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HPV E7 Oncoprotein Subverts Host Innate Immunity Via SUV39H1-Mediated Epigenetic Silencing of Immune Sensor Genes

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1 **HPV E7 Oncoprotein Subverts Host Innate Immunity Via SUV39H1-Mediated Epigenetic**
2 **Silencing of Immune Sensor Genes**

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16 **Running Head:** HPV E7 Subverts Innate Immunity Through SUV39H1

17

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27 **ABSTRACT**

28 Subversion of innate immunity by oncoviruses, such as human papillomavirus (HPV), favors
29 carcinogenesis because the mechanism(s) of viral immune evasion can also hamper cancer
30 immunosurveillance. Previously, we demonstrated that high-risk (hr) HPVs trigger simultaneous
31 epigenetic silencing of multiple effectors of innate immunity to promote viral persistence. Here, we
32 expand on those observations and show that the HPV E7 oncoprotein upregulates the H3K9-
33 specific methyltransferase, whose action shuts down the host innate immune response. Specifically,
34 we demonstrate that SUV39H1 contributes to chromatin repression at the promoter regions of the
35 viral nucleic acid sensors RIG-I, cGAS and the adaptor molecule STING in HPV-transformed cells.
36 Inhibition of SUV39H1 leads to transcriptional activation of these genes, especially RIG-I,
37 followed by increased IFN β and λ_1 production after poly(dA:dT) or RIG-I agonist M8 transfection,
38 Collectively, our findings provide new evidence that the E7 oncoprotein plays a central role in
39 dampening host innate immunity and raise the possibility that targeting the downstream effector
40 SUV39H1 or the RIG-I pathway may be a viable strategy to treat viral and neoplastic disease.

41

42 **IMPORTANCE**

43 High-risk HPVs are major viral human carcinogens responsible for approximately 5% of all human
44 cancers. The growth of HPV-transformed cells depends on the ability of viral oncoproteins to
45 manipulate a variety of cellular circuits, including those involved in innate immunity. Here, we
46 show that one of these strategies relies on E7-mediated transcriptional activation of the chromatin
47 repressor SUV39H1, which then promotes epigenetic silencing of RIG-I, cGAS and STING genes,
48 thereby shutting down interferon secretion in HPV-transformed cells. Pharmacological or genetic
49 inhibition of SUV39H1 restored the innate response in HPV-transformed cells, mostly through
50 activation of RIG-I signaling. We also show that IFN production upon transfection of poly(dA:dT)
51 or the RIG-I agonist M8 predominantly occurs through RIG-I signaling. Altogether, the reversible
52 nature of the modifications associated with E7-mediated SUV39H1 upregulation provides a

53 rationale for the design of novel anticancer and antiviral therapies targeting these molecules.

54

55 INTRODUCTION

56 Human papillomaviruses (HPVs) are circular double-stranded DNA viruses with a small
57 genome of approximately 8 kb. Over 200 types of HPV have been identified and classified
58 according to whether they infect cutaneous or mucosal epithelium
59 (<http://www.ictv.global/report/papillomaviridae>; 1-3). Cancer-causing HPVs are classified as “high-
60 risk” (hr) types, among which the most commonly found are the α -genotypes HPV16 and HPV18,
61 well-known to be the causative agents of cervical and anogenital cancers and heavily implicated in
62 head and neck cancers (4, 5).

63 The development of HPV-associated cancers relies on the expression of two oncoproteins,
64 E6 and E7, which are the only viral genes consistently found in these tumors (6, 7). Although these
65 oncoproteins do not exhibit enzymatic function, their transforming activity is mediated primarily
66 through protein-protein interactions that ultimately favor the formation of a replication-competent
67 environment that eventually leads to cancer (8). Specifically, hrHPV E6 targets the p53 tumor
68 suppressor protein for degradation, thereby preventing p53 from mediating cell cycle arrest and
69 apoptosis in response to cellular stress signals (9, 10). In contrast, hrHPV E7 promotes degradation
70 of the retinoblastoma tumor suppressor (pRb) protein, thus eliciting E2F-mediated transcriptional
71 activation of S-phase genes (2, 10). Importantly, both HPV E6 and E7 trigger epigenetic changes in
72 chromatin by altering the expression or the enzymatic activity of a number of epigenetic modifiers,
73 such as histone deacetylases, histone demethylases, histone acetyltransferases, and histone
74 methyltransferases (11-19). Concomitantly, the oncogenic stimuli triggered by HPV oncoproteins
75 cause host cells to mount an antiviral innate immune response. Nonetheless, HPVs have evolved
76 strategies to subvert antiviral immunity in order to complete their viral life cycle and persist in the
77 host cell (20-24).

78 In recent studies, we demonstrated that in NIKSmcHPV18 keratinocytes carrying episomal
79 HPV18, as well as in HeLa cells harboring an integrated HPV18 genome, induction of both IFN β
80 and IFN λ_1 by DNA ligands is significantly impaired compared to parental cells (25). Furthermore,
81 we found that downregulation of stimulator of IFN genes (STING), cyclic GMP-AMP synthase
82 (cGAS) and retinoic acid-inducible gene I (RIG-I) mRNA levels occurs at the transcriptional level
83 through a novel epigenetic silencing mechanism, based on the accumulation of repressive
84 heterochromatin marks, especially H3Lys9me2 (H3K9me2), at the promoter region of these genes
85 (25). The incorporation of histone marks in chromatin represents a dynamic balance between
86 enzymes depositing the mark (writers) and other enzymes removing it (erasers) (26). In this regard,
87 SUV39H1, the human homolog of the *Drosophila* Su(var)3-9 histone methyltransferase, is the
88 prime histone code “writer” responsible for histone H3Lys9 trimethylation (H3K9me3), which
89 marks chromatin in a “closed” conformation (27, 28).

90 In this study, we show that SUV39H1 is involved in epigenetic silencing of RIG-I, cGAS
91 and STING genes in hrHPV-transformed cells. Importantly, pharmacological or genetic inhibition
92 of SUV39H1 restored the innate immune response to exogenous DNA, as reflected by the
93 production of both IFN β and λ_1 . SUV39H1 upregulation was dependent on E7 protein expression,
94 as demonstrated by either loss or gain of function experiments. In particular, we show that loss of
95 E7 expression in both HeLa and CaSki cells significantly enhanced IFN production upon
96 poly(dA:dT) or RIG-I agonist M8 transfection, predominantly through RIG-I signaling.

97

98 **RESULTS**

99 **SUV39H1 increases heterochromatin formation at the promoter regions of RIG-I,**
100 **cGAS and STING genes in HPV-transformed cells.** To determine which histone modifier
101 enzyme was responsible for HPV-driven epigenetic modifications of the innate immune response,
102 RNA extracts from NIKS, NIKSmcHPV18 or HeLa cells were analyzed for mRNA expression
103 levels of the three major H3K9-specific methyltransferases, G9a-like protein (Glp1), G9a, and

104 SUV39H1 (27). CaSki cells were also included in our analysis because they harbor an integrated
105 HPV16 genome, another high-risk alpha genotype (29, 30). As shown in Fig. 1A, SUV39H1
106 mRNA levels were significantly upregulated in HPV-transformed vs NIKS cells, especially in HeLa
107 and CaSki cells (8- and 6-fold, respectively), while Glp1 and G9a mRNA levels were only
108 marginally modulated. A similar increase in SUV39H1 protein was also seen in Western blot
109 analysis (Fig. 1B).

110 To further define the mechanistic role of SUV39H1, we assessed mRNA and protein
111 expression levels of RIG-I, cGAS and STING genes in cells treated in the presence or absence of
112 chaetocin, a pharmacological inhibitor of H3K9me3-specific methyltransferase (31, 32). For these
113 experiments, HeLa and CaSki cells were chosen because they displayed higher basal levels of
114 SUV39H1 protein, compared to NIKSmcHPV18. As shown in Fig. 1C, RIG-I mRNA levels were
115 significantly upregulated (15-fold) in both HeLa and CaSki cells after 24 h of chaetocin treatment,
116 while cGAS and STING mRNA expression levels were also increased but to a lesser extent (5- and
117 3-fold in HeLa cells; 4- and 2.4-fold in CaSki cells, respectively). In contrast, NIKS cells, with low
118 basal expression levels of SUV39H1 (Fig. 1A and B), did not show any significant variation in gene
119 expression following chaetocin treatment (Fig. 1C). The same trend was also observed at the
120 protein level for all three genes (Fig. 1D). Consistent with the aforementioned transcriptional
121 activation, a significant decrease in H3K9me2 and H3K9me3 marks (i.e. repressive chromatin)
122 associated with the promoter regions of RIG-I, cGAS and STING was observed by ChIP assay in
123 lysates of chaetocin-treated HeLa (Fig. 1E) and CaSki cells (Fig. 1F). These findings indicate that
124 the H3K9-specific methyltransferases SUV39H1, whose expression is significantly upregulated in
125 HPV-transformed cells, is involved in the modeling of the repressive chromatin structure
126 surrounding the RIG-I, cGAS and STING promoters. Furthermore, pharmacological inhibition of
127 SUV39H1 decreased the promoter-bound heterochromatin marks H3K9me2 and H3K9me3, likely
128 switching the chromatin structure from a repressive to a permissive state.

129 **Pharmacological and genetic inhibition of SUV39H1 activity restores IFN production**

130 **in HPV-transformed cells upon poly(dA:dT) stimulation.** Next, to determine if the drug-induced
131 gain-of-function of RIG-I, cGAS and STING increased IFN production upon stimulation with the
132 DNA agonist poly(dA:dT), NIKS, HeLa, and CaSki cells were treated with chaetocin or vehicle for
133 6 h, transfected with poly(dA:dT) for 24 h, and supernatants harvested to assess IFN production.
134 Consistent with the results above, IFN β production was significantly higher in both chaetocin-
135 treated HeLa and CaSki cells compared to vehicle- or poly(dA:dT)-treated cells (Fig. 2A, left
136 panel); a similar trend was also observed for IFN λ_1 (Fig. 2A, right panel). Consistent with the
137 observed lack of SUV39H1 upregulation in NIKS cells (Fig. 1A and B), chaetocin treatment did not
138 lead to a significant change in IFN production after poly(dA:dT) transfection (Fig. 2A).

