1 **CRISPR/Cas9-based inactivation of human papillomavirus oncogenes E6 and**

2 **E7 induces senescence in cervical cancer cells**

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11 **ABSTRACT**

12 Human papillomaviruses (HPVs) such as HPV16 and HPV18 can cause cancers of

13 the cervix, vagina, vulva, penis, anus and oropharynx. Continuous expression of the

14 HPV viral oncoproteins E6 and E7 are essential for transformation and maintenance

15 of cancer cells. Therefore, therapeutic targeting of E6 and E7 genes can potentially

16 be used to treat HPV-related cancers. Previous CRISPR/Cas9 studies on

17 inactivation of E6 and E7 genes confirmed cell cycle arrest and apoptosis. Here we

18 report that CRISPR/Cas9-based knockout of E6 and E7 can also trigger cellular

19 senescence in HPV18 immortalized HeLa cells. Specifically, HeLa cells in which E6

20 and E7 were inactivated exhibited characteristic senescence markers like enlarged

21 cell and nucleus surface area, increased β-galactosidase expression, and loss of

22 lamin B1 with detection of cytoplasmic chromatin fragments. Furthermore, the

23 knockout of HPV18 E6 and E7 proteins resulted in upregulation of p53/p21 and

24 pRb/p21 levels in senescent cells. These senescent cells were devoid of

25 characteristic apoptotic markers and re-introduction of codon-modified HPV18 E6

26 decreased p53 levels. Taken together, our study demonstrates that cellular

27 senescence is as an alternative outcome of HPV oncogene inactivation by the

28 CRISPR/Cas9 methodology.

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INTRODUCTION

31 Human papillomaviruses (HPVs) are small, non-enveloped viruses with circular

32

33 double-stranded DNA genome. HPV infections are mainly sexually transmitted and

34 the virus exhibits tropism for cutaneous or mucosal squamous epithelium (1). The

35 high-risk HPVs contribute to over 99% of cervical cancer cases and are also

36 identified to cause anogenital cancers as well as head and neck carcinomas (2).

37 More than 200 HPV types have been identified so far and among these the high-risk

38 HPV16 and HPV18 cause 71% of cervical cancer cases worldwide. Cervical cancer

39 is the fourth most frequent cancer in women with an estimated 570,000 new cases

40 and 311,000 deaths in 2018 (3).

41 Progression of persistent HPV infections into tumor development is an unfortunate

42 consequence for the host, considering that this is of no apparent benefit for the virus.

43 Being a small DNA virus of approximately 8 kilobase pairs in genome size, HPV

44 does not encode viral DNA polymerases but completely depends on the host cell

45 replication machinery. A productive HPV life cycle requires initially proliferating

46 epidermal or mucosal epithelial cells, where the viral DNA and early proteins are

47 maintained at low levels in daughter cells. When cells lose the proliferation potential

48 and differentiate, the viral DNA is amplified to very high levels along with increased

49 expression of late genes. Uninfected epithelial cells stop proliferation and undergo

50 terminal differentiation after a certain number of cell divisions. These cells are devoid

51 of DNA replication and therefore cannot support virus replication. To counteract this

52 problem, high-risk HPVs encode the two viral oncogenes E6 and E7, which prolong

53 epithelial cell differentiation and maintain infected cells in DNA synthesis phase for

54 the virus to use cell replication tools (4).

55   The high-risk HPV E7 protein interacts with retinoblastoma tumor suppressor protein

56   (pRb) and its related proteins p107 and p130 through a conserved Leu-X-Cys-X-Glu

57   (LXCXE) motif and disrupts the Rb/E2F transcriptional repressor complex (5,6). This

58   results in release and activation of E2F family of transcription factors that induce

59   unscheduled cell cycle progression. The E7 activated cell cycle progression results

60   in stabilization and accumulation of p53 protein, which causes cell cycle arrest,

61   senescence and apoptosis. The E6 protein from high-risk HPVs targets the p53

62   protein for proteasomal degradation by forming a trimeric complex with E6-

63   associated protein (E6AP) (7,8). In this way, the E6 and E7 proteins conspire to

64   induce and maintain cell transformation by interfering with p53 and pRb proteins that

65   control cell cycle, senescence and apoptosis. Because the E6 and E7 proteins are

66   crucial for the transformed cell, they can be exploited as potential therapeutic targets

67   to control HPV-linked cancers.

68   The HPV or bovine papillomavirus (BPV) E2 protein binds to regulatory regions of

69   E6/E7 viral promoters and represses transcription (9,10). As a result, expression of

70   HPV E2 protein can efficiently block HPV E6 and E7 transcription and expression.

71   Previous studies on inactivation of E6 and E7 by RNA interference or expression of

72   the HPV or BPV E2 protein resulted in induction of senescence and/or apoptosis

73   (11–14). However, application of HPV E2 or RNA interference methods has not been

74   available for clinical treatment. CRISPR (Clustered regularly interspaced short

75   palindromic repeats)/Cas9 is a dsDNA editing method where the Cas9 nuclease

76   from *Streptococcus pyogenes*, guided by small RNAs, can be used to edit target

77   DNA. Inactivation of E6 and E7 genes using CRISPR/Cas9 methodology has been

78   reported to induce arrested cell growth and apoptosis but not senescence (15–17).

79   However, application of CRISPR/Cas9 technology for treatment of cervical cancer

80   needs to be addressed in more detail for a successful clinical application.

81   Here we investigated the CRISPR/Cas9 effect of HPV oncogene inactivation in

82   cervical cancer cells. We found that CRISPR/Cas9 specific knockout of HPV18 E6

83   and E7 proteins rendered senescence like phenotype to cervical cancer cells. The

84   senescence phenotype in cervical cancer cells was confirmed by increase in cell

85   surface area, increased senescence associated β-galactosidase activity, loss of

86   lamin B1 protein and detection of cytoplasmic chromatin fragments. We show that

87   knockout of HPV18 E6 and E7 proteins resulted in activation of p53 and pRb

88   pathways that led to cellular senescence. These senescent cells are devoid of

89   apoptotic markers, and reintroduction of 18E6 protein into senescent cells decreased

90   the p53 levels confirming the robustness of using CRISPR/Cas9 to knock out 18E6

91   and 18E7.

92

93   **MATERIALS AND METHODS**

94

95   **Plasmids and constructs**

96   The *S. pyogenes* Cas9 expression plasmid pX330-U6-Chimeric_BB-CBh-hSpCas9

97   (PX459) (Addgene plasmid 62988) was digested with BbsI and the gRNAs targeting

98   E6 and E7 genes from HPV18 and HPV16 were designed using NEBuilder® HiFi

99   DNA Assembly cloning kit (NEW ENGLAND BioLabs). Briefly, a 71 nucleotide single

100   stranded DNA oligonucleotide containing a 21 base pair target sequence flanked by

101   a partial U6 promoter sequence and scaffold RNA sequence was designed and

102   purchased from Integrated DNA technologies. The ssDNA oligonucleotide and BbsI

103   digested vector were mixed with NEBuilder HiFi DNA Assembly Master Mix and

5

104 incubated for 1 h at 50°C followed by transformation into NEB® 5-alpha

105 Competent *E. coli* (NEB#C2987).

106   The small guide RNAs (gRNA) were designed by utilizing Benchling online tool

107 (Biology software 2019). Two gRNAs were designed against each target gene and

108 two control plasmids were used in every experiment. The px459 plasmid expressing

109 Cas9 alone was used as a common primary control in all experiments (Ctrl-1),

110 whereas plasmid expressing Cas9 with gRNA against HPV16 E6 or E7 was used as

111 secondary control in HeLa cell experiments (Ctrl-2).

