sites: Lys 4, Lys 9, Lys 27, Lys 36 and Lys 37 (Supplementary Information, Table S2). The purified GST–H3 peptides produced two bands when subjected to SDS–PAGE, which corresponded to an intact H3 peptide of residues 1–57 (plus SGRIVTD from the expression vector) and a truncated H3 of residues 1–55, as confirmed by MALDI-TOF mass spectrometry (Figure 2c). The methylation assay showed that vSET only methylated H3 with an intact Lys 27 site (Figure 2c). Using site- and state-specific antibodies against Lys-methylated histone H3 (Supplementary Information, Figure S1a, b), we confirmed that vSET catalyses H3K27 dimethylation (H3K27me₂) and, to a much less extent, mono- and

trimethylation. No methylation occurred at Lys 4, Lys 9 or Lys 36 sites in H3 (Figure 2d). These results are consistent with the kinetic analyses of vSET methylation of H3K27 peptide, showing that monomethylation and mono- to dimethylation are very rapid, whereas di- to trimethylation is about 10 times slower than mono- to dimethylation (Supplementary Information, Figure S1c, d). Finally, we confirmed that histone H3 is the major substrate of vSET in an *in vitro* methylation assay using heat-inactivated extracts from HeLa cells depleted of EZH2 by RNA interference (RNAi) and uninfected *Chlorella* cells (Supplementary Information, Figure S2a–d). Collectively, these results establish that vSET is a H3K27-specific methyltransferase.

We next assessed the effects of PBCV-1 infection on H3K27 methylation in *Chlorella* cells. Western blot analysis showed that H3K27me1 was unchanged, whereas H3K27me2 increased markedly as early as 30 min after infection, and H3K27me3 was slightly enhanced (Figure 2e). This observation correlates with *in vitro* vSET state-specific methylation activity at H3K27 (Figure 2d). Immunofluorescence showed that vSET was present in the nucleus of *Chlorella* cells after PBCV-1 infection (Figure 2f), and vSET colocalized with the H3K27me2 (Figure 2g). These results confirm that vSET is a *bona fide* HKMTase and causes increased H3K27me2 in *Chlorella* cells during virus replication.

Technical difficulties in manipulating the PBCV-1 genome and genetic transformation of the host *Chlorella* cells prevented us from conducting a cellular study of vSET in its native host. But given the highly conserved H3 sequence and H3K27 methylation in eukaryotes, we reasoned that mammalian cells could serve as a suitable model system to study the biological function of vSET, which has enzymatic activity similar to that of EZH2 (ref. ³).

If vSET participates in early suppression of host transcription, it must move to the host nucleus on viral infection. To assess whether a solvent-exposed KRMR motif in vSET (residues 85–88, see Figure 5b) functions as a nuclear localization signal (NLS), NIH-3T3 cells were transfected with a GFP–vSET or a triple mutant in which KR(M)R was changed to AA(M)A. Fluorescence microscopy showed that wild-type GFP–vSET was localized to the nucleus, whereas the triple mutant remained in the cytoplasm (Figure 3a), confirming that the KR(M)R motif is an NLS in vSET. vSET H3K27 methylation activity was not compromised in this mutant (Supplementary Information, Figure S3a–c). A classical NLS sequence is absent in most eukaryotic SET domains, except for yeast SET1, which has an RRIV motif located at a site analogous to the KRMR motif in vSET.

H3K27 methylation in eukaryotes is catalyzed by the EZH2 of PRC2 and recruits the PRC1 complex, leading to gene silencing.^{1, 14} To investigate whether vSET can methylate H3K27 in mammalian cells, we knocked down *EZH2* using the RNAi method in HeLa cells. When treated with *EZH2*-specific siRNA, *EZH2* expression was reduced by more than 90%, compared with control cells (Figure 3b). *EZH2* knockdown caused a reduction of SUZ12, a component of PRC2, as well as near complete loss of H3K27me2 and H3K27me3, and a slight reduction of H3K27me1. No significant changes were observed with RING1 of PRC1, H3K4, H3K9, and H3K36 dimethylation. These results confirm that EZH2 is responsible for H3K27 di- and trimethylation in HeLa cells. vSET treatment of the nuclear extract from *EZH2* knockdown HeLa cells restored H3K27me2 and H3K27me3, and resulted in a slight reduction of H3K27me1, possibly because of its conversion to H3K27me2 and H3K27me3 by vSET (Figure 3c). Similar results were obtained when tetracycline was used to induce vSET expression in the *EZH2* knockdown HeLa cells (Figure 3d). Thus, these results demonstrate that vSET can mimic EZH2 to methylate H3K27 in mammalian cells.

