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Title: Promoter-Targeted Histone Acetylation of Chromatinized Parvoviral Genome Is
Essential for the Progress of Infection

Year: 2016

Version:

Please cite the original version:

Mäntylä, E., Salokas, K., Oittinen, M., Aho, V., Mäntysaari, P., Palmujoki, L.,
Kalliolinna, O., Ihalainen, T. O., Niskanen, E. A., Timonen, J., Viiri, K., & Vihinen-Ranta,
M. (2016). Promoter-Targeted Histone Acetylation of Chromatinized Parvoviral
Genome Is Essential for the Progress of Infection. *Journal of Virology*, 90(8), 4059-
4066. <https://doi.org/10.1128/JVI.03160-15>

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1 Promoter targeted histone acetylation of chromatinized parvoviral genome is essential
2 for infection progress

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14 Running Title: Epigenetic regulation of parvoviral gene expression

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20 Keywords: Epigenetic regulation, parvovirus, viral genome, chromatinization,
21 promoters, histone acetylation

22 Abstract word count: 142; Importance word count: 103; Text word count: 3580

23 **ABSTRACT**

24 The association of host histones with parvoviral DNA is poorly understood. We
25 analyzed the chromatinization and histone acetylation of canine parvovirus DNA
26 during infection by confocal imaging and *in situ* proximity ligation assay combined
27 with chromatin immunoprecipitation and high-throughput sequencing. We found that
28 at late infection parvovirus replication bodies were rich in histones bearing
29 modifications characteristic of transcriptionally active chromatin, i.e. histone H3 lysine
30 27 acetylation (H3K27ac). The H3K27ac, in particular, was located in close proximity
31 to the viral DNA-binding protein NS1. Importantly, our results show for the first time
32 that in the chromatinized parvoviral genome, particularly the two viral promoters were
33 rich in H3K27ac. Histone acetyltransferase (HAT) inhibitor efficiently interfered with
34 expression of viral proteins and infection progress. Altogether, our data suggest that
35 acetylation of histones on parvoviral DNA is essential for viral gene expression and
36 completion of viral life cycle.

37 **IMPORTANCE**

38 Viral DNA introduced into cell nuclei is exposed to cellular responses to foreign DNA
39 including chromatinization and epigenetic silencing, both of which determine the
40 outcome of infection. How the incoming parvovirus resists cellular epigenetic down-
41 regulation of its genes is not understood. Here, the critical role of epigenetic
42 modifications in regulation of parvovirus infection was demonstrated. We showed for
43 the first time that a successful parvovirus infection is characterized by deposition of
44 nucleosomes with active histone acetylation on the viral promoter areas. The results
45 provide new insights to regulation of parvoviral gene expression which is an important
46 aspect in the development of parvovirus-based virotherapy.

47 **INTRODUCTION**

48 Nuclear chromatin is composed of DNA and histone proteins (1). The histone proteins
49 assemble DNA into nucleosomes, the composition and spacing of which contribute to
50 higher order chromatin packing. The chromatin is organized into regions of less-
51 condensed actively transcribed chromatin (euchromatin) and highly-condensed
52 transcriptionally repressed chromatin (heterochromatin). Epigenetic modifications of
53 histone proteins have been shown to correlate with spatial distribution of active and
54 repressed chromatin (2,3). Acetylation of lysines 9 or 27 of histone H3 (H3K9ac and
55 H3K27ac) and tri-methylation of lysine 4 (H3K4me3) correlate with transcriptional
56 activity, while repressed chromatin is characterized by e.g. tri-methylation or di-
57 methylation of the same H3 lysine residues (H3K9me3 and H3K27me3; H3K9me2
58 and H3K27me2) (4-8).

59 Foreign DNA introduced into mammalian cells can be recognized as threat by the host
60 cell. The cellular responses to the foreign DNA, such as viral DNA, include
61 chromatinization of the entering DNA, leading to its transcriptional silencing (9–11).
62 How viruses resist cellular chromatinization and silencing is known for only few
63 viruses (9,12,13). These include herpes simplex viruses (HSVs) (14-16),
64 polyomaviruses (17, 18), adenoviruses (19), and cytomegaloviruses (20,21), all of
65 which encode proteins that promote transcriptionally activating histone modifications
66 on chromatinized viral genome during lytic infection.