112 A green fluorescent protein (GFP) expression plasmid was used as control for

113 transfection efficiency and puromycin selection. For generating codon modified

114 HPV18 E6, the nucleotide sequence of E6 gene was changed for modified codon

115 usage while the original amino acid sequence of the E6 protein is kept intact. The

116 codon modified HPV18 E6 gene was synthesized by GenScript Biotech

117 (Netherlands) and the gene was cloned into pcDNA3.1 vector.

118

119 **Cell lines and transfection**

120 HeLa (CCL-2) cell lines obtained from American Type Culture Collection were

121 cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum

122 and 100 units/ml penicillin G and 100 μg/ml streptomycin solution (Gibco) at 37°C,

123 5% CO_2. All experiments were performed within cell passage 7 to passage 17. The

124 cells were transfected using Turbofect transfection reagent (Thermo Scientific)

125 according to the manufacturer's instructions.

126

127 **DNA extraction and analysis**

6

128   Genomic DNA from Cas9 expressing cells was isolated using genomic DNA

129   extraction buffer (10 mM Tris pH 8.0, 2 mM EDTA, 0.2% Triton X-100 and 200 μg/ml

130   proteinase K). The extracted DNA was PCR amplified using primers targeting the

131   starting and ending regions of HPV18 E6 and E7 genes. The gel purified DNA

132   products were subjected to TA cloning reaction according to the manufacturer's

133   instructions, using TOPO TA cloning kit for sequencing (Invitrogen, 450030). The

134   colonies were sequenced for DNA encoding E6 and E7 to confirm mutations

135   introduced by CRISPR/Cas9. Primers targeting the starting and end regions of E6

136   and E7 DNA were used. The mutations were confirmed by sequence alignment to

137   HeLa control DNA samples.

138

139   **Senescence associated-β-galactosidase assay**

140   The senescence associated-β-gal assay was performed from puromycin selected

141   transfected cells as previously described (18). Briefly, the cells were washed twice

142   with ice cold PBS and fixed with fixation solution (2% formaldehyde (v/v), 0.2%

143   glutaraldehyde (v/v)) for 5 min at room temperature. The fixation solution was

144   removed and cells were washed again twice with PBS. Staining solution (40 mM

145   citric acid/Na_2HPO_4 buffer, 5 mM $K_4[Fe(CN)_6]$ 3 H_2O, 5 mM $K_3[Fe(CN)_6]$, 150 mM

146   NaCl, 2 mM $MgCl_2$ 6 H_2O and 5-Bromo-4-chloro-3-indolyl β-D-galactoside or X-gal

147   (Sigma, B4252, dissolved in dimethyl formamide) was used as 1 mg/ml stock

148   solution was added to the plates and incubated overnight at 37°C. Next day, plates

149   were washed twice with PBS, once with methanol and air-dried. The cells were

150   imaged by phase contrast microscope (Zeiss Axio Observer Inverted Microscope).

151

152   **Western blotting**

153 The Cas9 expressing cells with or without their respective gRNAs were positively

154 selected by puromycin dihydrochloride (Sigma, P8833, dissolved in water), used at a

155 final concentration of 1 μg/ml in HeLa cells for 5 to 8 days, until the gfp expressing

156 control cells were completely dead. As a positive control for apoptosis, HeLa cells

157 were treated with Staurosporine (Sigma, S4400, dissolved in DMSO) used at 0.4 μM

158 and 0.8 μM final concentrations. The cells were lysed in buffer containing 25 mM

159 Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1 % SDS,

160 supplemented with protease inhibitor (Complete Mini EDTA-free, Roche Applied

161 Sciences). The lysates were incubated on ice for 20 min, sonicated and cleared by

162 centrifugation at maximum speed for 10 min, 4°C (19). The supernatants were

163 quantified using Bradford assay and subjected to electrophoresis using 8%-15%

164 homemade gradient gels. The separated proteins were transferred to a 0.2 µm

165 nitrocellulose membrane (Amersham Protran, GE Life Sciences) using wet transfer

166 method for 2 h at 200 mA, 4°C. All membranes were blocked using Odyssey

167 blocking buffer (LI-COR) at room temperature for 1 h. All primary antibodies,

168 incubated overnight at 4°C and IRDye secondary antibodies (LI-COR), incubated at

169 room temperature for 30 min were diluted in Odyssey blocking buffer. The

170 membrane was washed three times in PBS with 0.05% tween 20 before and after

171 the incubation with IRDye secondary antibodies. The membranes were imaged by

172 Odyssey scanner (LI-COR) and quantified using Image Studio software, Version

173 5.2.5 (LI-COR).

174 Primary antibodies used in this study are anti-rabbit GAPDH (Santa Cruz; sc-47724),

175 anti-mouse GAPDH (Ambion; Am4300), anti-mouse HPV18 E6 (Arbor Vita

176 Corporation; AVC399), anti-mouse HPV18 E7 (Santa Cruz; sc-365035), anti-mouse

177 Flag (Sigma; M2, F1804), anti-mouse p53 (Santa Cruz; DO-1, sc-126), anti-rabbit

178   p21 (Cell Signaling; 2947), anti-mouse Rb (Santa Cruz; sc-102), anti-mouse

179   phosphor-Rb (Santa Cruz; sc-271930), anti-rabbit PARP (Gene Tex; GTX100573),

180   anti-rabbit Caspase 3 (Gene Tex; GTX110543), anti-goat Actin (Santa Cruz; sc-

181   1616).

182

Immunofluorescence and quantification

184   Immunofluorescence microscopy was performed as described previously (20). In

185   brief, cells grown on coverslips were fixed in 4% paraformaldehyde for 15 min at

186   room temperature followed by permeabilization with 0.1 % Triton X-100 for 15 min.

187   Cells were washed three times with 2% BSA in PBS supplemented with 0.1%Tween

188   20 (PBST) and blocked with BSA/PBST for 1 h at room temperature. Cells were then

189   incubated with primary antibodies: anti-rabbit Lamin B1 (Abcam; ab16048), anti-

190   mouse α-Tubulin (Santa Cruz; sc-69969), anti-rabbit Phospho-Histone H2A.X (γ-

191   H2AX) (Cell Signaling; 9718), diluted in BSA/PBST overnight at 4°C. The next day,

192   Alexa Fluor-conjugated secondary antibodies (Life Technologies) were added after

193   three washes with BSA/PBST solution. The cells were incubated with secondary

194   antibodies in BSA/PBST for 1 h at room temperature and then washed three times

195   with BSA/PBST. The DNA was labeled by incubation of cells with DAPI solution

196   (Sigma, D9542, dissolved in dimethyl formamide) was used at a working

197   concentration of 300 nM in PBS for 3 min, and washed three times with PBS. The

198   cover slips were then mounted on slides with ProLong Diamond Antifade Mountant

199   (Life Technologies) and imaged using Nikon eclipse 90i microscope. Quantification

200   of signal intensity and cell measurements was done with NIS-elements AR software

201   (Nikon).