139 We next used a lentiCRISPR-based approach to disrupt SUV39H1 in both HeLa and CaSki
140 cells and confirmed protein loss by immunoblotting (Fig. 2B). Accordingly, H3K9me3 expression
141 levels were decreased in SUV39H1 KO cells vs. control cells (4- and 2.5-fold in HeLa and CaSki,
142 respectively), while H3K27me3 levels remained unchanged (Fig. 2B). Consistent with the results
143 observed in chaetocin-treated cells (Fig. 1C and D), upregulation of RIG-I mRNA expression levels
144 upon poly(dA:dT) transfection was higher in SUV39H1 KO cells when compared to parental cells
145 (2-fold in both HeLa and CaSki cells) (Fig. 2C). In contrast, poly(dA:dT) transfection failed to
146 significantly induce both cGAS and STING mRNA levels in cells lacking SUV39H1 (Fig. 2C). The
147 same trend was confirmed at the protein level, where RIG-I was upregulated by 1.5-fold in both
148 poly(dA:dT)-transfected HeLa and CaSki cells compared to their normal counterparts similarly
149 treated (Fig. 2D).

150 Next, cells were treated with poly(dA:dT) to assess the innate immune response in terms of
151 IFN released in the culture supernatants. As shown in Figure 2E, IFN β production increased by 4-
152 and 2-fold in poly(dA:dT)-treated SUV39H1 KO HeLa and CaSki cells, respectively, in
153 comparison with their stimulated parental cells. A similar trend was observed for IFN λ_1 in both cell
154 lines. Altogether, this observation demonstrates that pharmacological or genetic inhibition of
155 SUV39H1 expression leads to modifications in the chromatin structure of the RIG-I, cGAS and

156 STING promoters, switching them from a repressive to a permissive status. The recovery of gene
157 expression, especially in the case of RIG-I, was then able to restore the innate immune response to
158 DNA ligands, as judged by the increased production of IFNs.

159 **HPV E7 regulates SUV39H1 expression levels.** To determine which viral oncoprotein was
160 responsible for the increase in SUV39H1 activity in hrHPV-transformed cells, E6 and E7 proteins
161 were either silenced in HeLa and CaSki cells or overexpressed in HEK293 cells. Because E6 and
162 E7 are transcribed as a single bicistronic pre-mRNA undergoing extensive alternative splicing, we
163 used an siRNA targeting the intron 1 region (siE6/E7_{#1}), only present in unspliced RNA, which
164 would have allowed us to knock down E6 expression in HeLa cells, while only marginally affecting
165 E7 expression (Fig. 3A). In addition, an exon 2-specific siRNA (siE6/E7_{#2}) was also used to
166 simultaneously disrupt E6 and E7 expression in the same cell (33). As shown in Fig. 3B, SUV39H1
167 protein levels were downregulated in siE6/E7_{#2}-transfected HeLa cells but not in cells silenced with
168 siE6/E7_{#1}, unable to inhibit E7 expression, or siCtrl, suggesting that E7 but not E6 regulates
169 SUV39H1 protein expression in these cells. Consistent with SUV39H1 inhibition, total H3K9me3
170 protein levels were only reduced in E7-silenced cells (Fig. 3B). Assessment of the mRNA
171 expression levels of the SUV39H1 gene confirmed that depletion of HPV oncoproteins, mainly E7
172 in the case of HPV18, determined a significant transcriptional inhibition of this gene (Fig. 3C).

173 The same siRNA sets were also used in CaSki cells, in which selected ablation was however
174 not achieved given that both siE6/E7_{#1} and siE6/E7_{#2} were able to knock down both oncoproteins,
175 albeit to different extents (Fig. 3B). As expected, transfection of either siE6/E7_{#1} or siE6/E7_{#2}, both
176 capable of shutting down E7 protein expression, but not siCtrl, resulted in downregulation of
177 SUV39H1 protein levels. Fittingly, H3K9me3 protein expression was significantly inhibited in both
178 E7-silenced cells in comparison with siCtrl-transfected cells.

179 Altogether, these findings indicate that E7 plays a major role in SUV39H1 transcriptional
180 activation and in the ensuing epigenetic silencing of the innate response. Accordingly, mRNA
181 expression levels of RIG-I, cGAS and STING genes were significantly increased after poly(dA:dT)

182 transfection in siE6/E7_{#2}-silenced HeLa cells (Fig. 3D, upper panels). Of note, transcriptional
183 activation of the RIG-I gene was induced 70-fold in unstimulated and 15-fold in siE6/E7_{#2}-
184 transfected HeLa cells (Fig. 3D, upper panels). A similar trend was also observed in E6/E7-silenced
185 CaSki cells: 9-fold induction in unstimulated and 110-fold induction in stimulated siE6/E7_{#1}-
186 transfected CaSki cells (Fig. 3D, lower panels). Importantly, siE6/E7_{#1} generally led to a more
187 robust transcriptional activation of all three genes when compared to cells transfected with
188 siE6/E7_{#2}, in good agreement with the stronger SUV39H1 inhibition shown in Figure 3A and B.

189 Consistent with the restoration of PRR expression, IFN β and IFN λ_1 production was
190 significantly higher in siE6/E7_{#2}- vs. siCtrl-transfected HeLa cells following poly(dA:dT)
191 stimulation (18-fold for IFN β and 10-fold for IFN λ_1 , respectively) (Fig. 3E, upper panels). IFN
192 production was also significantly enhanced in siE6/E7_{#1}-transfected CaSki cells stimulated with
193 poly(dA:dT) compared to similarly treated siCtrl cells (140-fold for IFN β and 2.5-fold for IFN λ_1 ,
194 respectively) (Fig. 3E, lower panels).

195 In parallel, HEK293 cells expressing either E6 or E7 from HPV18 or HPV16 were evaluated
196 for SUV39H1 expression and global H3K9 trimethylation. Interestingly, SUV39H1 expression was
197 increased in all E7-expressing cells compared to control cells (2- and 2.4-fold induction in HEK293
198 cells expressing HPV18 and HPV16, respectively) (Fig. 3F), while SUV39H1 expression was
199 unchanged in E6-expressing cells. Consistently, E7 but not E6, reproducibly increased total
200 H3K9me3 marks (Fig. 3F). Furthermore, SUV39H1 was upregulated at the mRNA level in E7-
201 expressing cells, indicating that the induction occurred at the transcriptional level (Fig. 3G). Lastly,
202 poly(dA:dT)-mediated IFN β and IFN λ_1 production was significantly inhibited in both HPV18 E6-
203 and E7-expressing cells, although to much higher extent in E7- vs. E6-expressing cells (Fig. 3H, 1st
204 and 2nd panels). By contrast, a clear-cut picture emerged in the case of HPV16, where only the E7
205 protein significantly downregulated the release of both IFN β and IFN λ_1 upon poly(dA:dT)
206 transfection as compared to control cells (Fig. 3H, 3rd and 4th panels).

207 **RIG-I is essential to regain the innate immune response in hrHPV-transformed cells.**

208 To further test the hypothesis that RIG-I activation in response to E7-mediated downregulation of
209 SUV39H1 is responsible for restoration of IFN inducibility, we asked whether knock-down of RIG-
210 I expression by lenti-CRISPR would prevent IFN gene upregulation and protein secretion in HeLa
211 and CaSki cells. RIG-I disruption (RIG-I KO) was confirmed by immunoblotting under basal
212 conditions or upon poly(dA:dT) transfection (Fig. 4A). In addition, cells were also stimulated with
213 the sequence-optimized 5'pppRNA RIG-I specific agonist M8 (34-36). IFN transcriptional
214 activation and secretion was then measured under basal conditions or in E6/E7-silenced cell in the
215 presence or absence of the aforementioned stimuli (Fig. 4B and C). Notably, M8 turned out to be a
216 much stronger IFN inducer than poly(dA:dT) in either cell line, especially in the case of IFN β .
217 Similar to what observed for poly(dA:dT), M8-treatment of E6/E7-depleted cells induced higher
218 levels of both IFN β and IFN λ_1 at either the mRNA or protein levels as compared to wild type
219 (WT) cells. Consistent with the results reported in Fig. 3D, E7 silencing by siE6/E7 $_{\#2}$ in HeLa or
220 siE6/E7 $_{\#1}$ in CaSki re-established agonist-mediated IFN β and IFN λ_1 transcription and secretion
221 (Fig. 4B and C, respectively). In contrast, IFN inducibility in response to either agonist in RIG-I
222 KO HeLa failed to restore, indicating that RIG-I is required for IFN induction in HPV-transformed
223 cells. Intriguingly, siE6/E7 $_{\#1}$ -transfected RIG-I KO CaSki cells displayed a significant reduction in
224 IFN production by M8 in comparison with siCtrl-transfected or siE6/E7 $_{\#1}$ -transfected WT CaSki
225 cells, especially with regard to IFN β (Fig. 4C, lower panels). On the other hand, following
226 poly(dA:dT) transfection, siE6/E7 $_{\#1}$ -treated RIG-I KO CaSki cells showed levels of IFN β /IFN λ_1
227 secretion comparable to those observed in treated WT CaSki cells (Fig. 4C, lower panels), implying
228 that the absence of RIG-I signaling might be compensated by the cGAS-STING pathway.

229 Similar results were obtained by directly silencing the SUV39H1 gene in both parental and
230 RIG-I KO cells (Fig. 4D). Once again, M8-mediated induction of IFN β and IFN λ_1 secretion was
231 significantly enhanced in SUV39H1-silenced WT HeLa cells when compared to that of parental
232 cells and more abundant than that observed in poly(dA:dT)-transfected cells (Fig. 4D, upper

233 panels). A similar trend was observed in siSUV39H1-transfected WT CaSki cells for IFN λ_1 in
234 response to poly(dA:dT) or M8 transfection (Fig. 4D, lower right panel). In the case of IFN β (Fig.
235 4D, lower left panel), the enhancement in siSUV39H1-transfected WT CaSki cells was more
236 evident upon poly(dA:dT) transfection than M8 transfection. Consistent with the results shown in
237 Fig. 4C (upper panels), in RIG-I-KO HeLa cells, IFN production was almost abolished in response
238 to either poly(dA:dT) or M8 transfection; in siSUV39H1 RIG-I KO CaSki cells the activity of M8
239 was dramatically reduced, whereas that of poly(dA:dT)-treated cells remained similar to that
240 observed in siSUV39H1-transfected WT CaSki cells stimulated with poly(dA:dT) (Fig. 4D, lower
241 panels).