67 In parvovirus infection, the nuclear entry of viral single-stranded DNA is followed by
68 the formation of double-stranded replicative intermediates, nuclear accumulation of
69 viral proteins and DNA, and the formation of autonomous parvovirus-associated
70 replication (APAR) bodies (22–25). Viral gene expression and DNA replication are

71 dependent on the S-phase of the cell cycle. In infected cells transcription of viral non-
72 structural protein 1 (NS1) at 4 h p.i. is followed by viral genome replication (26).
73 Replication continues throughout the infection and leads to production of viral capsids
74 and their nuclear egress at 20-24 h p.i. Intranuclear chromatinization of parvoviral
75 genome, modification of the assembled histones and effect of these events on viral
76 gene expression are not well understood. To provide answers to these questions we
77 analyzed the infection of an autonomous protoparvovirus, canine parvovirus (CPV), by
78 confocal imaging techniques and *in situ* proximity ligation assays (PLA)
79 complemented with chromatin immunoprecipitation coupled with high-throughput
80 sequencing (ChIP-seq). Our results demonstrated enrichment of acetylated histones
81 (H3K27ac) in close proximity to viral DNA-binding NS1 in APAR bodies and,
82 especially H3K27ac accumulated in the viral promoters. Inhibition of histone
83 acetyltransferase (HAT) activity led to interruption of viral life cycle. These results
84 reveal that histone acetylation on chromatinized parvoviral genome is necessary for
85 expression of viral genes and successful progress of infection.

86 **MATERIALS AND METHODS**

87 **Cells, viruses and constructs.** Norden laboratories feline kidney (NLFK; Quality
88 Control of Pfizer Animal Health, Lincoln, NE) cells were grown in Dulbecco's
89 modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (Gibco,
90 Paisley, UK) at 37 °C in the presence of 5 % CO₂. CPV-2d isolates originated from an
91 infectious plasmid clone (a gift from C.R. Parrish, Cornell University, Ithaca, NY
92 (27)). The viruses had been isolated as described by Suikkanen et al. (28). For
93 infection, the cells were inoculated with CPV (MOI 1–2) and kept at 37 °C until
94 fixation. In order to synchronize infections, the cells were incubated on ice at 4 °C for

95 20 min prior to virus addition. The cells were then inoculated on ice to allow for the
96 virus adsorption to occur for 30 min. Next, the cells were rinsed at 4 °C with
97 phosphate-buffered saline (PBS) containing 0.3 % bovine serum albumin (BSA)
98 followed by addition of 37 °C medium. The plasmid encoding fluorescent H3-EGFP
99 was a generous gift from J. Langowski (German Cancer Research Center, Heidelberg,
100 Germany). A NLFK cell line stably expressing H3-EGFP was established by
101 transfection (TransIT-LT1 reagent; Thermo Fisher Scientific Inc, Waltham, MA) with
102 an expression vector at 24 h after seeding. After 2 days the DMEM was replaced by
103 DMEM containing 1 mg/ml of geneticin (Sigma Aldrich, St. Louis, MO). The cells
104 were then seeded at different intervals until a stable expression was observed by
105 microscopy.