202

203 **RESULTS**

204

205 **CRSIPR/Cas9 disruption of HPV18 E6 and E7 genes results in knockout of E6**

206 **and E7 proteins**

207 To study the effect of CRISPR/Cas9-based disruption of the genes encoding HPV18

208 E6 and E7, respectively, in HeLa cancer cell lines, we designed two gRNAs against

209 each coding region. The gRNAs were constitutively expressed using a PX459 vector

210 carrying the Cas9 gene and a puromycin resistance cassette. Throughout the study,

211 we used the PX459 vector expressing Cas9 (no gRNA) with puromycin resistance as

212 common control (Ctrl-1) and as a specificity control we used a PX459 vector

213 expressing gRNAs targeting HPV16 E6 or E7 genes (E6Ctrl-2 or E7Ctrl-2) in HPV18

214 positive HeLa cells. Thus, Cas9 expression alone or Cas9 with gRNAs targeted

215 against HPV16 E6 or E7 genes should not act on HPV18 E6 and E7 within HeLa

216 cells due to the sequence difference between HPV16 and HPV18 E6 and E7 genes.

217 HeLa cells were tested for the knockout of E6 and E7 proteins by extraction of whole

218 cell protein lysates from puromycin selected CRISPR/Cas9 expressing cells with

219 gRNAs against E6 and E7 regions of HPV18 and HPV16 types. As shown in figures

220 1A and 1C, western blot analysis using antibodies against HPV18 E6 and E7

221 proteins confirmed efficient knockout of E6 and E7 proteins in lysates with HPV18

222 specific gRNAs. The protein lysates expressing Cas9 without gRNA or with

223 nonspecific gRNA were positive for endogenous E6 and E7 proteins confirming

224 specificity of the CRISPR/Cas9 knockout. The intensity of endogenous E6 and E7

225 proteins from at least four different experiments were normalized to GAPDH levels

226 and compared with Ctrl-1 as standard (Figs. 1B and 1D). To validate if the knockout

227 of E6 and E7 proteins in HeLa cells was due to CRISPR/Cas9 cleavage of

10

228 endogenous DNA at predicted E6 and E7 regions, we isolated genomic DNA from

229 the cells and sequenced the regions coding for E6 and E7. We found that the

230 predicted CRISPR/Cas9 cleavage sites within the E6 and E7 regions contained

231 insertions or deletions that disrupted the functional E6 and E7 protein expression.

232 The analyzed clones from HPV18 E6 gRNA-expressing cells contained three

233 sequences with deletion mutations and one sequence with an insertion that resulted

234 in frameshift mutation (Fig. 1E). The analyzed clones from HPV18 E7 gRNA-

235 expressing cells contained deletion mutations and one sequence with an insertion.

236 Taken together, we show that site-specific CRISPR/Cas9 cleavage efficiently

237 removed endogenous E6 and E7 proteins from HPV18 HeLa cells (Fig. 1E).

238

239 **HeLa cells devoid of E6 or E7 proteins exhibit senescence-associated β-**

240 **galactosidase activity and altered cell morphology**

241 Positive selections of transfected cells by antibiotic usually results in single cell

242 colonies followed by removal of untransfected cells. To generate homogenous single

243 cell populations with CRISPR/Cas9 expression, the HeLa cells were transfected with

244 plasmids expressing gRNAs and Cas9 for 40 h and then selected on puromycin for

245 over 4 days to eliminate untransfected cells. When observed under light microscope,

246 the puromycin-selected cells with gRNAs against HPV18 E6 and E7 appeared

247 enlarged with distinct morphology whereas the control cells with Cas9 alone or with

248 unspecific HPV16 gRNAs formed actively growing compact single colonies (Fig. 2A).

249 The puromycin selected cells not only failed to form single colonies, but also stopped

250 proliferation and did not grow confluent. This was unexpected, since previous

251 CRISPR/Cas9 studies targeting HPV E6 and E7 oncogenes have not reported this

252 phenomenon. Senescent cells are known to exhibit distinct characteristics like

253   increased cell morphology, elevated levels of lysosomal endogenous β-

254   galactosidase activity detectable at pH 6.0 compared to normal growing cells (21).

255   To further test for senescence, we performed a β-galactosidase assay on

256   CRISPR/Cas9 treated cells. The morphologically enlarged E6 and E7 knockout cells

257   exhibited increased blue staining, which confirmed senescence associated β-

258   galactosidase activity. The control HeLa cells grew as compact colonies and were

259   negatively stained for β-galactosidase activity (Fig. 2A).

260   Enlarged cell body and irregular shape due to rearrangements in cytoskeleton is

261   considered one of the distinct features of senescent cells. In line with that, the HeLa

262   E6 and E7 knockout cells were detectably enlarged in size and with greater surface

263   area than control cells. To study the extent of changes in size and morphology, we

264   measured the cell surface area and cell nucleus surface area after performing

265   immunofluorescence microscopy. Cells expressing Cas9 with gRNAs against E6 and

266   E7 along with control gRNAs were permeabilized, fixed and analyzed for α-tubulin as

267   cytoplasmic marker and DAPI staining for nucleus boundaries. By using Nikon NIS

268   elements imaging software, individual cells were manually selected for surface area

269   measurements. The total surface area measurements of cells as well as the nucleus

270   were plotted separately (Figs. 2D and 2E). The total surface area of E6 and E7

271   inactivated HeLa cells appeared 4-fold greater when compared to control cells

272   expressing Cas9 alone or with HPV16 E6 or E7 gRNA. Furthermore, the nucleus

273   surface area of senescence positive cells was increased >2–fold as compared to the

274   control HeLa cells. In summary, specific inactivation of E6 or E7 genes by

275   CRISPR/Cas9 resulted in enhanced lysosomal β-galactosidase activity, altered cell

276   morphology and increased total cell and nuclear surface area consistent with a

277   senescence-like phenotype.

278

HPV18 E6 or E7 inactivated HeLa cells display further hallmarks of senescence

The senescence-associated phenotype in cells is variable and heterogeneous, and there is no single universal marker to detect senescence. With increase in complexity of senescence-associated phenotypes, confirmation of senescence with more than two markers has been recommended. It is therefore crucial to use a combination of methods to confirm senescence in cells. To further validate the senescent phenotype we probed for changes in nuclear envelope protein, lamin B1 and activation of DNA damage response with detection of γ-H2AX nuclear foci, two commonly used senescence markers (22,23). Recent studies have shown that loss of lamin B1 results in nuclear changes such as appearance of cytoplasmic chromatin fragments associated with DNA damage markers like γ-H2AX (24). Hence, we tested the cells for lamin B1 levels, γ-H2AX expression and presence of cytoplasmic chromatin fragments markers by immunofluorescence microscopy.