242 Altogether, these findings clearly indicate that the RIG-I pathway plays a functional role in
243 IFN production in hrHPV-transformed cells. Furthermore, RIG-I signaling can be substantially
244 upregulated by inhibiting either E7 or SUV39H1 expression.

245

246 **DISCUSSION**

247 We recently reported that downregulation of RIG-I, cGAS and STING mRNA levels in
248 hrHPV-harboring cells occurs at the transcriptional level through a novel epigenetic silencing
249 mechanism, as shown by the presence of repressive heterochromatin marks at the promoter region
250 of these genes (25). In the present study, we expand on those findings and show that in hrHPV-
251 transformed cells the increase in the repressive H3K9me2 and H3K9me3 marks is achieved through
252 transcriptional induction of the H3K9-specific methyltransferase SUV39H1 (37). Specifically, we
253 demonstrate that both pharmacological inhibition and gene silencing of SUV39H1 negatively
254 affects the binding of H3K9me2 and H3K9me3 to the promoter region of RIG-I, cGAS and STING
255 genes. The reduction of these two repressive marks at the promoter regions of the aforementioned
256 genes was closely followed by gene transcriptional activation even in the absence of any exogenous
257 stimulus. When SUV39H1 KO cells were treated with poly(dA:dT), the release of both IFN β and
258 IFN λ_1 was significantly increased when compared to stimulated parental cells. Of note, the impact

259 of SUV39H1 activity on chromatin structure in HeLa cells harboring an integrated HPV18 was
260 similar to that observed in CaSki cells containing an integrated HPV16, indicating that these two
261 high-risk genotypes, accounting for the majority of HPV-related cancers (2), have developed
262 evolutionarily conserved strategies in order to epigenetically overturn key players of the innate
263 immune response.

264 We also demonstrate that SUV39H1 upregulation is predominantly dependent on E7 protein
265 expression, as demonstrated by either loss- or gain-of-function experiments. In particular, we show
266 that loss of E7 expression in both HeLa and CaSki cells boosts the innate immune response through
267 inhibition of SUV39H1 activity and transcriptional activation of genes upstream of the IFN
268 cascade, especially RIG-I, which is followed by a substantial increase in IFN production upon
269 poly(dA:dT) transfection.

270 The importance of the RIG-I pathway in hrHPV-transformed cells also emerged when we
271 used a strong RIG-I agonist M8 (34-36). Under basal conditions, M8 treatment of HeLa and CaSki
272 cells was sufficient to achieve robust IFN production compared to poly(dA:dT)-stimulated cells,
273 indicating that the host immune response was strictly dependent on the intrinsic performance of the
274 agonist. Consistent with the results obtained in poly(dA:dT)-transfected cells, this induction was
275 further enhanced in SUV39H1- or E7-depleted cells. Knock-down of the RIG-I gene in hrHPV-
276 transformed cells ablates IFN induction after M8 but not poly(dA:dT) transfection, further
277 confirming that inhibition of SUV39H1 activity preferentially rescues the RIG-I pathway rather
278 than the cGAS-STING pathway.

279 Collectively, these findings demonstrate that drug-targeted activation of the RIG-I signaling
280 pathway may be a feasible option to trigger the innate immune response in HPV-transformed cells,
281 which could potentially improve the effectiveness of existing anticancer therapies (38-44). In
282 summary, the present study describes a novel mechanism whereby impairment of the innate
283 immune response in hrHPV-transformed cells occurs through E7-mediated transcriptional down-
284 regulation of RIG-I, cGAS and STING and is dependent on SUV39H1 activity. As summarized in

285 Fig. 5, our findings show an unprecedented role of SUV39H1 methyltransferase in switching the
286 chromatin status from permissive to repressive, which in turn dampens the innate immune response
287 in hrHPV-transformed cells.

288

289 MATERIALS AND METHODS

290 **Cell culture, plasmids, transfection and treatments.** The spontaneously immortalized
291 human keratinocyte cell line NIKS (Stratatech Corporation) were cultured in the presence of J2 3T3
292 fibroblast feeders as previously described (45). HeLa and HEK293 cells were grown in DMEM
293 (Sigma-Aldrich), and CaSki cells in RPMI (Thermo Fisher Scientific), both supplemented with
294 10% FBS (Sigma-Aldrich). NIKSmcHPV18 cells, stably harboring a high viral load of HPV18
295 episomal genomes, were obtained and cultured as previously described (25).

296 Chaetocin (150 nM) was obtained from Sigma-Aldrich. Poly(dA:dT) (1.25 µg/mL) (InvivoGen)
297 was transfected into cells using Lipofectamine 3000, according to the manufacturer's instruction
298 (Thermo Fisher Scientific). M8 5'pppRNAs (100 ng/mL) was generated as previously described
299 (35) and transfected using Lipofectamine RNAiMax transfection reagent as recommended by the
300 manufacturer (Thermo Fisher Scientific).

301 HeLa and CaSki cells were transfected with siRNA using Lipofectamine RNAiMax transfection
302 reagent (Invitrogen). The following siRNAs were used: SUV39H1 (M-009604-02-0005,
303 siGENOME SMARTpool siRNA), and control siRNA (D-001206-13-05, siGENOME Non-
304 Targeting siRNA Pool) were purchased from Dharmacon; siRNAs against RIG-I and E6/E7_{#1}
305 HPV18 were synthesized by Dharmacon, whereas siRNA against E6/E7_{#2} HPV18, E6/E7_{#1} HPV16,
306 and E6/E7_{#2} HPV16 were synthesized by Sigma-Aldrich. E6/E7_{#1} HPV18 siRNA sequences were
307 kindly provided by Lawrence Banks and available through him (46). The siRNA sequences are
308 available upon request.

309 HPV16 and HPV18 E6 or E7 genes were sub-cloned into pCI-neo mammalian expression vector
310 (Promega) within compatible SalI/ EcoRI or XbaI/EcoRI restriction enzyme sites, respectively. The

311 primer sequences are available upon request. All constructs were sequenced (Eurofins), and
312 overexpression was confirmed by Western blot analysis. HEK293 cells were transiently transfected
313 with pCI-neo vector expressing HPV16 or HPV18 E6, E7, or empty vector control (1 µg) using
314 Lipofectamine 3000 according to the manufacturer's instructions (Thermo Fisher Scientific).

315 **Quantitative nucleic acid analysis.** Real-time quantitative reverse transcription (qRT)-PCR
316 analysis was performed on a CFX96tm Real Time System (Bio-Rad Laboratories Srl). Total RNA
317 was extracted using TRI Reagent (Sigma-Aldrich), and 1 µg was retrotranscribed using iScript
318 cDNA Synthesis kit (Bio-Rad Laboratories Srl). Reverse-transcribed cDNAs were amplified in
319 duplicate using SensiFast SYBR (Bioline) for cellular genes. The glyceraldehyde 3-phosphate
320 dehydrogenase (GAPDH) housekeeping gene was used to normalize for variations in cDNA levels.
321 The reaction conditions consisted of a 30 s at 95°C enzyme activation cycle, 40 cycles of 10 s
322 denaturation at 95°C, and 10 s annealing at 60°C. The primer sequences are available upon request.

323 **Immunoblotting.** Whole-cell protein extracts were prepared and subjected to immunoblot
324 analysis as previously described (47). Nuclear acid extracts were obtained resuspending cell pellets
325 in 200 µl ice-cold lysis buffer (10mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT,
326 and 200 mM HCl supplemented with protease (Sigma-Aldrich) and phosphatase inhibitor cocktail
327 (Active Motif). Cells were kept on ice for 30 min, and then the histone-enriched supernatants were
328 collected by centrifugation at 4°C. Samples were subsequently precipitated with eight volumes of
329 acetone overnight, centrifuged, air dried, and pellets were resuspended in deionized water.

330 The following antibodies were used: rabbit monoclonal antibody anti-SUV39H1 (#702443; Thermo
331 Fisher Scientific, diluted 1:1000), rabbit polyclonal antibodies anti-cGAS (HPA031700; Sigma-
332 Aldrich, diluted 1:500), RIG-I (06-1040; Merck Millipore, diluted 1:10000), anti-H3K9me3 (07-
333 442; Merck Millipore, diluted 1:500), anti-H3K27me3 (07-449; Merck Millipore, diluted 1: 20000),
334 anti-HPV18 E6 (GTX132687; GeneTex, diluted 1:250), anti-HPV18 E7 (GTX133412; GeneTex,
335 diluted 1:500), anti-HPV16 E6 (GTX32686; GeneTex, diluted 1:500), anti-HPV16 E7
336 (GTX133411; GeneTex, diluted 1:500) or mouse monoclonal antibody (MAb) anti-STING

337 (MAB7169; R&D Systems, 1:1500). Mab against α -tubulin (39527; Active Motif, diluted 1:4000)
338 and rabbit antibody against unmodified histone H3 (06-755; Merck Millipore, diluted 1:15000)
339 were used as a control for protein loading. Immunocomplexes were detected using sheep anti-
340 mouse or donkey anti-rabbit immunoglobulin antibodies conjugated to horseradish peroxidase
341 (HRP) (GE Healthcare Europe GmbH) and visualized by enhanced chemiluminescence (Super
342 Signal West Pico; Thermo Fisher Scientific). Images were acquired, and densitometry of the bands
343 was performed using Quantity One software (version 4.6.9; Bio-Rad Laboratories Srl).
344 Densitometry values were normalized using the corresponding loading controls.

345 **ChIP assay.** ChIP assays were performed as previously described (45).
346 Immunoprecipitation was performed with 3 μ g of unmodified histone H3 (06-755), dimethyl-
347 histone H3 (Lys4; 07-030), dimethyl-histone H3 (Lys9; 07-441), trimethyl-histone H3 (Lys9; 07-
348 442), and trimethyl-histone H3 (Lys27; 07-449) antibodies, all purchased from Merck Millipore
349 (Merck Millipore SpA). Threshold cycle (CT) values for the samples were equated to input CT
350 values to provide percentages of input for comparison, and these were normalized to the enrichment
351 level of unmodified histone H3 for each cell line. The primers used to amplify RIG-I, cGAS, and
352 STING promoters are available upon request.