106 **Confocal microscopy** For immunolabeling, cells seeded on round coverslips were
107 infected with CPV and fixed at 8, 10, 12, 16, and 24 h p.i. with 4 % paraformaldehyde
108 (PFA; 15 min at room temperature). Viral proteins were detected with an NS1-specific
109 monoclonal antibody (MAb) (generous gift from Caroline Astell) (29), an intact capsid
110 MAb and a polyclonal capsid-protein VP2 antibody (Ab; generous gifts from Colin R.
111 Parrish) (27) followed by goat anti-mouse or anti-rabbit Alexa-555 or Alexa-633
112 conjugated secondary Abs (Molecular Probes, Life technologies, Grand Island, NY).
113 Modified histones were labeled with rabbit Abs against H3K9me3 and H3K27ac
114 (Abcam, Cambridge, MA) followed by goat anti-rabbit Alexa-633 conjugated
115 secondary Abs. For deacetylation and hyper-acetylation studies, cells were either
116 pretreated for 30 min prior to virus inoculation or treated at 0-6 h p.i. with 0.1-mM
117 anacardic acid prepared in 100 % dimethyl sulfoxide (DMSO) (Sigma Aldrich, St.
118 Louis, MO). Cell viability was assessed using propidium iodide (Sigma) and annexin V
119 Alexa-647 (Molecular Probes) staining following manufacturer instructions (data not

120 shown). All cells were fixed at 24 h p.i. with 4 % PFA and permeabilized with 0.1 %
121 Triton X-100 in PBS supplemented with 1 % BSA and 0.01 % sodium azide. Imaging
122 was done with an Olympus FV-1000 confocal microscope with the UPLSAPO 60x oil-
123 immersion objective (NA=1.35). EGFP was excited with a 488 nm argon laser and
124 fluorescence was collected with a 515/30 nm band-pass filter. Alexa-555 and the PLA
125 reagent 594 nm were excited with a 543 nm He-Ne laser and the fluorescence were
126 collected with a 570 nm to 620 nm and a 560IF band pass filters, respectively. Alexa
127 633 was excited with a 633 nm He-Ne laser and the fluorescence was collected with a
128 647 nm long-pass filter. Image size was between 512 x 512 and 1600 x 1600 pixels
129 with a pixel resolution of 66–69 nm. Deconvolution was performed with Huygens
130 Essential software (SVI, Netherlands). The point-spread function was averaged, and
131 the iterative deconvolution was performed with a signal-to-noise ratio of 5, and the
132 quality threshold was 0.01. Image analysis was done with ImageJ (30).

133 Correlation analysis was done with ImageJ using the JACoP plugin (31). Distance
134 analysis of the fixed-cell samples was done by first making a Euclidian distance map
135 from an image of interest and then plotting the original image against the distance map.
136 The data from every cell were combined and arranged to 0.1 μm wide bins based on
137 the distance of the voxels from the nuclear envelope. The average intensity was then
138 plotted for each bin. Distance maps were done in ImageJ with the Exact Euclidian
139 Distance Transform (3D) plugin, and the data analysis was done with an in-house Java
140 code. Student's t-test (two-tailed, unequal variance) was used to evaluate statistical
141 significance in the change of the recovery time point values.

142 **Analysis of protein interactions.** For *in-situ* proximity ligation assay (PLA) (32),
143 cells were grown on 8-well chamber slides (Nunc™ Lab-Tek™ II Chamber Slide™
144 System, Nalgene Nunc International, Penfield, NY) to 80-90 % confluence and fixed

145 with 4% PFA. PLA was done with a Duolink[®]II kit (Olink Bioscience, Uppsala,
146 Sweden) and primary Abs against NS1 and H3K27ac. After labeling the cells with the
147 primary Abs, the PLA probes (oligonucleotide-conjugated secondary anti-mouse and
148 anti-rabbit IgGs diluted in 3 % BSA in PBS) were incubated for 1 h at 37 °C in a
149 humidified chamber followed by ligation and amplification according to the
150 manufacturer's instructions. Samples were embedded in ProLong Gold antifade
151 mounting medium with 4,6-diamidino-2-phenylindole (DAPI). The specificity of the
152 assay was confirmed using positive, negative and technical control readings. Positive
153 and negative controls consisted of infected and non-infected cells, respectively, labeled
154 with anti-VP2 and intact capsids specific Abs, or anti-NS1 and anti-H3K27ac Abs. For
155 a technical control, non-infected cells were labeled only with PLA probes. Quantitative
156 analysis was done with ImageJ by determining the arithmetic mean of the total number
157 of signals per cell. The PLA signal was normalized as $PLA = NA - NT$, where NA is the
158 total number of nuclear PLA dots in infected and non-infected cells labeled with
159 H3K27ac Ab and NS1 Mab, and NT is the average number of nuclear PLA dots in the
160 technical control. Student's t-test (two-tailed, unequal variance) was used to evaluate
161 statistical significance.