We next examined the effect of vSET methylation activity on gene transcription in a luciferase-based reporter assay. In this assay, we first transfected the 293T cells with vSET fused to a

Gal4 DNA-binding domain (DBD) and a luciferase reporter gene encoding HSV-*tk* promoter (plus Gal4 DNA-binding sites). Wild-type vSET repressed luciferase gene expression by about 95%, whereas the inactive mutants Y105A or Y105F had no vSET gene-silencing ability (Figure 4a). Notably, expression of a Flag- or HA-tagged vSET in the 293T cells caused repression of approximately 60%, compared with the vectors containing only Flag- or HA-tag, or inactive mutant Y105F (Figure 4b). The lesser repressive activity of Flag- or HA-tagged vSET on host transcription, compared with that of vSET–Gal4–DBD may be due, at least in part, to the presence of H3K27ac and/or H3S28p in host cells (Figure 2e), which would prevent H3K27 methylation by vSET²⁶ (Figure 5c). In a luciferase-based assay, we also observed that Flag–vSET co-transfected with HIV-Tat in 293T cells (Supplementary Information, Figure S4a) or HeLa cells (Supplementary Information, Figure S4b) repressed transcription of the HIV *LTR* promoter,^{18, 19} confirming repression of chromatin-mediated transcription by vSET.

To examine the biological consequence of H3K27 methylation by vSET, we assessed Polycomb group protein occupation at the H3K27me site on tetracycline-induction of vSET in the *EZH2* knockdown HeLa cells. Although their protein levels were unaltered, vSET induction enhanced occupation of the PRC1 complex protein CBX8, but not CBX4 and CBX7 at the H3K27me2 and H3K27me3 sites (Figure 4c). This CBX8/H3K27me2/3 association is probably facilitated by the chromodomain of CBX8, which binds methyl-Lysine.^{27, 28} Interestingly, a search of the SMART database reveals that a virus PBCV-1 encoded protein (accession number NP_049022) seems to contain the chromodomain as in CBX8. Therefore, we conclude that vSET induces transcription repression by H3K27me, and facilitates accumulation of the PRC1 complex at the H3K27me site.

Using a quantitative RT–PCR assay, we examined whether vSET methylation activity can modulate Polycomb target genes.²⁹ The analysis was performed in HeLa cells on five representative Polycomb target genes *HOXA7*, *HOXA9*, *HOXB9*, *HOXD8* and *Hey1*, and three general housekeeping genes *GAPDH*, *RPS* and *tubulin*. The effect on the three housekeeping genes was negligible; however *EZH2* knockdown by siRNA caused a 2.5-fold increase in transcription of *HOXA9*, *HOXB9*, *HOXD8* and *Hey1*, and a 7.5-fold increase in *HOXA7* expression (Figure 4d). The increase in the Polycomb target gene expression was reversed by tetracycline-induced expression of vSET to a level similar to or slightly higher than that of the control cells. Moreover, vSET induction in the normal HeLa cells did not alter the expression level of the five HOX genes when compared to the controls.

We next characterized vSET-dependent transcriptional repression of the *HOXA7* promoter in luciferase gene expression and chromatin immunoprecipitation (ChIP) assays. Induction of vSET effectively repressed luciferase expression at the *HOXA7* promoter in both *EZH2* siRNA-treated and untreated HeLa cells (Figure 4e; Supplementary Information, Figure S4c). The ChIP analysis confirmed that vSET specifically di- and tri-methylates H3K27 at the *HOXA7* promoter, which recruits CBX8 of the PRC1 and causes gene repression (Figure 4f). We obtained similar results for the characterization of vSET-mediated transcription repression of HIV-Tat-dependent *LTR* promoter at the nucleosome Nuc2 (Supplementary Information, Figure S4d, e). These results establish that vSET can modulate transcription of Polycomb target genes through its H3K27 methylation activity.

We then examined the effect of vSET expression on the cell cycle. Flow cytometric analysis indicated that vSET caused cell accumulation at the G2/M phase in transiently transfected NIH-3T3 cells, whereas active-site mutants E100A and Y105A were less effective (Figure 4g). The vSET effect on the G2/M phase in the cell-cycle was confirmed in the HeLa cells that were stably co-transfected with vSET in a tetracycline-controlled expression vector (Figure 4h). Collectively, our data indicate that vSET H3K27 methylation activity results in the recruitment

of the Polycomb repressive complexes and modulation of their target genes, leading to transcription repression and accumulation of cells at the G2/M phase.

To determine whether vSET is unique to viral PBCV-1 or is present in other chlorella viruses, we hybridized genomic DNA from 36 other chlorella viruses with a *vset* gene probe. Thirty-one of these viruses hybridized to the probe (Figure 5a). The probe did not hybridize to DNA from 5 viruses or the host DNA. However, a *vset* gene was identified in 3 of these 5 viruses using low stringency hybridization. The vSET proteins from these three viruses, NY-2A, NY-2B and MA-1D, have 85% amino acid similarity to PBCV-1 vSET, including the conserved active-site residues in the SET domain family (Figure 5b). We suspect the remaining two viruses, NYS-1 and IL-5-2S1, also have a vSET gene but we did not examine them further.