353 **ELISA assay.** The cytokines secreted in the culture supernatants were analyzed using
354 Single Analyte Human ELISA kits for IFN β (DY814-05; DuoSet ELISA Human IFN β , R&D
355 Systems) and IFN λ_1 (DY7246; DuoSet ELISA Human IL-29/IFN λ_1 , R&D Systems) according to
356 the manufacturer's instructions. All absorbance readings were measured at 450 nm using a Victor
357 X4 Multilabel Plate Reader (Perkin Elmer).

358 **Generation of SUV39H1 and RIG-I knockout HeLa and CaSki cells.** SUV39H1 or RIG-
359 I (*DDX58*, *DExD-Hbox helicase 58*) knockout cells were generated with CRISPR/Cas9 technology
360 using single guide RNA (sgRNA) obtained from Applied Biological Materials Inc. (All-in-One
361 Lentivectors: cat. No. K2317005_SUV39H1; K0575405_DDX58; and K010_scrambled sgRNA).
362 To produce viral particles, HEK293T cells were transfected with an All-in-One Lentivector set

363 encoding Cas9 and SUV39H1, DDX58 or scrambled sgRNAs alongside 2nd Generation Packaging
364 System Mix (Applied Biological Materials, Inc.) using Lipofectamine 2000 (Invitrogen). Viral
365 supernatants were harvested at 72 h post-infection and used to transduce cells by infection in the
366 presence of 8 mg/ml polybrene. Transduced HeLa or CaSki cells were selected with puromycin (4
367 µg/ml) 48 h post infection over the course of 14 days post-transduction. After selection, successful
368 knockout was confirmed by immunoblotting.

369 **Statistical analysis.** All statistical tests were performed using Graph-Pad Prism version 5.00
370 for Windows (GraphPad Software). The data are presented as mean ± standard deviation (SD). For
371 comparisons consisting of two groups, means were compared using two tailed Student's t tests.
372 Differences were considered statistically significant at a *P* value of < 0.05.

373

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378

379 **REFERENCES**

380 1. Van Doorslaer K, Ruoppolo V, Schmidt A, Lescroël A, Jongsomjit D, Elrod M, Kraberger S,
381 Stainton D, Dugger KM, Ballard G, Ainley DG, Varsani A. 2017. Unique genome organization
382 of non-mammalian papillomaviruses provides insights into the evolution of viral early proteins.
383 *Virus Evol.* 3(2): vex027. doi:10.1093/ve/vex027.

384

385 2. Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, Stanley MA. 2012. The biology
386 and life-cycle of human papillomaviruses. *Vaccine.* 30 Suppl 5:F55-70.
387 doi:10.1016/j.vaccine.2012.06.083.

388

- 389 3. Egawa N, Egawa K, Griffin H, Doorbar J. 2015. Human Papillomaviruses; Epithelial Tropisms,
390 and the Development of Neoplasia. *Viruses*. 7(7):3863-90. doi:10.3390/v7072802.
- 391
- 392 4. Galloway DA, Laimins LA. 2015. Human papillomaviruses: shared and distinct pathways for
393 pathogenesis. *Curr Opin Virol*. 14:87-92. doi:10.1016/j.coviro.2015.09.001.
- 394
- 395 5. Groves IJ, Coleman N. 2015. Pathogenesis of human papillomavirus-associated mucosal disease.
396 *J Pathol*. 235(4):527-38. doi:10.1002/path.4496.
- 397
- 398 6. Hoppe-Seyler K, Bossler F, Braun JA, Herrmann AL, Hoppe-Seyler F. 2018. The HPV E6/E7
399 Oncogenes: Key Factors for Viral Carcinogenesis and Therapeutic Targets. *Trends Microbiol*.
400 26(2):158-168. doi:10.1016/j.tim.2017.07.007.
- 401
- 402 7. Moody CA, Laimins LA. 2010. Human papillomavirus oncoproteins: pathways to
403 transformation. *Nat Rev Cancer*. 10(8):550-60. doi:10.1038/nrc2886.
- 404
- 405 8. McLaughlin-Drubin ME, Münger K. 2009. Oncogenic activities of human papillomaviruses.
406 *Virus Res*. 143(2):195-208. doi:10.1016/j.virusres.2009.06.008.
- 407
- 408 9. Talis AL, Huibregtse JM, Howley PM. 1998. The role of E6AP in the regulation of p53 protein
409 levels in human papillomavirus (HPV)-positive and HPV-negative cells. *J Biol Chem*.
410 273(11):6439-45. doi:10.1074/jbc.273.11.6439
- 411
- 412 10. Mittal S, Banks L. 2017. Molecular mechanisms underlying human papillomavirus E6 and E7
413 oncoprotein-induced cell transformation. *Mutat Res Rev Mutat Res*. 772:23-35.
414 doi:10.1016/j.mrrev.2016.08.001.

415

416 11. Rincon-Orozco B, Halec G, Rosenberger S, Muschik D, Nindl I, Bachmann A, Ritter TM,
417 Dondog B, Ly R, Bosch FX, Zawatzky R, Rösl F. 2009. Epigenetic silencing of interferon-kappa in
418 human papillomavirus type 16-positive cells. *Cancer Res.* 69(22):8718-25. doi:10.1158/0008-
419 5472.CAN-09-0550.

420

421 12. McLaughlin-Drubin ME, Crum CP, Münger K. 2011. Human papillomavirus E7 oncoprotein
422 induces KDM6A and KDM6B histone demethylase expression and causes epigenetic
423 reprogramming. *Proc Natl Acad Sci U S A.* 108(5):2130-5. doi:10.1073/pnas.1009933108.

424

425 13. Durzynska J, Lesniewicz K, Poreba E. 2017. Human papillomaviruses in epigenetic regulations.
426 *Mutat Res Rev Mutat Res.* 772:36-50. doi:10.1016/j.mrrev.2016.09.006.

427

428 14. Soto D, Song C, McLaughlin-Drubin ME. 2017. Epigenetic Alterations in Human
429 Papillomavirus-Associated Cancers. *Viruses.* 9(9). doi:10.3390/v9090248.

430

431 15. Soto DR, Barton C, Munger K, McLaughlin-Drubin ME. 2017. KDM6A addiction of cervical
432 carcinoma cell lines is triggered by E7 and mediated by p21CIP1 suppression of replication stress.
433 *PLoS Pathog.* 13(10):e1006661. doi:10.1371/journal.ppat.1006661.

434

435 16. Gautam D, Johnson BA, Mac M, Moody CA. 2018. SETD2-dependent H3K36me3 plays a
436 critical role in epigenetic regulation of the HPV31 life cycle. *PLoS Pathog.* 14(10):e1007367.
437 doi:10.1371/journal.ppat.1007367.

438

- 439 17. Brehm A, Nielsen SJ, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T. 1999. The
440 E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. *EMBO*
441 *J.* 18(9):2449-58. doi:10.1093/emboj/18.9.2449
442
- 443 18. Langsfeld ES, Bodily JM, Laimins LA. 2015. The Deacetylase Sirtuin 1 Regulates Human
444 Papillomavirus Replication by Modulating Histone Acetylation and Recruitment of DNA Damage
445 Factors NBS1 and Rad51 to Viral Genomes. *PLoS Pathog.* 11(9):e1005181.
446 doi:10.1371/journal.ppat.1005181.
447
- 448 19. Munger K, Jones DL. 2015. Human papillomavirus carcinogenesis: an identity crisis in the
449 retinoblastoma tumor suppressor pathway. 89(9):4708-11. doi:10.1128/JVI.03486-14.
450
- 451 20. Hong S, Laimins LA. 2017. Manipulation of the innate immune response by human
452 papillomaviruses. *Virus Res.* 231:34-40. doi:10.1016/j.virusres.2016.11.004.
453
- 454 21. Westrich JA, Warren CJ, Pyeon D. 2017. Evasion of host immune defenses by human
455 papillomavirus. *Virus Res.* 231:21-33. doi:10.1016/j.virusres.2016.11.023.
456
- 457 22. Krump NA, You J. 2018. Molecular mechanisms of viral oncogenesis in humans. *Nat Rev*
458 *Microbiol.* 16(11):684-698. doi:10.1038/s41579-018-0064-6.
459
- 460 23. Chiang C, Pauli EK, Biryukov J, Feister KF, Meng M, White EA, Münger K, Howley PM,
461 Meyers C, Gack MU. 2018. The Human Papillomavirus E6 Oncoprotein Targets USP15 and
462 TRIM25 To Suppress RIG-I-Mediated Innate Immune Signaling. *J Virol.* 92(6).
463 doi:10.1128/JVI.01737-17.
464

- 465 24. Lau L, Gray EE, Brunette RL, Stetson DB. 2015. DNA tumor virus oncogenes antagonize the
466 cGAS-STING DNA-sensing pathway. *Science*. 350(6260):568-71. doi:10.1126/science.aab3291.
467
- 468 25. Albertini S, Lo Cigno I, Calati F, De Andrea M, Borgogna C, Dell'Oste V, Landolfo S, Gariglio
469 M. 2018. HPV18 Persistence Impairs Basal and DNA Ligand-Mediated IFN- β and IFN- λ (1)
470 Production through Transcriptional Repression of Multiple Downstream Effectors of Pattern
471 Recognition Receptor Signaling. *J Immunol*. 200(6):2076-2089. doi:10.4049/jimmunol.1701536.
472
- 473 26. Zhang T, Cooper S, Brockdorff N. 2015. The interplay of histone modifications - writers that
474 read. *EMBO Rep*. 16(11):1467-81. doi:10.15252/embr.201540945.
475
- 476 27. Fritsch L, Robin P, Mathieu JR, Souidi M, Hinaux H, Rougeulle C, Harel-Bellan A, Ameyar-
477 Zazoua M, Ait-Si-Ali S. 2010. A subset of the histone H3 lysine 9 methyltransferases Suv39h1,
478 G9a, GLP, and SETDB1 participate in a multimeric complex. *Mol Cell*. 37(1):46-56.
479 doi:10.1016/j.molcel.2009.12.017.
480
- 481 28. Peters AH, O'Carroll D, Scherthan H, Mechtler K, Sauer S, Schöfer C, Weipoltshammer K,
482 Pagani M, Lachner M, Kohlmaier A, Opravil S, Doyle M, Sibilia M, Jenuwein T. 2001. Loss of the
483 Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell*.
484 107(3):323-37. doi.org/10.1016/S0092-8674(01)00542-6
485
- 486 29. Meissner JD. 1999. Nucleotide sequences and further characterization of human papillomavirus
487 DNA present in the CaSki, SiHa and HeLa cervical carcinoma cell lines. *J Gen Virol*. 80 (Pt
488 7):1725-33. doi:10.1099/0022-1317-80-7-1725
489