162 **ChIP-seq and qPCR.** Cells were fixed with formaldehyde and nuclei were isolated,
163 lysed, and sonicated with a Covaris S220 ultrasonicator. The resulting nuclear extract
164 was incubated overnight at 4 °C with Dynal Protein G beads pre-incubated with 5µg of
165 H3K27ac (ab4729) or H3 (ab1791) Abs. Beads were washed and bound complexes
166 eluted, and cross-links were reversed by heating at 65 °C. IP and input DNA were then
167 purified by a treatment with RNase A, proteinase K, and phenol-chloroform
168 extraction. Before moving forward to ChIP-seq, precipitated DNA was analyzed by
169 qPCR using primers specific for gp1, gp2 and gp5 regions. Libraries were constructed

170 from IP and input DNA by NEBnext® Ultra™ DNA-library preparation kit for
171 Illumina. DNA in the range 150–350 bp was gel-purified after PCR amplification. The
172 library was quantified using Agilent bioanalyzer and subjected to 50 bp single-end read
173 sequencing with Illumina Hiseq 2000 at EMBL Genecore, Heidelberg. Quality metrics
174 for sequenced reads were gathered with FastQC (33). Adapter sequences were
175 removed with cutadapt (34). Reads were then aligned with Bowtie2 (35) to cat (ICGSC
176 Felis_catus 6.2) and CPV (NCBI Reference Sequence NC_001539.1) reference
177 genomes. Sequencing reads of H3K27ac ChIP-seq from infected cells were normalized
178 to CPV reads per genomic content (RPGC). File conversions to the BAM format were
179 done with SAMtools (36), and visualizations with deepTools (37).

180 **RESULTS**

181 **Temporal changes in localization of modified histones in infection.** To analyze
182 chromatinization of CPV genome at various times post infection (p.i.), we used H3-
183 EGFP-expressing cells to identify the distribution of histone H3 and APAR bodies
184 represented by NS1. The line profiles showed that at 12 h p.i. H3 was mostly located
185 distinctly from NS1, whereas at 24 h p.i. increased colocalization of H3 with NS1 was
186 observed (Fig. 1A and 1B). Similarly, Pearson correlation coefficient (PCC) analysis
187 indicated correlation of H3-EGFP with NS1 at 16 h p.i. (0.61 ± 0.29 , $n=22$, student's
188 T-test p-values at 16/24 h p.i. in comparison to 10 h p.i., $p<0.05$) and 24 h p.i. ($0.77 \pm$
189 0.21 , $n=26$, $p<0.01$), but not at earlier times (Fig. 1G). To assess how H3-associated
190 active and repressed chromatin is distributed in infected cells, we used H3K27ac and
191 H3K9me3 as markers of active and repressed chromatin, respectively. Immunolabeling
192 indicated that at 12 h p.i., distinct NS1 foci began to emerge, while H3K27ac and
193 H3K9me3 showed a thorough nuclear distribution. The line profiles of H3K27ac and
194 H3K9me3 with NS1 at 12 h p.i. indicated only a weak colocalization (Fig. 1C and 1E).