Using histones H2A, H2B, H3 and H4 as substrates, we established that the SET proteins from these three viruses selectively methylate H3 (data not shown). As with vSET, these viral SET proteins only methylated a H3 peptide containing residues 15–30 but not one containing H3 residues 1–20 (Figure 5c). They showed little methyltransferase activity towards H3 peptide that was previously dimethylated at Lys 27 or phosphorylated at Ser 28, suggesting a preference for the H3K27 site. The selective methylation at H3K27 was confirmed using GST–H3 (residues 1–57), in which these viral SET proteins had robust methylation activity on wild-type H3 but diminished activity on the K27R mutant (Figure 5d). These results establish that SET proteins from the chlorella viruses possess selective H3K27 methylation activity, and suggest that vSET function in host chromatin modification and transcription repression is conserved among the chlorella viruses.

Histone modifications provide epigenetic control of gene transcription. Viruses recruit host proteins to either integrate their genomes into host chromatin^{16, 17} or to facilitate viral transcription and replication.^{18, 19} Here, we show for the first time that a virus regulates the host transcriptional machinery through direct modifications of chromatin. Our study reveals that vSET is packaged in the PBCV-1 virion and can directly methylate host H3K27, an important epigenetic modification in eukaryotes that is functionally linked to *Hox* gene silencing,^{4, 5, 30, 31} X-inactivation,⁸ and stem-cell pluripotency.^{9, 10} Our results establish that vSET H3K27 methylation promotes the recruitment of the PRC1 complex through a molecular interaction between CBX8 and di- and/or trimethylated H3K27. This interaction results in modulation of Polycomb target genes, transcription repression and cell accumulation at the G2/M phase of the cell cycle in continuously dividing cells. Our study supports the view that vSET proteins, which are encoded by all chlorella viruses tested, function to repress transcription in infected chlorella cells. Collectively, our findings suggest a unique and powerful mechanism by which some viruses commandeer the host transcription machinery through direct modifications of histones and in this way govern host chromatin-mediated cellular processes.

Methods

Growth of cells and viruses, and preparation of *Chlorella* cell extracts

Growth of *Chlorella* NC64A on MBBM medium, plaque assay, production of viruses and isolation of viral DNA were carried out as described previously.³² Actively growing *Chlorella* NC64A cells, concentrated to 2×10^8 cells ml⁻¹, were mock-infected or infected with PBCV-1 at a multiplicity of infection (MOI) of 5 for the indicated times. Samples were pelleted, disrupted in lysis buffer by vortexing with glass beads and clarified by centrifugation. Purified PBCV-1 virus particles (200 μ l of 6 mg ml⁻¹ total virus) were treated with 200 U ml⁻¹ sequencing-grade modified trypsin (Promega) before further purification.

RNA analysis of infected *Chlorella*

Actively growing *Chlorella* NC64A cells ($1-2 \times 10^7$ ml⁻¹) were incubated with ³H-adenine (5 μl ml⁻¹, 37 MBq ml⁻¹) for 10 min and centrifuged. Cells were then resuspended in 50 mM Tris-HCl, pH 7.8, (5×10^7 cells ml⁻¹) containing PBCV-1(MOI, 5). Samples of 1 ml were collected at the appropriate times and mixed with equal volumes of ice-cold 10% trichloroacetic acid (TCA). Cells were collected on glass filters, washed three times with 5% TCA, once with 80% ethanol and dried. RNA was hydrolyzed by placing the filters in 5 ml of NaOH (0.5 M) overnight at 37 °C, collected by filtering through a fresh filter, neutralized with TCA, and analyzed by scintillation counting. Total RNA was isolated from PBCV-1 infected *Chlorella* cells at various times after viral infection using the Trizol reagent (Invitrogen). Total RNA was electrophoresed and hybridized with a ³²P-labelled gene probe using a random-primer DNA labeling kit (Invitrogen). Viral DNAs used for dot blots were denatured, applied to nylon membranes, fixed by UV cross-linking and hybridized with the gene probe used for the northern analysis.

DNA cloning, protein purification and HKMTase assays

Full-length histone H3 from *Chlorella* NC64A was cloned with degenerate primers based on H3 from *Chlamydomonas reinhardtii*. The SET gene was isolated from chlorella viruses MA-1D, NY-2A and NY-2B using an established protocol.³³ The DNA bands that hybridized weakly with the PBCV-1 vSET probe were excised from the gel, recovered with a QIAEX II gel extraction kit (Qiagen) and cloned into pGEM-7Zf⁺. Primers were designed according to the vSET homologues identified from these hybridized DNA fragments. The vSET homologues from these three viruses were amplified by PCR and subcloned into pET-15b (Novagen). All DNA constructs and vSET mutants prepared using the QuikChange mutagenesis kit (Stratagene) were confirmed by DNA sequencing. Recombinant proteins were expressed in *Escherichia coli* strain BL21. The *in vitro* HKMTase reaction was performed by following methyl transfer from *S*-adenosyl-¹⁴C-methyl-L-methionine (Amersham) to histone peptide or nucleosome substrates, or by mass spectroscopy analysis of the histone peptide substrates.²⁶ Upstream and downstream primer sequences used for histone H3 are 5'-ATGGCCCGCACCAAG and 5'-TTAGGCGCGCTCGCC, respectively.