490 30. Xu F, Cao M, Shi Q, Chen H, Wang Y, Li X. 2015. Integration of the full-length HPV16
491 genome in cervical cancer and Caski and Siha cell lines and the possible ways of HPV integration.
492 *Virus Genes*. 50(2):210-20. doi:10.1007/s11262-014-1164-7.
493

494 31. Kaniskan HÜ, Konze KD, Jin J. 2015. Selective inhibitors of protein methyltransferases. *J Med*
495 *Chem*. 58(4):1596-629. doi:10.1021/jm501234a.
496

497 32. Greiner D, Bonaldi T, Eskeland R, Roemer E, Imhof A. 2005. Identification of a specific
498 inhibitor of the histone methyltransferase SU(VAR)3-9. *Nat Chem Biol*. 1(3):143-5.
499 doi:10.1038/nchembio721
500

501 33. Tang S, Tao M, McCoy JP Jr, Zheng ZM. 2006. The E7 oncoprotein is translated from spliced
502 E6*I transcripts in high-risk human papillomavirus type 16- or type 18-positive cervical cancer cell
503 lines via translation reinitiation. *J Virol*. 80(9):4249-63.
504

505 34. Goulet ML, Olganier D, Xu Z, Paz S, Belgnaoui SM, Lafferty EI, Janelle V, Arguello M,
506 Paquet M, Ghneim K, Richards S, Smith A, Wilkinson P, Cameron M, Kalinke U, Qureshi S,
507 Lamarre A, Haddad EK, Sekaly RP, Peri S, Balachandran S, Lin R, Hiscott J. 2013. Systems
508 analysis of a RIG-I agonist inducing broad spectrum inhibition of virus infectivity. *PLoS Pathog*.
509 9(4):e1003298. doi:10.1371/journal.ppat.1003298.
510

511 35. Chiang C, Beljanski V, Yin K, Olganier D, Ben Yebdri F, Steel C, Goulet ML, DeFilippis VR,
512 Streblow DN, Haddad EK, Trautmann L, Ross T, Lin R, Hiscott J. 2015. Sequence-Specific
513 Modifications Enhance the Broad-Spectrum Antiviral Response Activated by RIG-I Agonists. *J*
514 *Virol*. 89(15):8011-25. doi:10.1128/JVI.00845-15.
515

- 516 36. Beljanski V, Chiang C, Kirchenbaum GA, Olganier D, Bloom CE, Wong T, Haddad EK,
517 Trautmann L, Ross TM, Hiscott J. 2015. Enhanced Influenza Virus-Like Particle Vaccination with
518 a Structurally Optimized RIG-I Agonist as Adjuvant. *J Virol.* 89(20):10612-24.
519 doi:10.1128/JVI.01526-15.
- 520
- 521 37. Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K,
522 Ponting CP, Allis CD, Jenuwein T. 2000. Regulation of chromatin structure by site-specific histone
523 H3 methyltransferases. *Nature.* 406(6796):593-9. doi:10.1038/35020506.
- 524
- 525 38. Patel SA, Minn AJ. 2018. Combination Cancer Therapy with Immune Checkpoint Blockade:
526 Mechanisms and Strategies. *Immunity.* 48(3):417-433. doi:10.1016/j.immuni.2018.03.007.
- 527
- 528 39. Grivennikov SI, Greten FR, Karin M. 2010. Immunity, inflammation, and cancer. *Cell.*
529 140(6):883-99. doi:10.1016/j.cell.2010.01.025.
- 530
- 531 40. Hopcraft SE, Damania B. 2017. Tumour viruses and innate immunity. *Philos Trans R Soc Lond*
532 *B Biol Sci.* 372(1732). pii: 20160267. doi:10.1098/rstb.2016.0267.
- 533
- 534 41. Shekarian T, Valsesia-Wittmann S, Brody J, Michallet MC, Depil S, Caux C, Marabelle A.
535 2017. Pattern recognition receptors: immune targets to enhance cancer immunotherapy. *Ann Oncol.*
536 28(8):1756-1766. doi:10.1093/annonc/mdx179.
- 537
- 538 42. Elinav E, Nowarski R, Thaiss CA, Hu B, Jin C, Flavell RA. 2013. Inflammation-induced
539 cancer: crosstalk between tumours, immune cells and microorganisms. *Nat Rev Cancer.*
540 13(11):759-71. doi:10.1038/nrc3611.
- 541

542 43. Langsfeld E, Laimins LA. 2016. Human papillomaviruses: research priorities for the next
543 decade. Trends Cancer. 2(5):234-240. doi:10.1016/j.trecan.2016.04.001

544

545 44. Smola S. 2017. Immunopathogenesis of HPV-Associated Cancers and Prospects for
546 Immunotherapy. Viruses. 9(9). doi: 10.3390/v9090254.

547

548 45. Lo Cigno I, De Andrea M, Borgogna C, Albertini S, Landini MM, Peretti A, Johnson KE,
549 Chandran B, Landolfo S, Gariglio M. 2015. The Nuclear DNA Sensor IFI16 Acts as a Restriction
550 Factor for Human Papillomavirus Replication through Epigenetic Modifications of the Viral
551 Promoters. J Virol. 89(15):7506-20. doi:10.1128/JVI.00013-15.

552

553 46. Ganti K, Massimi P, Manzo-Merino J, Tomaić V, Pim D, Playford MP, Lizano M, Roberts S,
554 Kranjec C, Doorbar J, Banks L. 2016. Interaction of the Human Papillomavirus E6 Oncoprotein
555 with Sorting Nexin 27 Modulates Endocytic Cargo Transport Pathways. PLoS Pathog.
556 12(9):e1005854. doi:10.1371/journal.ppat.1005854.

557

558 47. Gugliesi F, Mondini M, Ravera R, Robotti A, de Andrea M, Gribaudo G, Gariglio M, Landolfo
559 S. 2005. Up-regulation of the interferon-inducible IFI16 gene by oxidative stress triggers p53
560 transcriptional activity in endothelial cells. J Leukoc Biol. 77(5):820-9. doi:10.1189/jlb.0904507

561

562 **FIGURE LEGENDS**

563 **FIG 1** Pharmacological inhibition of the H3K9-specific histone methyltransferase SUV39H1
564 decreases heterochromatin in hrHPV-transformed cells. (A) Transcript levels of the indicated genes
565 were assessed by qPCR, and values were normalized to those for GAPDH, with NIKS value set to
566 1. Data are presented as mean values of biological triplicates. Error bars indicate SD * $P < 0.05$; ** P
567 < 0.01 (unpaired t test). (B) NIKS, NIKSmcHPV18, HeLa and CaSki total cell extracts were

568 subjected to immunoblot analysis with anti-SUV39H1 and anti-tubulin antibodies. The
569 densitometry values of SUV39H1 were normalized to those of tubulin. Values are representative of
570 three independent experiments. Error bars indicate SD * $P < 0.05$; ** $P < 0.01$ (unpaired t test). (C)
571 NIKS, HeLa and CaSki cells were treated with chaetocin (150 nM) or vehicle (DMSO). After 24 h,
572 transcript levels of the indicated genes were assessed by qPCR, and the values were normalized to
573 those for GAPDH, with each vehicle-treated value set to 1. Data are presented as mean values of
574 biological triplicates. Error bars indicate SD * $P < 0.05$; ** $P < 0.01$ (unpaired t test). (D) NIKS,
575 HeLa, and CaSki cells were treated with chaetocin (150 nM) or vehicle (DMSO). After 24 h, total
576 cell extracts were subjected to immunoblot analysis with anti-RIG-I, cGAS, STING, and anti-
577 tubulin antibodies. The intensities of the bands for each antibody were quantified by densitometry,
578 and ratios of the abundance of these proteins relative to that of tubulin were calculated. Values are
579 representative of three independent experiments. Error bars indicate SD * $P < 0.05$; ** $P < 0.01$
580 (unpaired t test). (E) Extracts were prepared from HeLa or (F) CaSki cells treated for 24 h with
581 chaetocin (150 nM) or vehicle (DMSO). ChIP assay was carried out using antibodies specific for
582 unmodified histone H3 (PAN-H3), trimethylated lysine 4 of H3 (H3K4me3), dimethylated lysine 9
583 of H3 (H3K9me2), trimethylated lysine 9 of H3 (H3K9me3), trimethylated lysine 27 of H3
584 (H3K27me3), or IgG as control. Immunoprecipitated promoter sequences were measured by qPCR,
585 and CT values for the samples were equated to input CT values. Values are represented as relative
586 binding activity from three independent experiments. Error bars indicate SD * $P < 0.05$; ** $P < 0.01$
587 (unpaired t test).