195 However, at 24 h p.i. H3K27ac concentrated in the enlarging APAR bodies (Fig. 1D).
196 Similarly, PCC of NS1 with H3K27ac was significantly higher at 16 h p.i. ($0.83 \pm$
197 0.22 , $n=18$, $p<0.01$) and at 24 h p.i. (0.87 ± 0.08 , $n=23$, $p<0.01$) in comparison to
198 earlier times (Fig. 1G). No correlation of H3K9me3 with NS1 was observed by the line
199 profile and PCC analyses (Fig. 1E, 1F and 1G,). Colocalization of NS1 with H3 and
200 H3K27ac correlated time-dependently with enlargement of APAR bodies. Quantitative
201 3D distribution analysis of H3K27ac and H3K9me3 as a function of distance from the
202 nuclear rim indicated that the H3K27ac signal was located in the nuclear center in both
203 infected (24 h p.i.) and non-infected cells ($n=20$, 1000 spots/cell counted; Fig. 1H). In
204 infected cells, the $0.7 \mu\text{m}$ -thick region at the nuclear periphery, in particular, was
205 enriched in H3K9me3, and its intensity decreased toward the nuclear center. In non-
206 infected cells, the H3K9me3 signal was distributed throughout the nucleus ($n=20$, 1000
207 spots/cell counted; Fig. 1I).

208 In summary, these data showed that in the infected cells both H3K27ac and H3-EGFP
209 were enriched in APAR bodies. At the same time, H3K9me3 was found to accumulate
210 in the nuclear periphery, an area known to harbor the layer of marginalized cellular
211 chromatin. This suggests that the histones associated with the viral genome in the
212 APAR area bear modifications that are characteristic of active gene expression.

213 **Time-dependent intranuclear interplay of H3K27ac and NS1.** In order to address
214 the nuclear interaction of H3K27ac with viral DNA-bound NS1, *in situ* PLA was
215 performed. PLA is an immunodetection technique that generates a fluorescent signal
216 only when two antigens of interest are within 40 nm of each other (32). To assess the
217 nuclear interactions of H3K27ac and NS1, we analyzed infected cells by PLA at 8–24
218 h p.i. Non-infected control cells exhibited only a faint nuclear signal (PLA signal per
219 cell 1.1 ± 0.19 , $n=111$). At 8, 10, 12, and 16 h p.i. time-dependent increase in amount of

220 punctuate intranuclear PLA signals was detected (0.14 ± 0.16 , $n=105$; 0.81 ± 0.30 , $n=92$;
221 3.43 ± 0.91 , $n=110$; 19.46 ± 3.39 , $n=90$) with a maximal signal at 24 h p.i. (36.49 ± 3.97 ,
222 $n=99$, Fig. 2A) in the low DAPI nuclear interior (Fig. 2B). The infection-induced
223 compaction of the host chromatin and its dislocation into the nuclear periphery and
224 around the nucleolus at 24 h p.i., were visualized with DAPI.

225 In summary, these results demonstrated a time-dependent increase in interaction or
226 close proximity of H3K27ac with NS1 after 10 h p.i. followed by extensive interaction
227 at 24 h p.i. This finding is consistent with the results of immunofluorescence and PCC
228 analyses of infected cells, which showed increased colocalization and correlation of
229 H3K27ac and NS1 at late-stage infection.

230 **CPV promoters are rich in acetylated histones.** The prominent colocalization and
231 interaction of NS1 with H3K27ac in the nucleus at 24 h p.i. prompted us to study
232 whether the CPV genome *per se* is chromatinized with H3K27ac-enriched
233 nucleosomes. To this end, ChIP-seq with H3K27ac Ab for the infected cells was
234 performed. First, the success of ChIP was assessed with genome-wide occupancy of
235 H3K27ac in infected cells showing a typical occupancy of this histone marker at ± 2 kb
236 around the transcription start sites (TSSs) of ~ 35 % of the genes (data not shown). We
237 found that 9.23 % of the total reads (2.23×10^7 reads) were aligned with the viral
238 genome in a unique manner (0.00% in non-infected cells) (38). Alignment of these
239 reads with the genome, after normalization to reads per CPV-genomic content,
240 revealed that H3K27ac is mostly enriched in the TSSs of P4 promoter driven
241 transcriptional units for NS1 and NS2 (gp1 loci) and P38 driven transcriptional units
242 for VP1 and VP2 (gp2 loci) (Fig. 3A) (39).