Western blot and histone immunoprecipitation analyses

PBCV-1 infected *Chlorella* cells were collected at various times after infection. Western blot analysis of the immunoprecipitated protein was performed using anti-vSET antibodies (Supplementary Information, Table S1), generated in rabbits against recombinant vSET (Covance). Histone immunoprecipitation was performed on *Chlorella* cells collected at 0–120 min after PBCV-1 infection. The assay was carried out using antibodies against histone H3, H3K27me1, H3K27me2, H3K27me3, H3K27ac, and H3S28p (Millipore) with cells cross-linked with paraformaldehyde and disrupted by sonication. To assess the PRC proteins at H3K27, HeLa cells stably transfected with vSET were induced with tetracycline, subjected to cross-linking and immunoprecipitated using antibodies against EZH2 and Polycomb complex proteins, RING1, SUZ12, CBX4, CBX7, and CBX8, followed by western blot analyses using anti-H3 antibodies (see Information, Supplementary Table S1).

Nuclear localization and co-localization

vSET nuclear localization was analyzed in NIH-3T3 cells transiently transfected with a pEGFP-N1 vector (Clontech) that encodes a C-terminal GFP fusion to vSET. The analysis in chlorella cells include fixation, permeabilization, and immunofluorescence microscopy. Typically, the exponentially growing chlorella cells were collected and suspended in 2% paraformaldehyde in PBS buffer of pH 7.4 at 4 °C for 6 h. The fixed cells were pelleted by centrifugation, resuspended in 5 ml chilled methanol, washed with PBS, spotted onto a slide

and air-dried. Cells that adhered to the slides were incubated for 15 min at 4 °C in DMSO (0.5% vol/vol in PBS). For fluorescence microscopy analysis, cells were blocked with 0.5% bovine serum albumin in PBS and incubated with anti-vSET and anti-histone antibodies (Abcam) for 1 h at 25 °C. Cells were washed with PBS and incubated with secondary fluorescein-labeled antibody (Alexa 488), mounted on a coverslip and analyzed using a Zeiss Axioplan2 microscope. For the colocalization assay, cells were stained for rabbit polyclonal anti-vSET and anti-rat monoclonal H3K27me2 antibodies. The respective immunocomplexes were detected using Alexa488 and 594 (Molecular Probes).

Transcription reporter assay and cell-cycle analysis

A luciferase reporter gene transcription assay was performed as described previously³⁴ (see Supplementary Information, Methods). Briefly, 293T cells were co-transfected with 2 µg of pcDNA3-Gal4-DBD-vSET (or the mutants) and 1 µg of HSV-*tk*-promoter plus Gal4 binding site and luciferase gene constructs. After 48 h, cells were lysed and assayed for luciferase activity using the Stop-Glo luciferase assay system (Promega). A luciferase assay on *HOX7A* promoter was also performed after co-transfection with *Renilla* luciferase in HeLa cells with or without *EZH2* knockdown by siRNA, as described below. Each assay was performed in duplicate and repeated five times. The vSET effect on the cell cycle was analyzed in NIH-3T3 cells transiently transfected with vSET in a pCMV-tag2B vector (Stratagene) and a Us9-GFP encoding plasmid, or HeLa cells stably co-transfected with vSET in a tetracycline-controlled vector pcDNA4/TO (Invitrogen) and the Us9-GFP plasmid. Post-transfection cells were treated with tetracycline (1 µg ml⁻¹), collected after 24 h by trypsinization, washed with PBS and fixed in chilled 70% ethanol in PBS. One hour before acquiring the data, cells were washed again with PBS and stained with propidium iodide. Cell-cycle analysis was performed with a Calibur flow cytometer (Becton Dickinson) after GFP and propidium iodide staining.