588 **FIG 2** Pharmacological and genetic inhibition of SUV39H1 activity restores IFN production upon
589 poly(dA:dT) transfection in HPV-transformed cells. (A) ELISA quantitation of IFN β and IFN λ_1
590 protein in supernatants from cells treated with chaetocin (150 nM) or vehicle (DMSO) for 6 h and
591 mock-transfected or transfected with poly(dA:dT) for 24 h. Data are presented as mean values of
592 biological triplicates. Error bars indicate SD * $P < 0.05$; ** $P < 0.01$ (unpaired t test). (B) Acid
593 extracts from SUV39H1-deficient (KO) HeLa and CaSki or wild type (WT) cells were subjected to

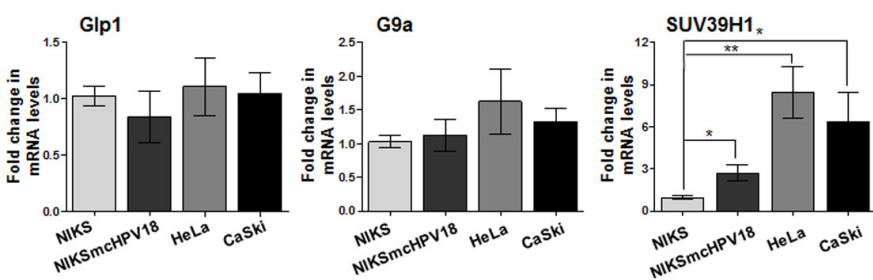
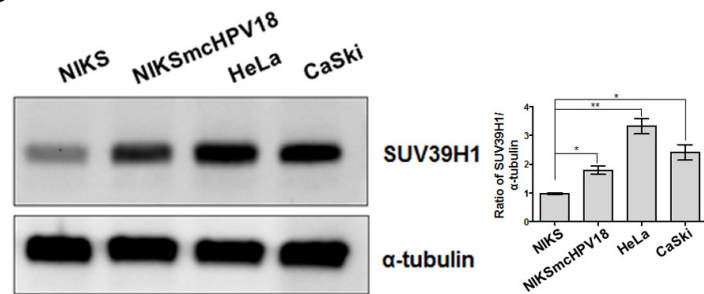
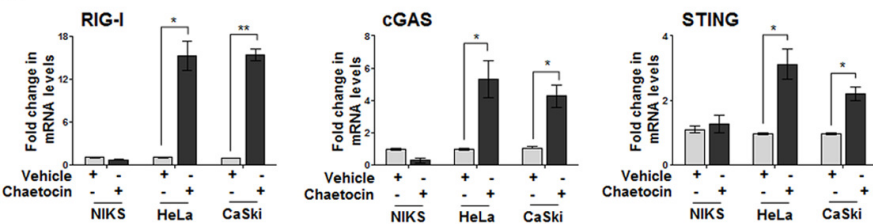
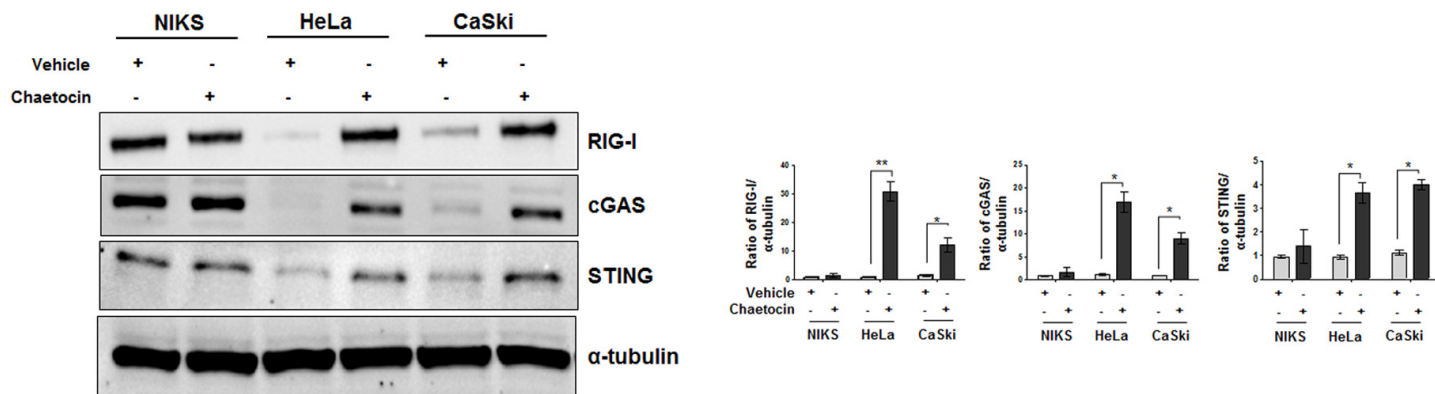
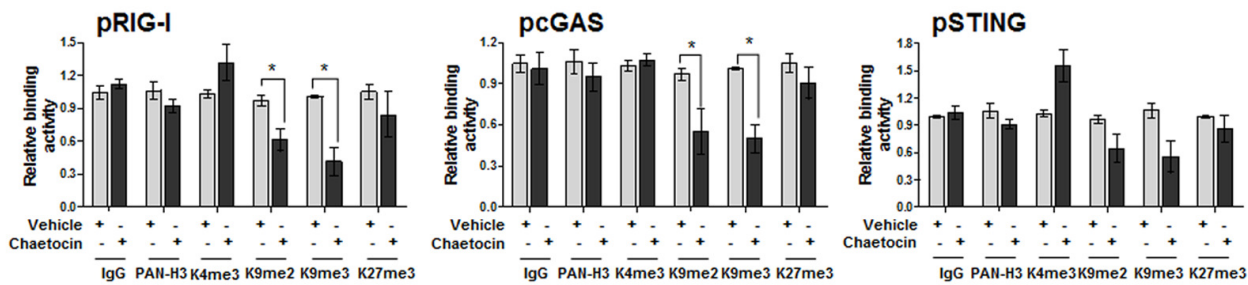
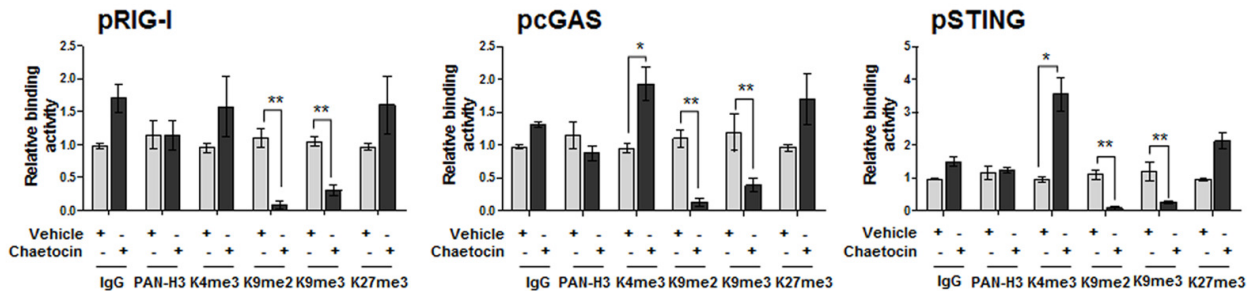
594 immunoblot analysis with anti-SUV39H1, anti-H3K9me3, anti-H3K27me3 or anti-PAN-H3
595 antibodies. The densitometry values of protein bands were normalized to those of PAN-H3. Values
596 are representative of three independent experiments. Error bars indicate SD * $P < 0.05$; ** $P < 0.01$
597 (unpaired t test). (C) Transcript levels of the indicated genes were assessed by qPCR in cells
598 described in panel B, and values were normalized to those of GAPDH, with WT mock-transfected
599 cells value set to 1. Data are presented as mean values of biological triplicates. Error bars indicate
600 SD * $P < 0.05$ (unpaired t test). (D) HeLa and CaSki SUV39H1 KO or control cells were subjected
601 to immunoblot analysis with anti-RIG-I, cGAS, STING or anti-tubulin antibodies. The
602 densitometry values of protein bands were normalized to those of tubulin. Values are representative
603 of three independent experiments. Error bars indicate SD * $P < 0.05$ (unpaired t test). (E) ELISA
604 quantitation of IFN β and IFN λ_1 protein in supernatants from HeLa and CaSki SUV39H1 KO or
605 control cells mock-transfected or transfected with poly(dA:dT) for 24 h. Data are presented as mean
606 values of biological triplicates. Error bars indicate SD * $P < 0.05$ (unpaired t test).

607 **FIG 3** The HPV E7 oncoprotein regulates SUV39H1 expression levels. (A) Diagrams of HPV16
608 and HPV18 E6 and E7 ORFs (boxes with E6 and E7 labels) and bicistronic pre-mRNA transcripts
609 with exons (boxes) and introns (lines between boxes). Numbers above the ORFs and bicistronic
610 transcripts are nucleotide positions in each viral genome. Red boxes indicate siRNA oligo target
611 sites. (B) Total extracts from HeLa or CaSki cells transfected with siE6/E7 $_{\#1}$, siE6/E7 $_{\#2}$ or siCtrl for
612 72 h were subjected to immunoblot analysis with anti-E6, anti-E7 or anti-tubulin, and acid extracts
613 from the same set of samples were probed with anti-SUV39H1, anti-H3K9me3 or anti-PAN-H3.
614 Densitometry values of protein bands were normalized to those of PAN-H3 (acid extracts) or
615 tubulin (total extracts). Values are representative of three independent experiments. Error bars
616 indicate SD * $P < 0.05$ (unpaired t test). (C) Transcript levels of the SUV39H1 were assessed by
617 qPCR in the cells described in panel B. Values were normalized to those of GAPDH, with siCtrl
618 value set to 1. Data are presented as mean values of biological triplicates. Error bars indicate SD * P
619 < 0.05 ; ** $P < 0.01$ (unpaired t test). (D) Transcript levels of the indicated genes were assessed by

620 qPCR in HeLa (upper panels) or CaSki cells (lower panels) transfected with siE6/E7_{#1}, siE6/E7_{#2} or
621 siCtrl for 48 h and then mock-transfected or transfected with poly(dA:dT) for 24 h. Values were
622 normalized to those of GAPDH, with siCtrl-mock-transfected cells value set to 1. Values are
623 representative of three independent experiments. Error bars indicate SD **P* < 0.05; ***P* < 0.01
624 (unpaired *t* test). (E) ELISA quantification of IFN β and IFN λ_1 protein in supernatants from the cells
625 described in panel B, mock-transfected or transfected with poly(dA:dT) for 24 h. Data are presented
626 as mean values of biological triplicates. Error bars indicate SD **P* < 0.05; ***P* < 0.01 (unpaired *t*
627 test). (F) Total or acid extracts from HEK293 cells transfected with pCI-neo, pCI-neo HPV18 E6,
628 pCI-neo HPV18 E7, pCI-neo HPV16 E6 or pCI-neo HPV16 E7 for 72 h were subjected to
629 immunoblot analysis with anti-E6, anti-E7 or anti-tubulin antibodies (all total extracts), while anti-
630 SUV39H1, anti-H3K9me3 or anti-PAN-H3 were used for acid extracts. Densitometry values of
631 protein bands were normalized to those of PAN-H3 (acid extracts) or tubulin (total extracts). Values
632 are representative of three independent experiments. Error bars indicate SD **P* < 0.05 (unpaired *t*
633 test). (G) Transcript levels of the SUV39H1 were assessed by qPCR in HEK293 cells transfected
634 with pCI-neo, pCI-neo HPV18 E6, pCI-neo HPV18 E7, pCI-neo HPV16 E6 or pCI-neo HPV16 E7
635 for 72 h. Values were normalized to those of GAPDH, with pCI-neo-transfected value set to 1. Data
636 are presented as mean values of biological triplicates. Error bars indicate SD **P* < 0.05 (unpaired *t*
637 test). (H) ELISA quantification of IFN β and IFN λ_1 protein in supernatants from the cells described
638 in panels F and G, mock-transfected or transfected with poly(dA:dT) for 24 h. Data are presented as
639 mean values of biological triplicates. Error bars indicate SD **P* < 0.05; ***P* < 0.01; ****P* < 0.001
640 (unpaired *t* test).