243 The viral genome was mostly devoid of this histone marker towards the right-hand end
244 (gp5 loci). The location of gp1 loci was very similar to that of P4 promoter of the
245 parvovirus minute virus of mice (MVM, nucleotides 1–260) and the location of gp2
246 was similar to that of CPV P38 promoter (1355–2260) (40). The targeted ChIP-qPCR
247 of H3 with gp1, gp2 and gp5-specific primers showed that the CPV genome is
248 thoroughly chromatinized with uniformly distributed H3 (Fig. 3B, data not shown).
249 Finally, targeted ChIP-qPCR analysis confirmed occupancy of H3K27ac in the
250 genome (Fig. 3C).

251 In conclusion, our data verified that the parvoviral genome is chromatinized with
252 histones at late-stage infection. Importantly, acetylated histones were observed to be
253 enriched in viral promoters to allow for transcriptional activation of the viral NS and
254 VP genes.

255 **Viral histone acetylation is important for infection progress.** In order to determine
256 the importance of histone acetylation of parvoviral genomes during early stages of
257 infection, the effect of histone HAT inhibitor anacardic acid on viral protein synthesis
258 was determined. Treatment with anacardic acid at very early infection (-0.5–1 h p.i.)
259 resulted in decrease in the percentage of cells with nuclear NS1 (~5 %) and VP2 (~2
260 %) at 24 h p.i. in comparison to non-treated infected cells (68.0 and 50.3 %
261 respectively, $n \geq 250$). This suggests that early inhibition of histone acetylation induced
262 an almost complete block of infection. While the early treatment exerted a maximal
263 effect, anacardic acid was able to inhibit the infection progress when introduced later,
264 up to three hours post infection (Fig. 4A). Here, we cannot rule out the possibility that
265 histone acetylation was not the only factor affecting viral protein production. Also cell
266 cycle progression could be involved. Of note, the treatment did not affect cell viability
267 (data not shown). Our results suggest that histone acetylation is required at very early

268 infection for the production of viral proteins necessary for efficient progress of
269 infection.

270 Next, we studied if the anacardic acid-induced inhibition of histone acetylation
271 affected late infection. For this we divided the infected cells in four categories based
272 on intracellular localization of capsids at 24 h p.i. The categories were: (I) capsids
273 localized into cytoplasmic endocytic vesicles due to inhibition of infection or
274 secondary infection (24); (II) capsids localized in discrete foci scattered throughout the
275 nucleus; (III) capsids in enlarged APAR bodies enriched with NS1; (IV) capsids both
276 in APAR bodies and in the cytoplasm (egress initiated) (Fig. 4B). Next, the
277 localization of capsids was determined at 24 h p.i. in cells exposed to anacardic acid at
278 6 h p.i. Studies indicated that category II, III and IV cells were reduced in treated cells
279 (4.9 %, 12.5 %, 21.2 %; $n \geq 250$) in comparison with that of the non-treated infected
280 control cells (7.9 %, 26.8 %, 26.8 %; $n \geq 250$). Moreover, an increase in category I cells
281 was detected in treated cells (61.1 %; $n \geq 250$) in comparison to control cells (36.6 %;
282 1.9 %, $n \geq 250$; Fig. 4B). These results demonstrated that efficient progress of infection
283 correlates with histone acetylation in APAR area at late stages of infection.

284 Finally, we studied whether the timing of viral histone acetylation was critical for
285 progress of late-stage infection. Here, the distribution of H3K27ac and NS1, positioned
286 on viral genome, was analyzed at 24 h p.i. in cells treated with anacardic acid. The
287 inhibitor treatment at 16 h p.i. resulted in exclusion of H3K27ac from the NS1-positive
288 APAR bodies (Fig. 4C). This indicated that the viral genomes produced after drug
289 treatment at 16 h p.i. were not acetylated. However, H3K27ac colocalized with NS1
290 after exposure to inhibitor at 20 h p.i. (Fig. 4C). These results suggested that histone
291 acetylation on majority of newly synthesized viral DNA occurs until 16 - 20 h p.i.