EZH2 RNAi knockdown, quantitative RT-PCR and ChIP analyses

EZH2 knockdown by RNAi was performed in HeLa cells that were transfected with target-specific and smart-pool siRNAs (Dharmacon) according to manufacturer's instructions (Supplementary Information, Table S3). *EZH2* RNAi knockdown was evaluated 72 h after transfection by western blot analysis using an anti-*EZH2* antibody (Cell Signaling). Methylation states of Lys 4, Lys 9, Lys 27, and Lys 36 in histone H3 were evaluated using various antibodies obtained from Millipore and Abcam. For RT-PCR analysis, RNA was extracted using RNAeasy kit (Qiagen) from HeLa cells that were transfected with *EZH2* siRNA and/or vSET. The primers for the select Polycomb target genes and house-keeping genes for analysis were designed based on the published sequences.³⁵ cDNA was generated by RT-PCR using the affinity script from Stratagene. Reactions were determined using the SYBR Green I detection chemistry system (Applied Biosystems) with an ABI Prism 7300 Sequence Detection System. Chromatin immunoprecipitation (ChIP) analysis was performed for the *HOXA7* gene using the EZ-ChIP kit (Millipore) following the manufacturer's instruction.¹³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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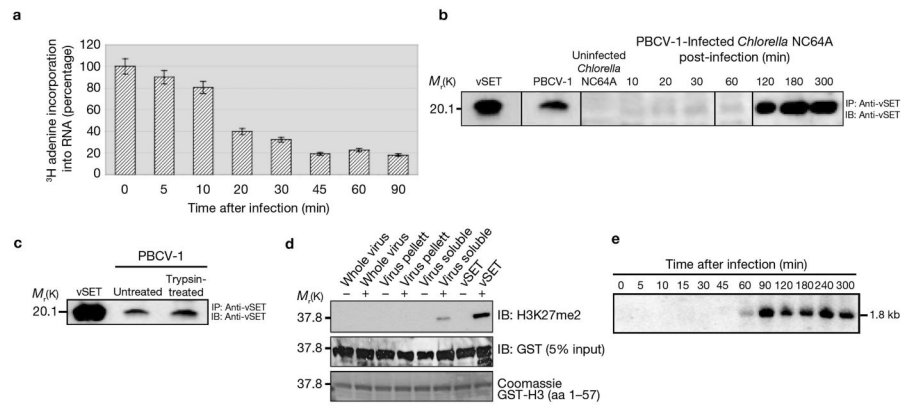


Figure 1. Presence of vSET in PBCV-1 virions

(a) Inhibition of host transcription by virus infection. Incorporation of ³H-adenine into RNA in PBCV-1 infected *Chlorella* NC64A cells, compared with mock-infected cells. The data are mean ± s.d. of triplicate assays. (b) Detection of vSET in PBCV-1 virions and virus-infected *Chlorella* cells by immunoprecipitation (IP) and then immunoblotting (IB) with anti-vSET antibodies. (c) The presence of vSET in PBCV-1 virions is not due to external contamination, as demonstrated by trypsin pre-treatment of purified PBCV-1 virus particles. (d) H3K27 methylation activity of vSET in the lysate from disrupted PBCV-1 virions as demonstrated by western blotting with anti-H3K27me2 antibodies. (e) Northern blot analysis establishes that *vset* is expressed as a transcript of about 1.8 kb in PBCV-1 infected *Chlorella* NC64A cells 60 min after infection. For full scans of western blots in b and d, see Supplementary Information, Figure S5.