Antibodies against lamin B1 and γ-H2AX were used to detect specific signals from CRISPR/Cas9 expressing cells and the mean signal intensity was quantified using Nikon NIS elements imaging software. The mean intensity of the quantified lamin B1 and γ-H2AX signals were plotted and shown in figures 2F and 3. As evident from the quantification, the E6 and E7 knockout HeLa cells exhibited reduced lamin B1 levels and increased γ-H2AX nuclear foci signal relative to control treated HeLa cells. Loss of lamin B1 would compromise the integrity of nucleus, which leads to detection of cytoplasmic chromatin fragments (19,20). To test this, we checked for presence of cytoplasmic chromatin fragments by DAPI stained fragments in the cytoplasm (Fig. 3). Parts of these DAPI-stained cytoplasmic fragments were found associated with γ-H2AX. On the other hand, the overall DAPI stained cytoplasmic fragments and γ-

13

303   H2AX nuclear foci were relatively absent in control treated cells (Fig. 3). Taken

304   together, these results indicate that oncogene inactivation-induced senescent cells

305   display decrease in lamin B1 protein, increased γ-H2AX nuclear foci, and presence

306   of DAPI stained cytoplasmic fragments associated with γ-H2AX nuclear foci.

307

308   **p53/p21 signaling is activated in HPV18 E6 knockout cells**

309   The significant role of the HPV18 E6 protein is to promote proteasomal degradation

310   of p53 protein by recruitment of the E3 ubiquitin ligase E6AP. Continuous expression

311   of E6 protein in HeLa cells results in decreased endogenous p53 protein levels.

312   Therefore, CRISPR/Cas9 mediated knockout of endogenous E6 protein would

313   activate p53 signaling pathways. To test this, the whole cell protein lysates from

314   HPV18 E6 knockout cells were made and analyzed for endogenous p53 and p21

315   proteins. As expected, the endogenous p53 and p21 protein levels were restored

316   and increased expression was observed in HPV18 E6 gRNA expressed cells (Figs.

317   4A-4C). The Cas9 protein expression in the puromycin-selected cells was verified by

318   using anti-Flag antibody, which detects the Flag-Cas9 protein. In the control cells the

319   p53 and p21 protein signals remained lower confirming the presence of active

320   endogenous HPV18 E6 protein (Figs. 4A-4C). Inactivation of HPV18 E6 restored p53

321   protein levels resulting in activated expression of p21 downstream signaling that

322   could lead to cellular senescence.

323

324   **pRb/p21 signaling is activated in HPV18 E7 knockout cells**

325   The HPV18 E7 protein binds to tumor suppressor protein pRb and thereby disrupts

326   the Rb/E2F transcriptional repression. Further, the HPV16 E7 protein has been

327   shown to destabilize and degrade Rb family proteins (25,26). In combination to

14

328   disruption of Rb/E2F complex, high-risk HPV E7 proteins can directly interact and

329   inactivate p21 and p27 proteins to maintain cell proliferation (27,28). Therefore,

330   knockout of HPV18 E7 protein might destabilize the pRb and hence decrease the

331   phosphorylated pRb levels in the cells. Western blot analysis of HPV18 E7 knockout

332   HeLa cell lysates demonstrated that the pRb protein levels remained unchanged

333   compared to control treated HeLa cells (Fig. 4D). The stable pRb protein levels in

334   control E7gRNA-treated cells could imply pRb protein not being destabilized in HeLa

335   cells. Notably, pRb proteins showed drastically reduced phosphorylation in HPV18

336   E7 knockout cells compared to control cells. The knockout of E7 protein correlates

337   with decreased inactive pRb, which could relate to hypophosporylated Rb being

338   available to bind E2F resulting in cell cycle arrest. The endogenous p53 protein

339   levels in HPV18 E7 knockout cells remained relatively unchanged, but the p21 levels

340   were elevated compared to control cells (Figs. 4D and 4E). The p53 independent

341   increase in p21 expression could be due to the direct effect of the E7 protein on p21

342   protein expression. Altered pRb levels in combination with increased p21 expression

343   can result in cellular senescence in E7 knockout HeLa cells (29). Altogether, we

344   could confirm that knockout of either HPV18 E6 or HPV18 E7 protein levels in HeLa

345   cells could restore endogenous p53/p21 and pRb/p21 signaling to induce

346   senescence.

347

348   **Apoptotic markers are absent in HPV18 E6 and E7 knockout senescent cells**

349   Previous studies on CRISPR/Cas9 application for HPV18 E6 and E7 inactivation

350   reported apoptosis rather than senescence as downstream result. We therefore

351   tested if the CRISPR/Cas9 inactivated HPV18 E6 or E7 cells that showed

352   senescence (Fig. 2), also exhibited apoptosis signatures. As a positive control for

353　apoptotic markers we treated HeLa cells with staurosporine at increasing

354　concentrations and collected the protein lysates. The protein lysates were subjected

355　to western blot analysis and probed for apoptotic markers using antibodies against

356　PARP1 and caspase-3, which are cleaved to activate apoptosis (30,31). Cell lysates

357　treated with staurosporine displayed increased levels of PARP fragments and

358　decreased levels of full-length PARP (Figs. 5A and 5C). We did not observe any

359　cleavage products of PARP or caspase-3 in either HPV18 E6 or HPV18 E7 knockout

360　cell lysates which might indicate that these cells are resistant to apoptosis. In case of

361　HPV18 E6 knockout cells we noticed a total decrease in PARP levels when

362　compared to HPV18 E7 knockout cells. When probed for HPV18 E7 protein levels,

363　we observed decreased endogenous E7 protein levels in lysates treated with

364　staurosporine while the control cells retained normal E7 protein levels. The

365　endogenous HPV18 E6 levels remained unaltered with staurosporine treatment. In

366　conclusion, the HPV18 E6 or E7 knockout cells were negative for characteristic

367　markers of apoptosis (Figs. 5A-5D)

368

369　**Re-introduction of HPV18 E6 protein in senescent cells decreases p53 levels**

370　To confirm the specific effect of HPV18 E6 knockout by CRISPR/Cas9, we re-

371　introduced the HPV18 E6 protein into HPV18 E6 knockout senescent cells.

372　Repression of endogenous E6 protein levels resulted in enhanced endogenous p53

373　levels (Fig. 6). At the same time, introduction of exogenous HPV18 E6 into E6 gRNA

374　induced senescent cells lowered the total endogenous p53 levels. The alteration of

375　endogenous p53 expression with inactivation and reintroduction of HPV18 E6 protein

376　correlated with endogenous p21 protein expression in these cells. The switch of p53

377   levels with respect to E6 protein levels confirms the robustness of CRISPR/Cas9

378   system for specific inactivation of HPV E6.