641 **FIG 4** RIG-I is crucial for the innate immune response in hrHPV-transformed cells. (A) Total
642 extracts from HeLa and CaSki RIG-I-deficient (RIG-I KO) or wild type (WT) cells, mock-
643 transfected or transfected with poly(dA:dT) for 24 h, were subjected to immunoblot analysis with
644 anti-RIG-I or anti-tubulin antibodies. One representative Western blot of three independent
645 triplicates is shown. (B) Transcript levels of IFN β and IFN λ_1 genes were assessed by qPCR in RIG-

646 I KO HeLa (upper panels) and CaSki (lower panels) or WT cells transfected with siE6/E7_{#1},
647 siE6/E7_{#2} or siCtrl for 48 h and then mock-transfected or transfected with poly(dA:dT) or M8 for 24
648 h. Values were normalized to those of GAPDH, with siCtrl-mock-transfected cells value set to 1.
649 Values are representative of three independent experiments. Error bars indicate SD **P* < 0.05; ***P*
650 < 0.01 (unpaired *t* test). (C) ELISA quantitation of IFN β and IFN λ_1 protein in supernatants from the
651 cells described in panel B. Data are presented as mean values of biological triplicates. Error bars
652 indicate SD **P* < 0.05; ***P* < 0.01; ****P* < 0.001 (unpaired *t* test). (D) ELISA quantification of
653 IFN β and IFN λ_1 protein in supernatants from RIG-I KO HeLa (upper panels) and CaSki (lower
654 panels) or WT cells, transfected with siCtrl or siSUV39H1 for 48 h and mock-transfected or
655 transfected with poly(dA:dT) or M8 for 24 h. Data are presented as mean values of biological
656 triplicates. Error bars indicate SD **P* < 0.05; ***P* < 0.01; ****P* < 0.001, (unpaired *t* test).
657 **FIG 5** Schematic model representing the impact of SUV39H1 activity on the promoter region of
658 RIG-I, cGAS, and STING genes under basal conditions or after transfection with poly(dA:dT) or
659 the RIG-I agonist M8. In the lower panel, the same cellular circuits are illustrated under conditions
660 where E7 or SUV39H1 are inhibited.