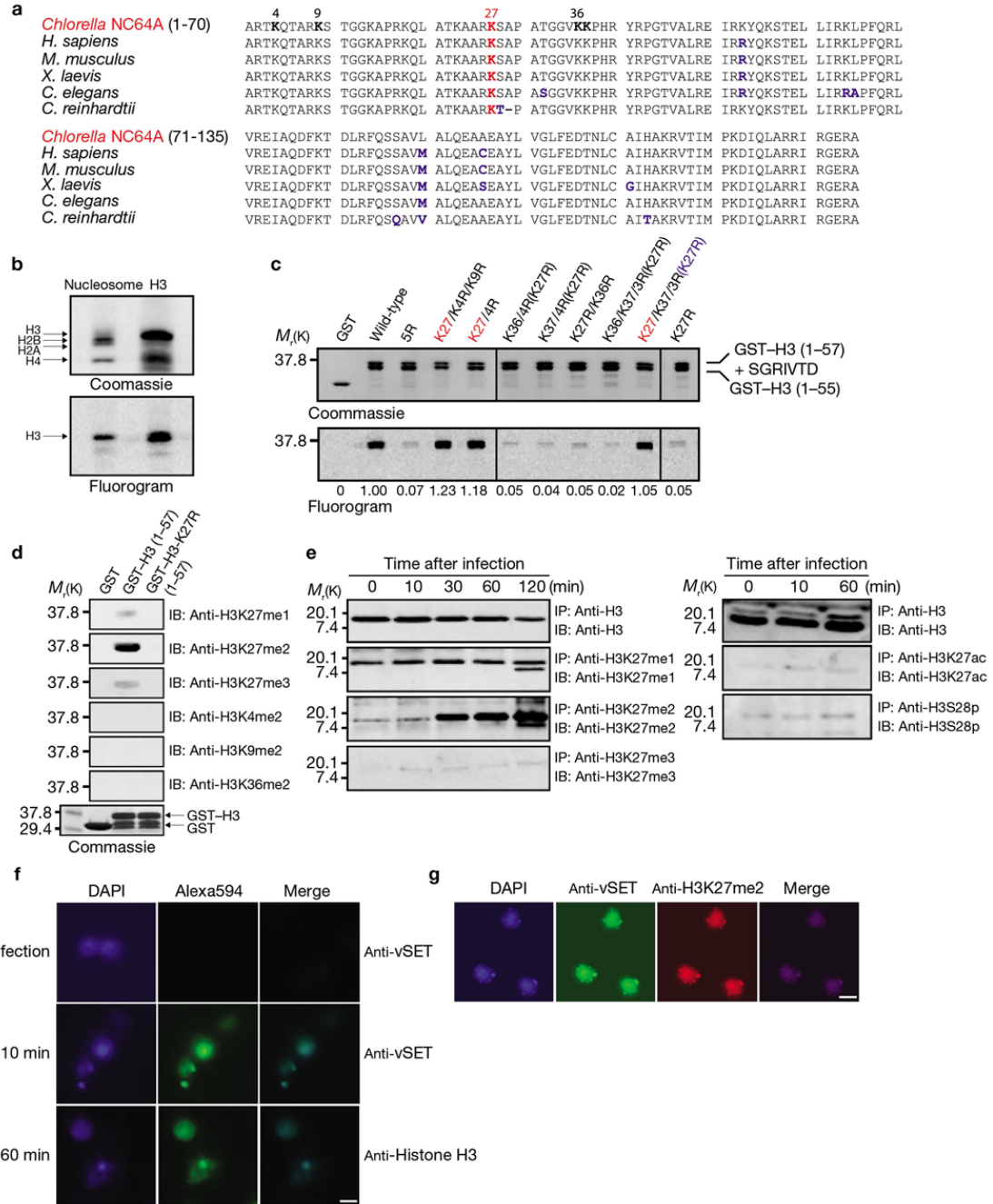


Figure 2. Methylation of *Chlorella NC64A* histone H3K27 by vSET
(a) Amino acid sequence alignment of H3 from *Chlorella NC64A*, *Homo sapiens*, *Mus musculus*, *Xenopus laevis*, *Caenorhabditis elegans*, and *Chlamydomonas reinhardtii*. Residues that differ in H3 are shown in blue. **(b)** vSET methylates H3 in nucleosomes. Native histone H3 was used as a control (right lane). **(c)** HKMTase activity of vSET targets H3K27, as illustrated by methyl transfer from ¹⁴C-methyl-SAM to GST-fusion H3 peptides (residues 1–57). Fluorescent values relative to wild-type enzyme activity are indicated under the lower panel. Relative amounts of GST–H3 in the assays are shown in SDS–PAGE (upper panel). **(d)** Western blot analysis of vSET-treated GST, GST–H3 wild-type and K27R mutant peptides using anti-H3K27me1, 2, 3, anti-H3K4me2, anti-H3K9me2, and anti-H3K36me2 antibodies.

(e) Western blot analyses of *Chlorella* NC64A H3 before and after PBCV-1 infection at specified times using antibodies against histone H3 with different modifications. (f) vSET is localized in the nucleus of PBCV-1 infected *Chlorella* cells collected 60 min after infection, as illustrated by immunofluorescence microscopy. *Chlorella* cells (uninfected or 10 and 60 min after infection) were probed with anti-vSET (uninfected and 10 min) or anti-H3 antibodies (60 min). (g) vSET and host *Chlorella* H3K27me2 colocalize in the nucleus 60 min after infection, as shown by immunofluorescence microscopy. *Chlorella* cells were probed with anti-vSET and anti-H3K27me2 antibodies and visualized by immunofluorescence microscopy using Alexa488 and Alexa594 dyes, respectively. Scale bars are 10 μ m. For full scans of western blots in **d** and **e** see Supplementary Information, Figure S5. H3 peptide sequences of **c** are shown in Supplementary Information, Table S2.