379

380   **DISCUSSION**

381

382   Continuous expression of high-risk HPV E6 and E7 oncogenes is essential for

383   maintenance and survival of cervical cancer cells. The cervical cancer cells are "viral

384   oncogene addicted" meaning that any disruption of these oncogenes is detrimental

385   to the cells. At present, no treatment options other than surgery are available for

386   HPV-induced cancers. Even after surgical treatment, risk of recurrence due to

387   persistent infection compromises the chances to cure. Hence, there is an urgent

388   need for development of treatment options to treat HPV related cancers and

389   persistent infections. With recent advancements in DNA editing technology,

390   CRISPR/Cas9 has evolved as a powerful genome-editing tool with precision and

391   specificity. Using the CRISPR/Cas9 expression system we specifically knocked out

392   integrated E6 and E7 regions from HPV18 induced HeLa cancer cells, which

393   resulted in cellular senescence and stopped cell proliferation (32).

394

395   Integration of E6 and E7 regions within the host cell in combination with non-viral risk

396   factors facilitate progression of HPV infections to invasive cancer. Recent work has

397   shown that inactivation of high-risk HPV E6 and E7 genes by CRISPR/Cas9 systems

398   restored the p53 and pRb networks resulting in induction of cell cycle arrest and

399   apoptosis as final outcome (15–17). Studies from Jubair et al., indicated that

400   systemic administration of Cas9/gRNAs against HPV16 E7 Caski cell and HeLa cell

401   tumors in mice were cleared by apoptosis (17). Similarly, lentiviral transduction of

402   Cas9/gRNA against HPV 18 E6 and E7 genes for a 10-day period induced cell death

403   in HeLa cells (15). In line with these two studies, we transfected Cas9/gRNA

404   expressing plasmid to target HPV18 E6 and HPV18 E7 genes and positively

405   selected the transfected cells using puromycin. Within 4 to 6 days of selection, we

406   noticed senescent cells with enlarged morphology instead of compact colonies as in

407   control treated cells. Remarkably, we identified DAPI stained chromatin fragments in

408   cytoplasm of senescent cells (Figs. 3A and 3B) and to our knowledge this the first

409   reported example of detection of cytoplasmic chromatin fragments in HPV18 E6 and

410   E7 knockout senescent cells. Since previous CRISPR/Cas9 studies on HPV18

411   oncogenes did not report senescence or assayed for senescence markers, it is

412   difficult to exclude senescence as a potential outcome. It is possible that cells might

413   undergo either senescence or apoptosis or both depending on the extent of intrinsic

414   and extrinsic cellular stress. We hypothesize that the technical reasons behind the

415   differences in alternative outcomes could be anything from differences in expression

416   levels of Cas9, selection procedure, gRNA design, and experimental outline.

417

418   Before the advent of CRISPR/Cas9 technology, the HPV oncogene inactivation was

419   achieved by expression of human or bovine papillomavirus E2 protein, a virus-

420   encoded negative regulator of E6 and E7, or by RNA interference (RNAi). Inhibition

421   of E6 and E7 expression in cervical cancer cells by E2 protein or RNAi resulted in

422   cell cycle arrest with senescence (12–14) or apoptosis as end point (11). The

423   induction of senescence was reported to be due to activation of dormant tumor

424   suppressor pathways, like the p53 and pRb pathways repressed by E6 and E7

425   (12,33). In agreement with the above-mentioned studies, we could successfully show

426   that CRISPR/Cas9 knockout of HPV18 E6 and E7 genes can induce cellular

18

427   senescence in HeLa cells (Figs. 2 and 3). The p21 protein is one of the key

428   mediators of therapy-induced senescence and high expression of p21 protein is

429   often associated with senescence (29,34). With the knock down of HPV18 E6 and

430   E7 proteins we observed the increase in p21 levels both in p53-dependent and p53-

431   independent environments leading to senescence (Fig 4).

432

433   Cellular senescence, like apoptosis, appears to act as a defense mechanism against

434   viruses. Expression of HPV18 E6 and E7 proteins prevents senescence in terminally

435   differentiated cells like keratinocytes, which if untransformed undergo replicative

436   senescence. Therefore, it is reasonable that inactivation of HPV18 E6 and HPV18

437   E7 genes could result in senescence. Another explanation for induction of

438   senescence in HPV-positive cancer cells upon E6 and/or E7 repression could be due

439   to activation of the mTOR signaling pathway. This model suggests that continued

440   activation of mTOR signaling mediates the conversion of reversible cell growth that

441   occurs due to repression of E6 and E7 oncogenes into irreversible growth arrest or

442   senescence (35,36).

443

444   From a functional importance and proof of concept point, our results highlight an

445   alternative outcome pointing senescence as permanent block of cancer cell growth.

446   These results might be extended to develop clinically applicable viral vector or

447   delivery systems with potential to treat HPV-linked cancers. Furthermore, senescent

448   HeLa cells generated by inactivation of HPV18 E6 or E7 oncogenes can act as a

449   robust cell model system to understand the details of senescence mechanisms and

450   for screening senolytic agents to stop ageing.

451

Conflict of Interest

The authors declare no conflict of interest

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594

595 **Figure legends**

596

597 **Fig. 1 HPV18 E6 and E7 protein knockout as a result of Cas9 specific cleavage**

598 **at predicted HPV genome sites.** (A) Western blot analysis of puromycin selected

599 HeLa cells expressing Cas9 (Ctrl-1), Cas9 with HPV18 E6 specific gRNAs (E6gRNA-

600 1 and E6gRNA-2) and Cas9 with HPV16 E6 specific gRNA (Ctrl-2). The blot was

601 probed for antibodies against HPV18 E6 and GAPDH. Representative image of four

25

602   independent experiments is shown. (B) Quantification of HPV18 E6 signal intensity

603   normalized with GAPDH and compared with Ctrl-1. (C) HeLa cells expressing Cas9

604   (Ctrl-1), Cas9 with HPV18 E7 specific gRNAs (E7gRNA-1 and E7gRNA-2) and Cas9

605   with HPV16 E7 specific gRNA (Ctrl-2) were analyzed by western blot. The blot was

606   probed for HPV18 E7 and GAPDH antibodies. Representative image of four

607   independent experiments is shown. (D) Quantification of HPV18 E7 signal intensity

608   normalized with GAPDH and compared with Ctrl-1. Error bars represent mean

609   values with standard deviation; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$,

610   two-tailed student's *t*-test was used to assess statistical significance. (E) DNA

611   sequence alignment of HPV18 E6 and E7 sequences retrieved from HeLa cells

612   treated with Cas9 and the respective gRNAs as indicated. The proto-spacer-adjacent

613   motif (PAM) is denoted in bold and the red bold letters indicate the nucleotide

614   insertions.

615

616   **Fig. 2 HPV18 E6 and E7 inactivated HeLa cells display senescence phenotype.**

617   (A) Light microscopic images showing senescence –associated β-galactosidase

618   assay of HeLa cells expressing Cas9 (Ctrl-1), Cas9 with 18E6 specific gRNAs

619   (E6gRNA-1, E6gRNA-2) or 18E7 specific gRNAs (E7gRNA-1, E7gRNA-2) and Cas9

620   with 16E6 or 16E7 specific gRNA (Ctrl-2) treated HeLa cells. Scale bar 100μm.