292 These inhibition studies provided evidence that histone acetylation of intranuclear viral
293 genomes at early infection plays an essential role in production of viral proteins.
294 Similarly, acetylation of histones on newly synthesized viral genomes during active
295 virus replication is necessary for efficient accomplishment of infection. In summary,
296 our findings demonstrate that viral histone acetylation is essential for both early and
297 late steps in parvoviral life cycle.

298 **DISCUSSION**

299 Although much is known about nuclear replication and gene expression of
300 parvoviruses, little is known about chromatinization of parvoviral genome and histone
301 modifications and effect of these events on progress of infection. For some DNA
302 viruses, such as herpesviruses and adenoviruses, epigenetic mechanisms, including
303 histone modifications play an important role in the regulation of viral gene expression.
304 During herpesvirus lytic infection, the viral genomes are associated with histones
305 immediately after injection into the nucleus, and viral proteins are required to enhance
306 histone acetylation to allow for efficient viral gene expression (41-45). Moreover,
307 studies of infection by a adenovirus have shown that viral proteins mediate
308 transcriptional activation of viral promoter regions (46-48). In this work, we first
309 observed that the progress of CPV infection was accompanied by enrichment of H3
310 histones in the enlarged APAR body area. This is consistent with earlier studies
311 showing nuclear chromatinization of adeno-associated parvoviruses, used as gene
312 therapy vectors, and MVM genomes (49-52). Next, we addressed the existence of
313 histone modifications in the parvoviral APAR bodies and revealed accumulation of
314 histones with modifications characteristic of transcriptionally active chromatin
315 (H3K27ac). Moreover, our analysis demonstrated that H3K27ac was located in close
316 proximity to the viral NS1 protein in the APAR bodies. This interaction is likely to be

317 caused by accumulation of NS1 on viral genome because of its involvement in
318 transcription and replication (53-55). NS1 of autonomous parvoviruses not only
319 controls the viral activities but also regulates the host-gene expression through histone
320 acetylation in cancer cells by recruiting innate proteins with HAT-activity (56,57). To
321 date, evidence for involvement of NS1 in regulation of histone acetylation on
322 parvoviral promoters has not been reported. Here, our PLA studies revealed that CPV
323 NS1 was located in close proximity to H3K27ac in the enlarged APAR bodies. Our
324 earlier studies indicated that CPV NS1 has two distinct binding sites in the viral
325 genome (54,55,58). It is tempting to speculate that NS1 is involved in histone
326 acetylation of viral P4 and P38 promoters by recruiting host proteins with acetyl-
327 transferase activity. However, the specific role and interactions of NS1 in viral histone
328 modifications remains to be determined. Our ChIP-seq analyses demonstrated for the
329 first time that in the extensively chromatinized parvoviral genome H3K27ac
330 accumulated in viral P4 and P38 promoter areas. This suggests that parvoviral gene
331 expression is regulated by histone acetylation in promoter areas. The importance of
332 histone acetylation for the progress of infection was supported by our results showing
333 that inhibition of histone acetylation was accompanied by extensive repression of
334 infection. Earlier studies have shown that formation of parvoviral gene transcription
335 templates and intermediates for genome replication are temporally phased in infection
336 (59-62). In line with these, our results suggest that parvoviruses regulate their gene
337 expression via histone acetylation in a temporal fashion.

338 In summary, CPV genome is chromatinized inside the nuclei of infected cells and
339 histone modifications associated with transcriptional activation are enriched in viral
340 promoters. Our results highlight a critical role of epigenetic modification in
341 progression of the parvoviral life cycle.

FUNDING INFORMATION

342 This work was financed by the Academy of Finland under the award numbers 135609
343 (EAN), 267471 (TOI), 138388 (MVR), 265575 (KV), the Graduate School of the
344 University of Jyvaskyla (VA), Sohlberg foundation (KV, MO), and Jane and Aatos
345 Erkkko Foundation (JT, MVR).