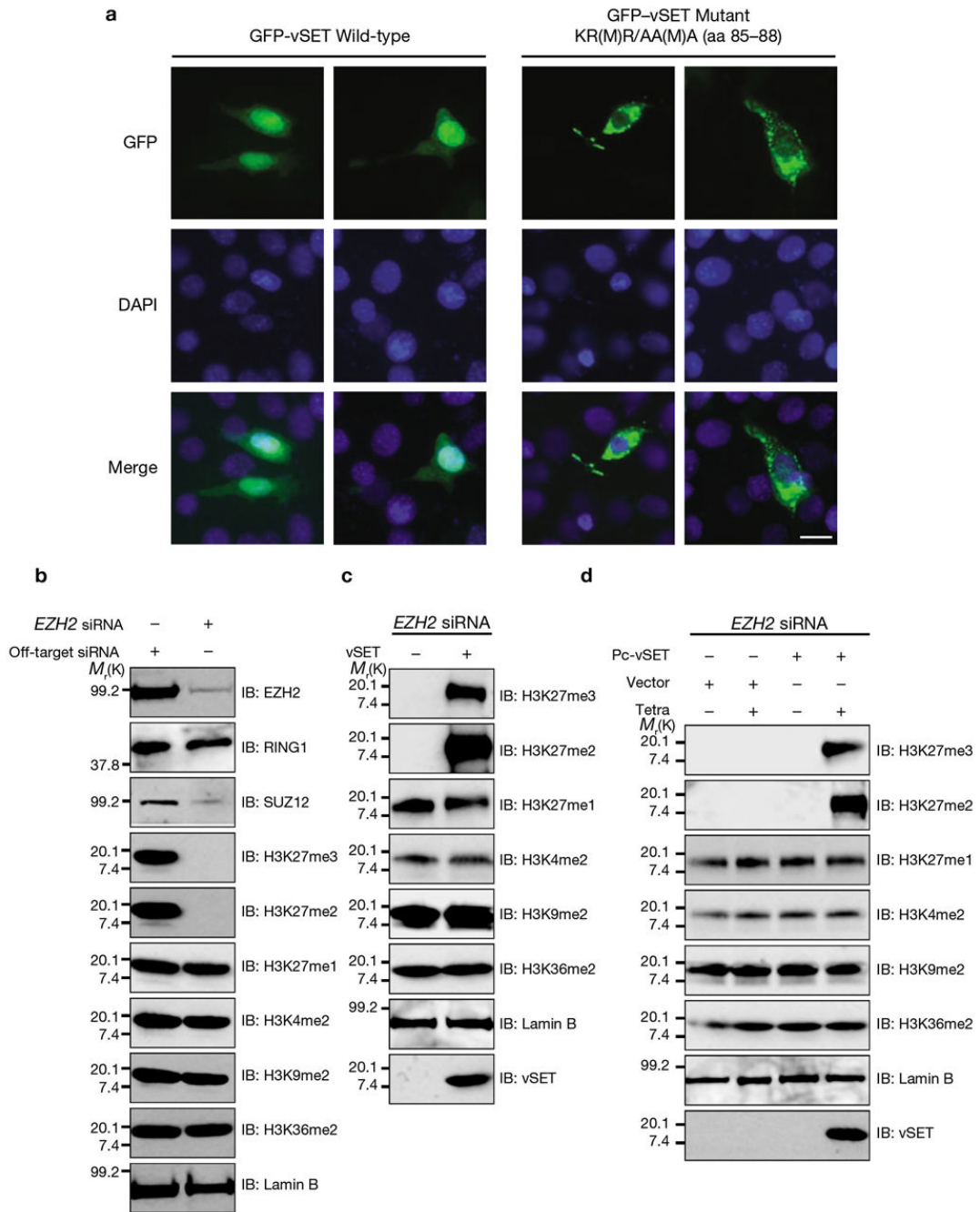


Figure 3. Nuclear localization and H3K27 methylation activity of vSET

(a) Nuclear localization of GFP-vSET in transiently transfected NIH-3T3 cells. Fluorescence microscopy images show that wild-type vSET is located in the nucleus, whereas a triple mutant, KR(M)R/AA(M)A (amino acids 85–88) is in the cytoplasm. Cells were counterstained with DAPI to highlight nuclei. Scale bar is 10 μ m. (b) Western blot analysis of HeLa cells showing effects of *EZH2*-specific- and mock-siRNAs treatment on protein expression of EZH2, RING1, SUZ12, and histone H3 with different modifications. Lamin B was used as a control. (c) Western blot analysis of the nuclear extract from HeLa cells treated with *EZH2* siRNA and subjected to *in vitro* methylation by vSET. (d) Western blot analysis showing di- and trimethylation of H3K27 after tetracycline-induced expression of stably transfected vSET in

HeLa cells 72 h after *EZH2* RNAi knockdown. For full scans of western blots in **d** see Supplementary Information, Figure S5. *EZH2* siRNA sequences are shown in Supplementary Information, Table S3.