621   Representative image of three independent experiments is shown. (B) and (C)

622   Indirect immunofluorescence microscopy of HeLa cells expressing Cas9 (Ctrl-1),

623   Cas9 with 18E6 specific gRNAs (E6gRNA-1, E6gRNA-2) or 18E7 specific gRNAs

624   (E7gRNA-1, E7gRNA-2), and Cas9 with nonspecific HPV16E6 and E7 gRNAs (Ctrl-

625   2). Shown from left to right are DAPI stained nucleus, tubulin stained cytoplasm

626   along with lamin B1 and merge of images. Representative image of three

627   independent experiments is shown. Scale bar 20μm. (D) The cell surface area

628   measurements of tubulin staining cells from panel B and C plotted for the respective

629   experiment. Mean values from three independent experiments were shown. The Y-

630   axis is in log$_2$ scale. (E) Quantitative measurements of cell nucleus surface area from

631   DAPI stained cells from panel B. Mean values from three independent experiments

632   were shown. The Y-axis is in log$_2$ scale. (E) The lamin B1 signal from HeLa cells in

633   panel B and C were quantified from three independent experiments and mean

634   values are shown from each experiment. The error bars indicate mean values with

635   standard deviation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, nested one-

636   way analysis of variance (ANOVA) with Turkey's multiple comparison test was used

637   to assess statistical significance.

638

639   **Fig. 3 HPV18 E6 and E7 inactivated cells are positive for senescence markers.**

640   Immunofluorescence microscopy of cells with DAPI stained nucleus, γ-H2Ax and α-

641   tubulin stained cytoplasm after transfection with Cas9 (Ctrl-1), Cas9 with HPV18 E6

642   specific gRNAs (E6gRNA-1, E6gRNA-2) or E7 specific gRNAs (E7gRNA-1,

643   E7gRNA-2) and Cas9 with nonspecific gRNAs (Ctrl-2). Panel (A) represents cells

644   with HPV18 E6 gRNAs and controls. Panel (B) shows cells with HPV18 E7 specific

645   gRNAs and control cells. White arrows indicate cytoplasmic chromatin fragments.

646   Scale bar 20μm. Representative image of three independent experiments is shown

647   in both panels A and B.

648

649   **Fig. 4 HPV18 E6 and E7 knockout restores p53 and pRb tumor suppressor**

650   **pathways.** (A) HeLa cells expressing only Cas9 (Ctrl-1), Cas9 with 18 E6 specific

651   gRNAs (E6gRNA-1 and E6gRNA-2) and Cas9 with non-specific gRNA (Ctrl-2) were

652 analyzed by western blot. The blot was probed to detect Flag-tagged Cas9 protein,

653 p53, p21, HPV18 E6 and GAPDH. Representative image of three independent

654 experiments is shown. Quantification of endogenous p53 and p21 levels were

655 performed and relative intensities were plotted for the respective experiment in panel

656 (B) and (C). (D) Western blot analysis of protein lysates from cells treated similarly

657 as explained in panel (A), but the gRNAs expressed were targeted for endogenous

658 HPV18 E7 protein and the antibodies were against Flag-Cas9, retinoblastoma

659 protein (pRb), p53, p21, and GAPDH. The lysates were re-run to detect

660 phosphorylated-Rb (phosp-Rb). (E, F, G) The mean band intensities from three

661 independent experiments were compared with those from Ctrl-1 and plotted versus

662 the respective experiment. Relative intensities of (E) p53 (F) p21 (G) phosp-Rb were

663 plotted. The error bars represent mean values with standard deviation. *$P < 0.05$,

664 **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, two-tailed student's t-test was used to

665 assess statistical significance, ns indicate not significant.

666

667 **Fig. 5 The senescent HeLa cells does not undergo apoptosis.** (A) The senescent

668 HeLa cells lysates knocked out for HPV18 E6 protein was analyzed by western blot.

669 The blot was probed for apoptotic markers to detect PARP, cleaved-PARP,

670 Caspase-3 and cleaved caspase-3 proteins. HeLa cells treated with staurosporine

671 (Sts) at final concentrations of 0.4μM and 0.8μM was used as positive control for

672 apoptosis. Representative image of three independent experiments is shown. (B)

673 The cleaved PARP1 and cleaved caspase-3 band intensity from HPV18 E6 knockout

674 cells were normalized with GAPDH and the mean band intensities were plotted

675 versus the respective experiment. (C) The senescent HeLa cells lysates knocked out

676 for HPV18 E7 protein was analyzed by western blot. The blot was probed in same

677   way as explained in panel A. (D) The cleaved PARP1 and cleaved caspase-3 band

678   intensity from HPV18 E7 knockout cells was normalized with GAPDH and the mean

679   band intensities were plotted versus the respective experiment.

680

681   **Fig. 6 Re-introduction of HPV18 E6 in senescent HeLa cells.**

682   HPV18 E6 knock out senescent cells were transfected with either codon modified

683   18E6 expressing plasmid or empty plasmid followed by western blot analysis. The

684   blot was probed for antibodies against p53, p21, 18E6 protein and actin.

685   Representative image of two independent experiments is shown. (B) The mean p53

686   signal from two independent experiments was normalized with loading control actin