A**B****C****D****E****F****Figure 1**

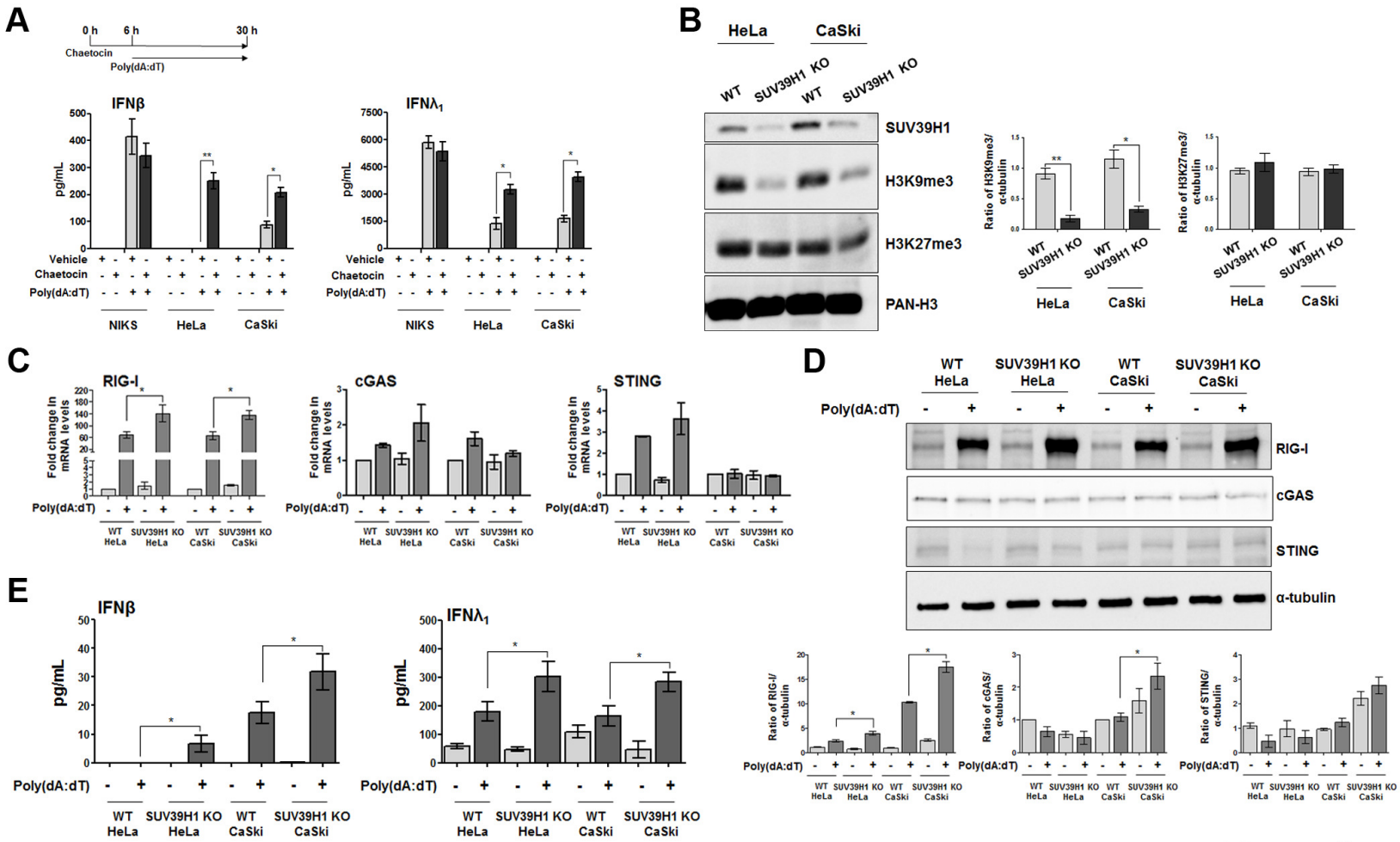


Figure 2

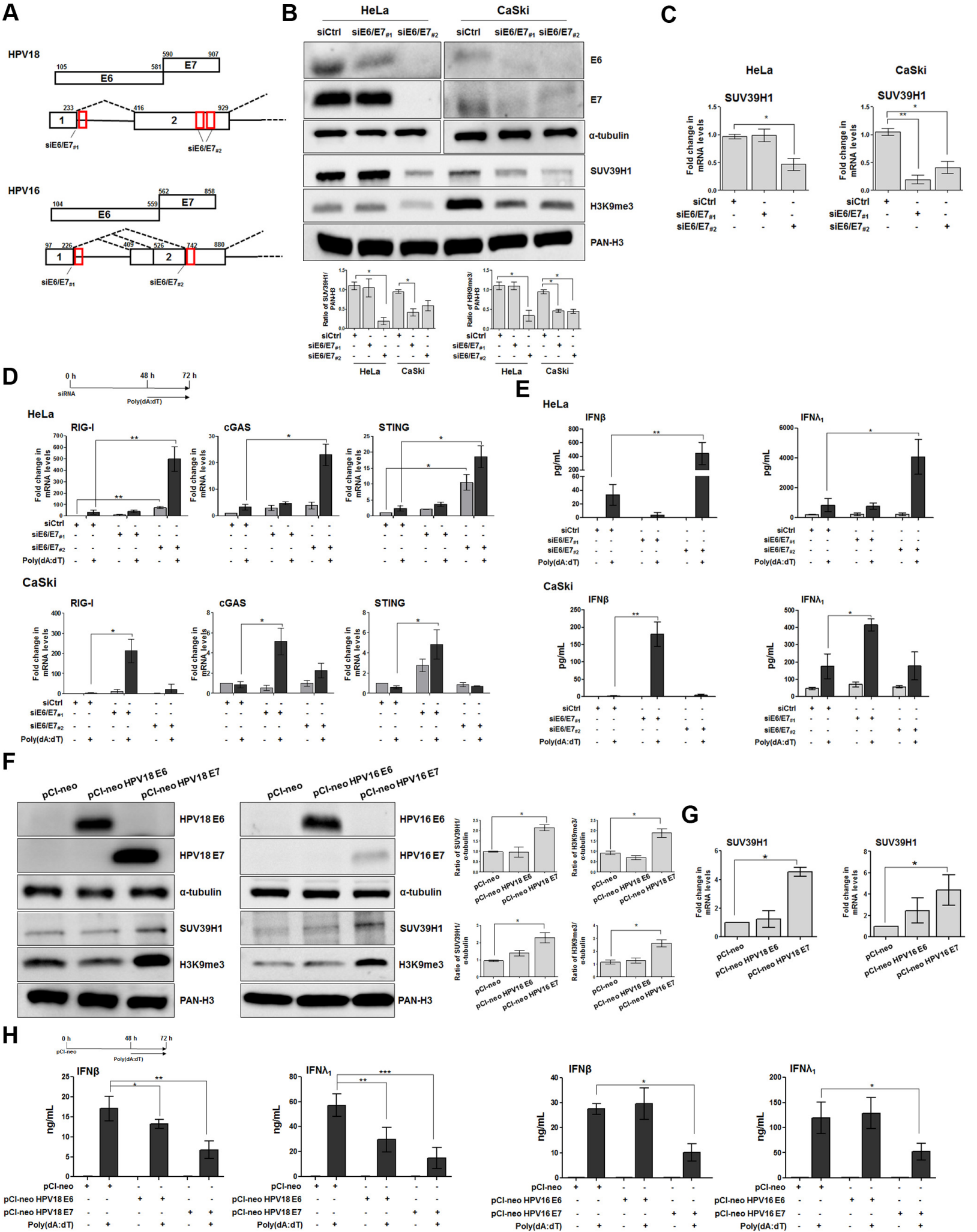
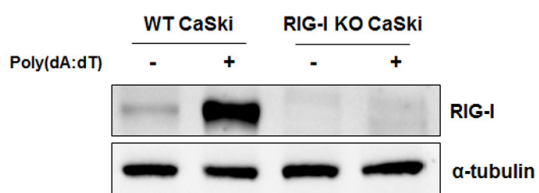
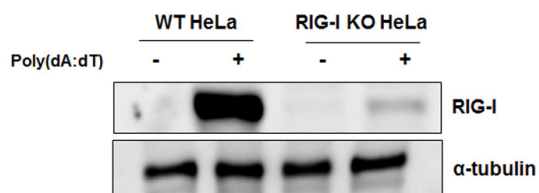
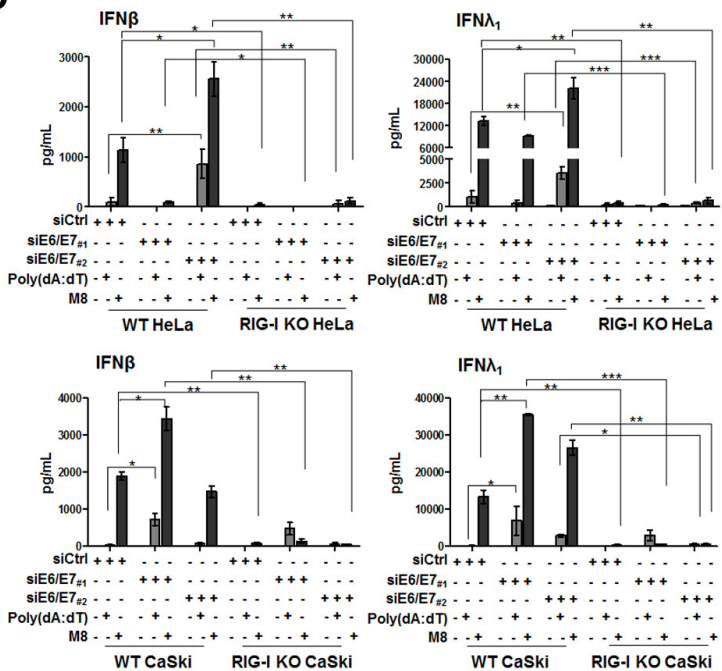
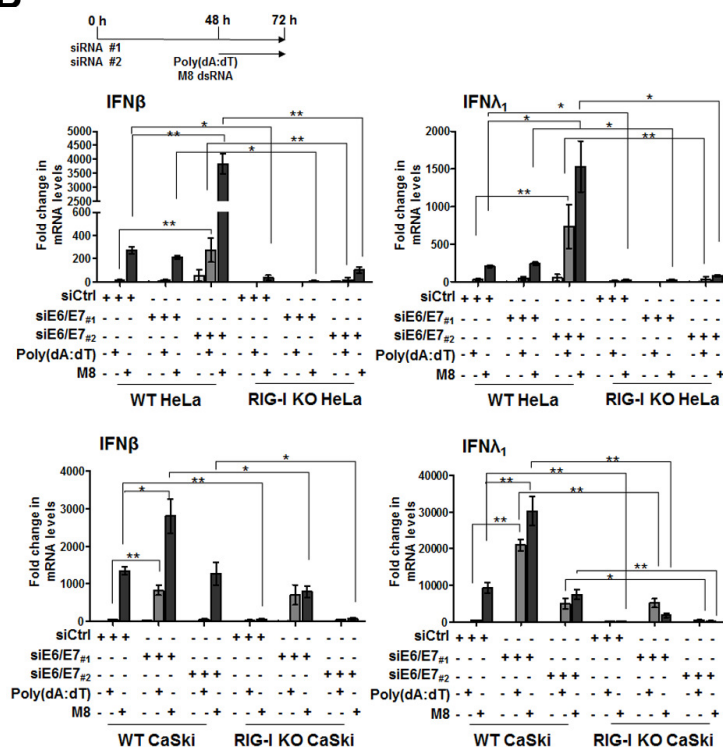
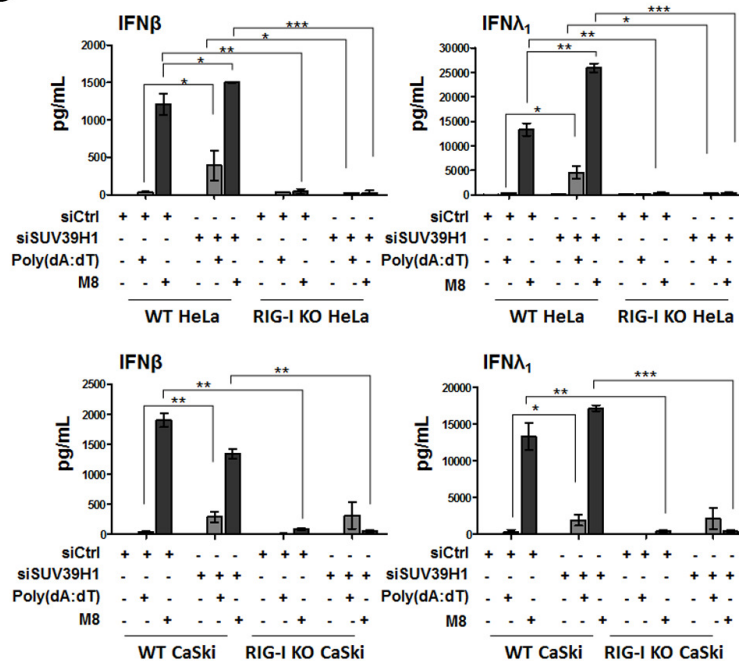


Figure 3

A**C****B****D****Figure 4**

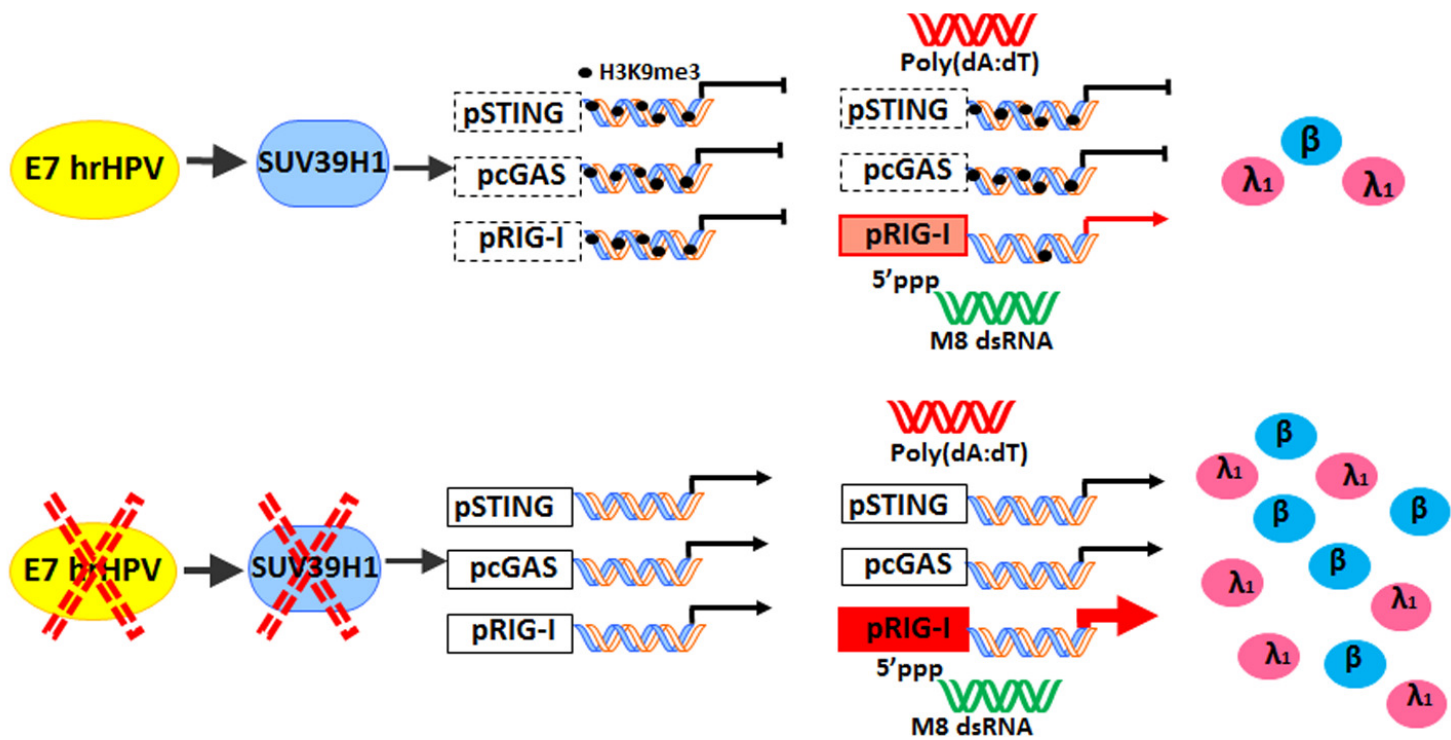


Figure 5