ACKNOWLEDGEMENTS

347 We are grateful to Klaus Hedman for comments on the manuscript.

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536 **FIGURE LEGENDS**

537 **Figure 1**

538 **Intranuclear distribution of H3-EGFP, modified histones and viral NS1 protein.**

539 Confocal microscopy images of cells stably expressing H3-EGFP (cyan) (A) at 12 h
540 and (B) at 24 h pi labeled with NS1 (yellow) antibody. Infected cells at 12 h p.i. and 24
541 h p.i. labeled with antibodies for H3K27ac (cyan) (C, D), H3K9me3 (cyan) (E, F) and
542 NS1 (yellow). In order to clarify the changes in colocalization, pseudo-colour images
543 with intensity increasing from blue to yellow are shown. Fluorescence line-profile
544 analysis of the intensity of H3-EGFP/H3K27ac/H3K9me3 (cyan) and NS1 (yellow) in
545 a single optical section through the center of nucleus is shown beside each image.
546 Analysis was performed with ImageJ and Plot RGB Profile-plugin. Scale bars, 5 μ m.
547 (G) Quantitative colocalization analysis of H3K27ac (dove grey), and H3K9me3 (dark
548 grey) and H3-EGFP (black) with NS1. The mean values of PCCs with standard
549 deviation (SD) are shown. Statistical significance of colocalization at 16 and/or 24 h
550 p.i. in comparison to 10 h p.i. is shown (Student's T-test p-values: * $P < 0.05$; ** $P <$
551 0.01). Plots of intensity of H3K27ac (H) and H3K9me3 (I) in infected cells at 24 h p.i.
552 (dark grey) and in non-infected (dove grey) as a function of distance from the nuclear
553 envelope.

554 **Figure 2**

555 ***In situ* PLA of H3K27ac interaction with NS1 in infected cells.** (A) Number of PLA

556 signals in infected cells at 8, 10, 12, 16 and 24 h p.i. The mean values of PLA signals
557 with \pm standard error from \sim 100 cells per time point are shown. The negative control,
558 PLA signal in non-infected cells, is shown by a dashed line. Statistical significance of
559 PLA signals per cell at 12, 16 and 24 h p.i. in comparison to negative control is shown

560 (Student's T-test p-values: *P<0.05;**P< 0.01). (B) Distribution of PLA signals in
561 DAPI-stained nucleus at 24 h p.i. Scale bar, 10 μ m.

562

563 **Figure 3**

564 **Enrichment of acetylated histones on gp1 and gp2 transcription start sites and**
565 **effect on gene expression.** (A) Histogram depicts occupancy of H3K27ac at CPV
566 genome. Sequencing reads of H3K27ac ChIP-seq from infected cells at 24 h p.i. are
567 normalized to RPGC. Bent arrows indicate TSSs of transcriptional units for NS1 and
568 NS2 (gp1), VP1 and VP2 (gp2) and right-hand terminal gp5 loci and red bars depict
569 regions where qPCR primers anneal. ChIP-qPCRs for targeted measurements of (B)
570 H3 and (C) H3K27ac level at gp1 and gp2 TSSs and VP2-gp5 inter-region is shown as
571 enrichment of DNA relative to input DNA. Two primer pairs for each of the three
572 regions were used; columns represent the means and \pm SD of triplicate wells of two
573 independent ChIP experiments.

574

575 **Figure 4**

576 **Importance of the acetylation on progress of infection.** (A) Percentage of cells
577 showing viral NS1 and VP proteins in the nucleus in the cells exposed to HAT
578 inhibitor, anacardic acid (0.1 mM). Cells were either pretreated for 30 min with the
579 drugs prior to infection or treated at 0-6 h pi, and the treatment continued until fixation
580 at 24 h p.i. (B) Effect of anacardic acid treatment on localization of viral capsids.
581 Images of cells representing four type categories based on intracellular localization of
582 capsids at 24 h p.i. and percentages of cell showing various types after treatments at 6

583 or 16 h p.i. The mean values with \pm SD are shown. (C) Distribution of H3K27ac in
584 comparison to NS1 at 24 h p.i. in cells treated with anacardic acid at 16 or 20 h p.i.
585 Scale bars, 10 μ m.