687   and the relative intensity of p53 with Ctrl-1 was plotted versus the respective
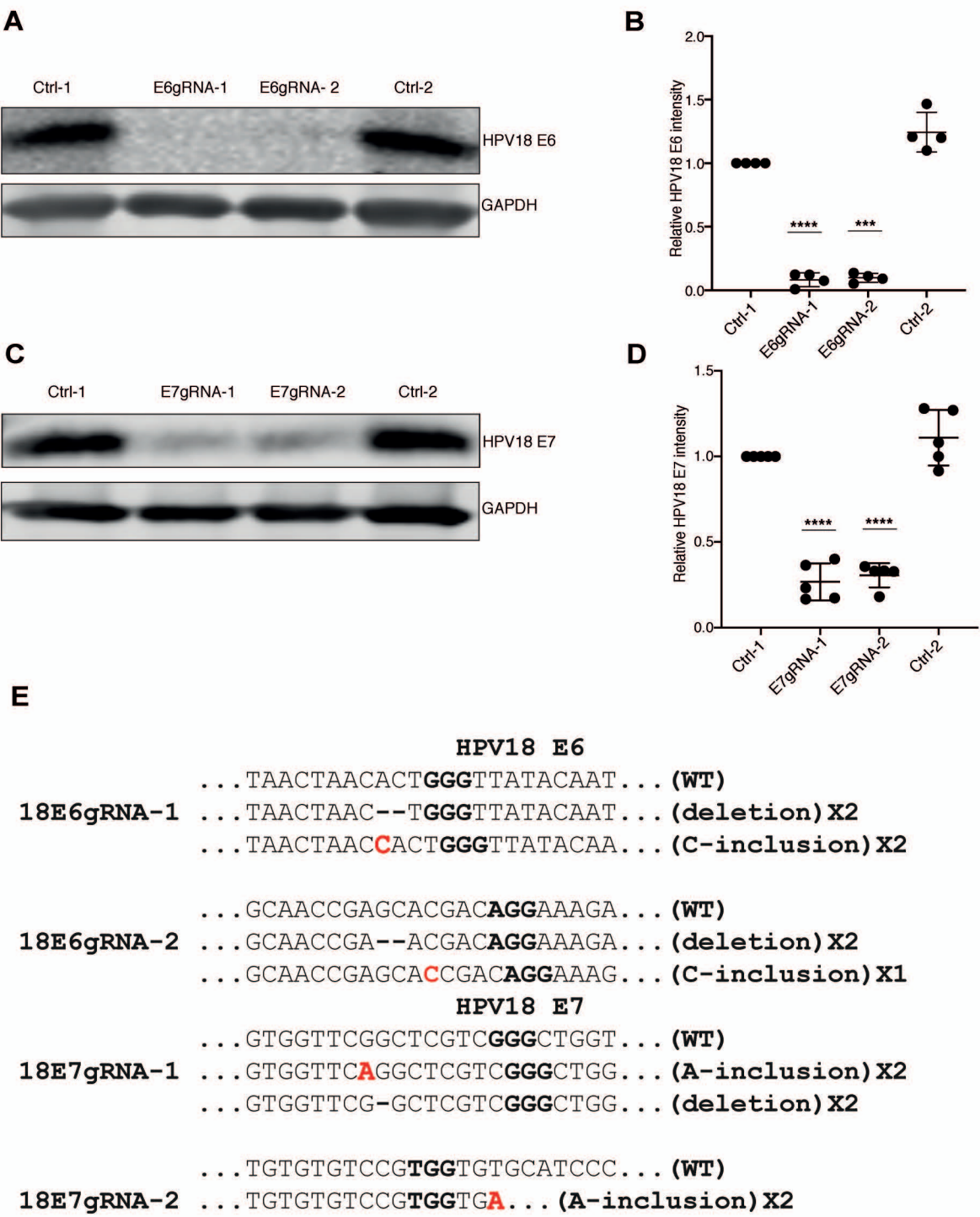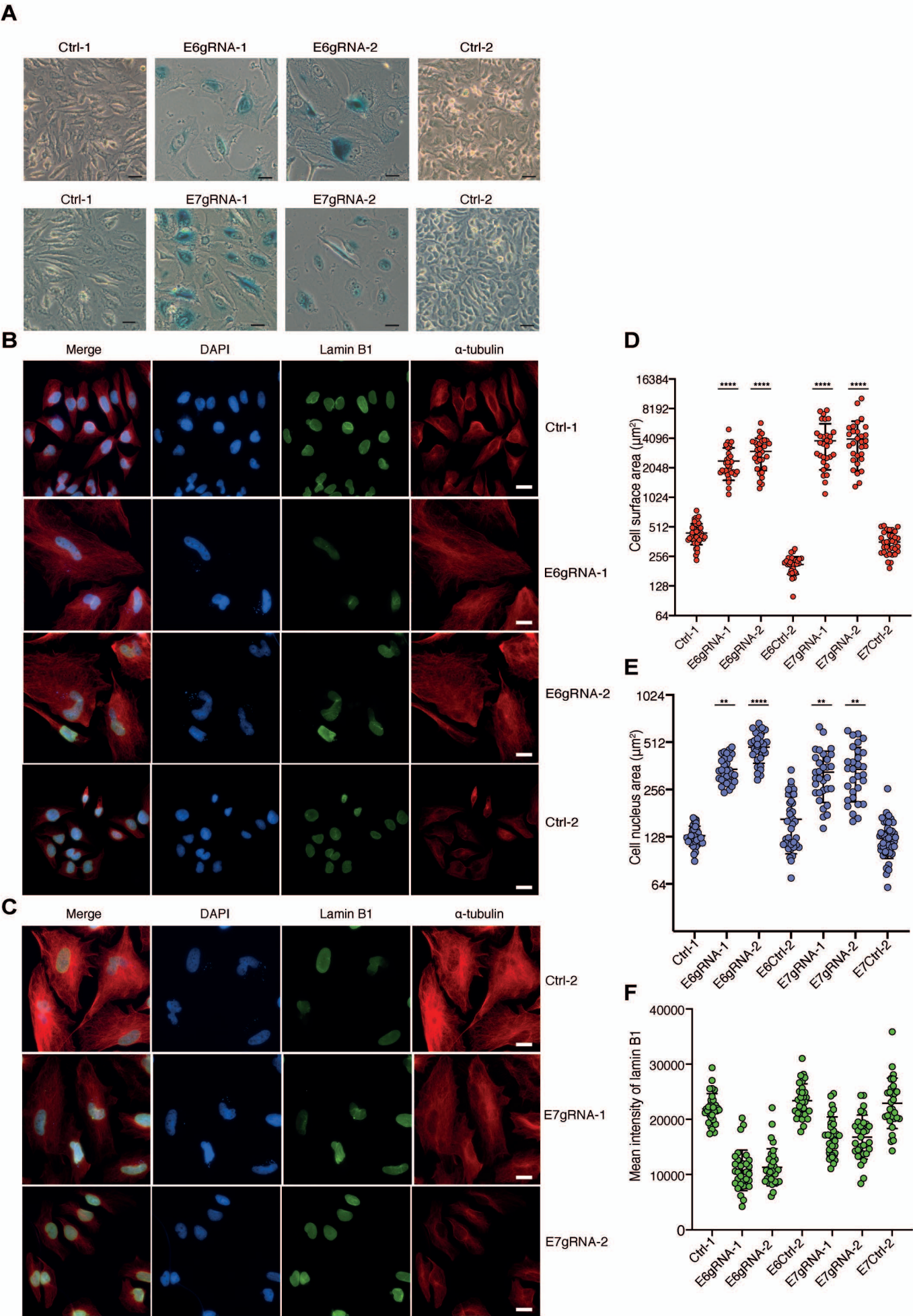
688   experiment.