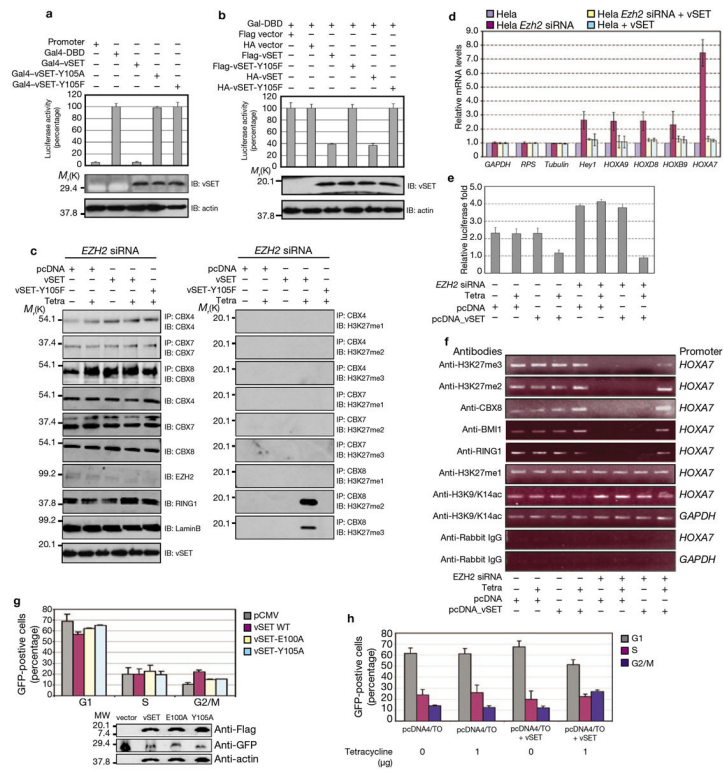


Figure 4. Repression of gene transcription by vSET H3K27 methylation activity
(a) Transcriptional repression of a reporter luciferase gene by vSET and its inactive mutants Y105A or Y105F in 293T cells; vSET was fused to the Gal4–DNA binding domain. **(b)** Transcriptional repression of the reporter luciferase gene by Flag- or HA-tagged vSET and its inactive mutant Y105F transiently transfected in 293T cells. Data are mean \pm s.d. of triplicate assays **(a, b)**. **(c)** Western blot analysis of PRC1 and PRC2 protein association with methylated H3K27 after induction of stably transfected vSET in the *EZH2* knockdown HeLa cells. The inactive vSET-Y105F mutant was used as a control. **(d)** Quantitative RT–PCR measurements showing relative mRNA levels of five Polycomb target genes of *Hey1*, *HOXA9*, *HOXD8*, *HOXB9*, and *HOXA7*, and three house keeping genes (*GAPDH*, *RPS*, *tubulin*) in normal HeLa cells (grey) and *EZH2* knockdown HeLa cells with (yellow) or without (red) induction of the transiently transfected vSET, as well as HeLa cells transfected with vSET (blue). **(e)** Luciferase-based reporter assay of *HOXA7* promoter in the presence of tetracycline-induced vSET in normal and *EZH2* depleted HeLa cells. Data are mean \pm s.d. of triplicate assays **(d, e)**. **(f)** ChIP analysis at the *HOXA7* promoter on induction of vSET in the HeLa cells with and without *EZH2* siRNA treatment. **(g)** vSET induces G2/M phase cell accumulation in NIH-3T3 cells co-transfected with vSET and Us9–GFP expression plasmids. Western blots showing relative equal expression of vSET and its mutants. **(h)** G2/M accumulation of HeLa cells stabilized on vSET induction with tetracycline. The DNA content of the gated GFP-positive cells was determined by propidium iodide staining and FACS analysis. DNA content of cell-cycle phases is shown **(g, h)**, mean \pm s.d. of at least three independent transfection experiments). For full scans of western blots in **a–c** see Supplementary Information, Figure S5. RT–PCR and ChIP primer sequences of **d** and **f** are available in Supplementary Information, Table S3.

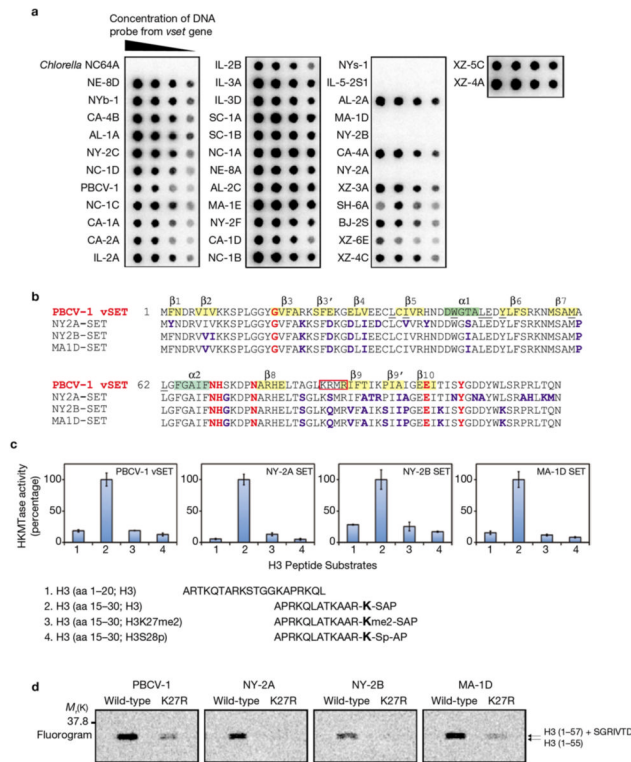


Figure 5. HKMT activity of SET proteins from chlorella viruses

(a) Southern dot blot analysis of 37 chlorella viruses confirmed the presence of a *vset*-like gene in 32 viruses. *Vset*-like genes from three of the five viruses that did not exhibit positive hybridization signals: NY-2A, NY-2B and MA-1D were detected using low stringency Southern hybridization (data not shown). (b) Sequence alignment of vSET and SET proteins from chlorella viruses NY-2A, NY-2B and MA-1D. Positions of α helices (grey) and β strands (yellow) are highlighted in the vSET sequence. Residues conserved among all known SET proteins are in red; residues in the three viral SET proteins that differ from vSET are in blue. Residues of vSET at the dimer interface are underlined. The NLS sequence in PBCV-1 is boxed. (c) Methylation activity of the SET proteins from viruses NY-2A, NY-2B and MA-1D measured with H3 peptides using ^{14}C -methyl-SAM. Data are mean \pm s.d. of triplicate assays. (d) HKMTase activity of the viral SET proteins using wild-type H3 (residues 1–57) or the K27R mutant. The fluorogram shows that NY-2A, NY-2B and MA-1D SET proteins methylate wild-type H3 but not the K27R mutant. H3 modified by vSET was used as a control.