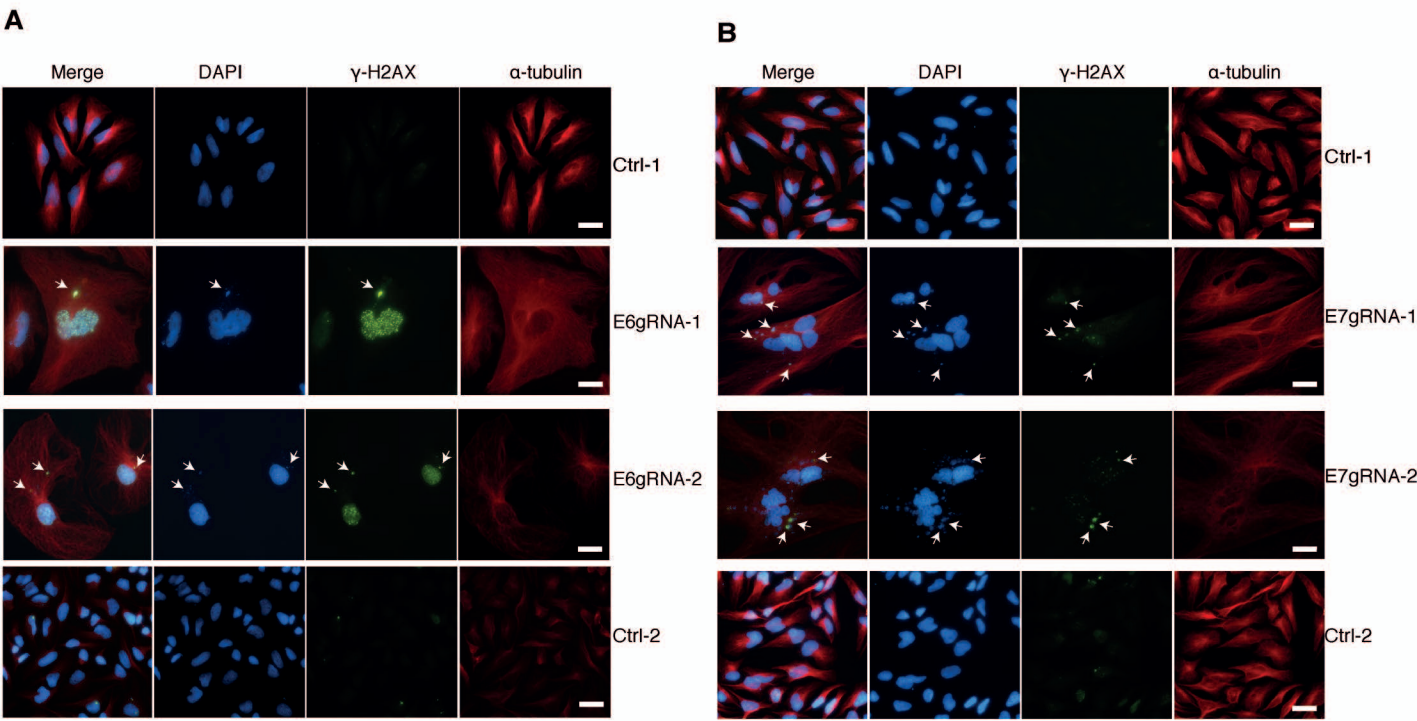29

E

```
                    HPV18 E6
            ...TAACTAACACTGGGTTATACAAT...(WT)
18E6gRNA-1  ...TAACTAAC--TGGGTTATACAAT...(deletion)X2
            ...TAACTAACCACTGGGTTATACAA...(C-inclusion)X2

            ...GCAACCGAGCACGACAGGAAAGA...(WT)
18E6gRNA-2  ...GCAACCGA--ACGACAGGAAAGA...(deletion)X2
            ...GCAACCGAGCACCGACAGGAAAG...(C-inclusion)X1
                    HPV18 E7
            ...GTGGTTCGGCTCGTCGGGCTGGT...(WT)
18E7gRNA-1  ...GTGGTTCAGGCTCGTCGGGCTGG...(A-inclusion)X2
            ...GTGGTTCG-GCTCGTCGGGCTGG...(deletion)X2

            ...TGTGTGTCCGTGGTGTGCATCCC...(WT)
18E7gRNA-2  ...TGTGTGTCCGTGGTGA...(A-inclusion)X2
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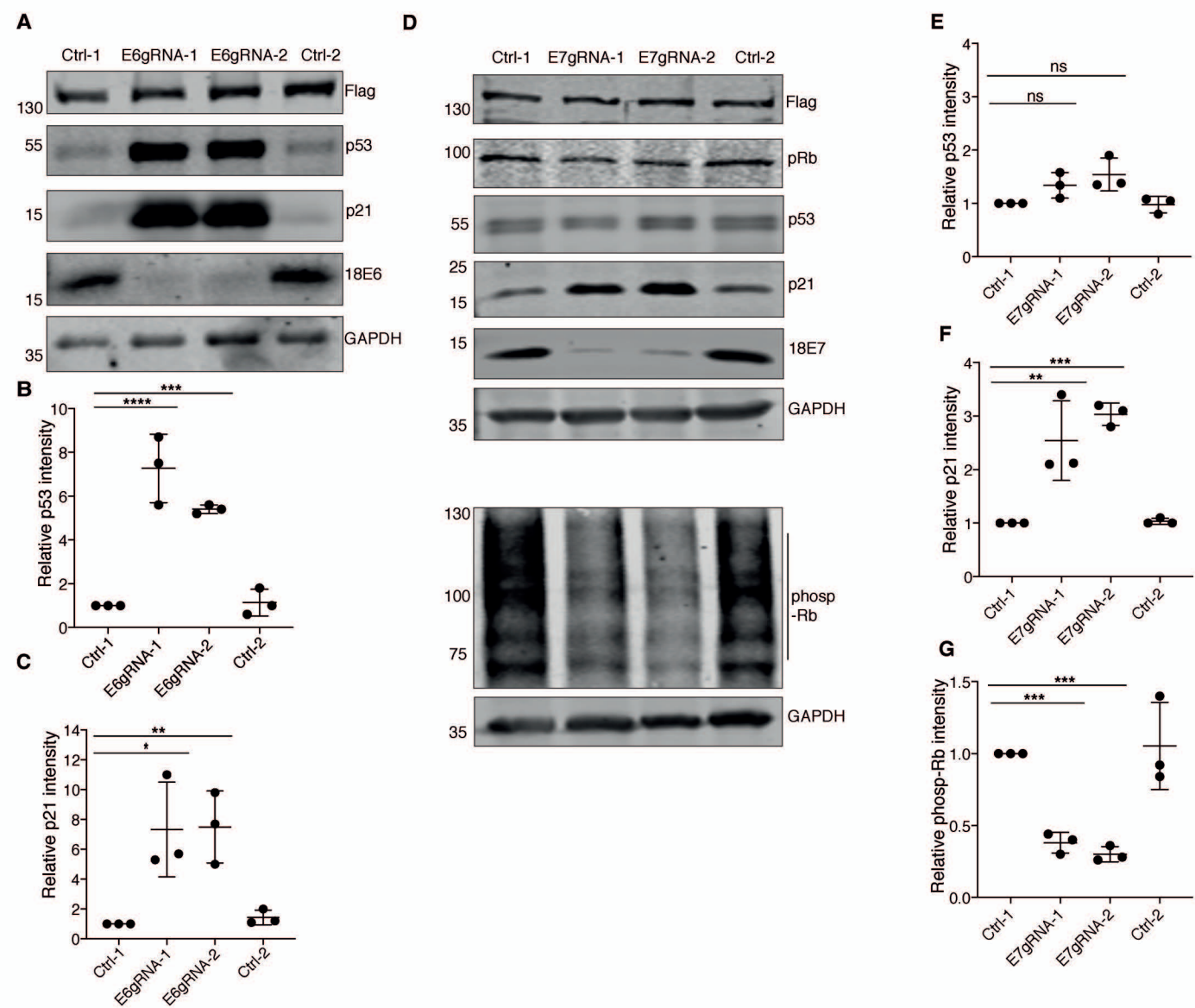
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6