

1 **A critical role for E2-p53 interaction during the HPV16 life cycle**

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8

9 **Abstract**

10 Human papillomaviruses (HPV) are causative agents in ano-genital and oral cancers; HPV16 is
11 the most prevalent type detected in human cancers. The HPV16 E6 protein targets p53 for
12 proteasomal degradation to facilitate proliferation of the HPV16 infected cell. However, in HPV16
13 immortalized cells E6 is predominantly spliced (E6*) and unable to degrade p53. Here we
14 demonstrate that human foreskin keratinocytes immortalized by HPV16 (HFK+HPV16), and
15 HPV16 positive oropharyngeal cancers, retain significant expression of p53. In addition, p53
16 levels can be increased in HPV16+ head and neck cancer cell lines following treatment with
17 cisplatin. Introduction of full-length E6 into HFK+HPV16 resulted in attenuation of cellular growth
18 (in hTERT immortalized HFK, E6 expression promoted enhanced proliferation). An understudied
19 interaction is that between E2 and p53 and we investigated whether this was important for the
20 viral life cycle. We generated mutant genomes with E2 unable to interact with p53 resulting in
21 profound phenotypes in primary HFK. The mutant induced hyper-proliferation, but an ultimate
22 arrest of cell growth; β -galactosidase staining demonstrated increased senescence, and COMET
23 assays showed increased DNA damage compared with HFK+HPV16 wild type cells. There was
24 failure of the viral life cycle in organotypic rafts with the mutant HFK resulting in premature
25 differentiation and reduced proliferation. The results indicate that the E2-p53 interaction is critical
26 during the HPV16 life cycle, and that disruption of this interaction has anti-viral potential. We
27 discuss potential mechanisms to explain these phenotypes.

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33 **Importance**

34 Human papillomaviruses are causative agents in around 5% of all cancers. There are currently
35 no antivirals available to combat these infections and cancers, therefore it remains a priority to
36 enhance our understanding of the HPV life cycle. Here we demonstrate that an interaction
37 between the viral replication/transcription/segregation factor E2 and the tumor suppressor p53 is
38 critical for the HPV16 life cycle. HPV16 immortalized cells retain significant expression of p53,
39 and the critical role for the E2-p53 interaction demonstrates why this is the case. If the E2-p53
40 interaction is disrupted then HPV16 immortalized cells fail to proliferate, have enhanced DNA
41 damage and senescence, and there is premature differentiation during the viral life cycle. Results
42 suggest that targeting the E2-p53 interaction would have therapeutic benefits, potentially
43 attenuating the spread of HPV16.

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60 **Introduction:**

61 HPV16 infection causes half of cervical cancers and up to 90% of HPV-positive oropharyngeal
62 cancers (1). Despite advances in vaccination, the prevalence in HPV-associated oropharyngeal
63 cancer continues to rise, contributing to an ongoing public health crisis without any effective anti-
64 viral therapies (2-5).

65 HPV infects basal epithelial cells and delivers its circular 8kbp DNA genome into the nucleus of
66 the host. Consequently, cellular host factors initiate transcription from the viral long control region
67 (LCR) (6). The viral mRNA is expressed from a single transcript which is then processed, spliced
68 and translated into individual viral proteins. In high-risk HPV infection, the viral oncoproteins E6
69 and E7 contribute to cellular transformation and cancer progression by targeting several cellular
70 proteins, including tumor suppressors p53 and pRb respectively (7-11).

71 HPV uses two proteins to initiate replication of the viral episome. The E2 protein homodimerizes
72 via a carboxyl terminus domain and binds to four 12-bp palindromic sequences within the viral
73 LCR and origin (12). Via its amino terminus, E2 recruits the viral helicase E1 to the origin which
74 forms a di-hexamer that replicates the viral genome with the assistance of host polymerases (13,
75 14). Upon initial infection, the HPV genome replicates to 20-50 copies and maintains this copy
76 number as the infected cell migrates through the epithelium. As the infected cell differentiates and
77 reaches the upper layers, the viral genome amplifies and expresses the L1 and L2 capsid proteins
78 that promote virus assembly and shedding (15-17).

79 E2 has additional roles in the viral life cycle. E2 can promote or repress viral transcription
80 depending on protein concentration (18). E2 binds to host mitotic chromatin to facilitate viral
81 segregation, resulting in sorting of viral episomes into daughter nuclei following mitosis (19). E2
82 also regulates expression of host genes important in the viral life cycle and cancer progression
83 (20-24).

84 The tumor suppressor p53 primarily functions as a transcription regulatory factor during cellular
85 stress and DNA-damage, leading to cell cycle arrest, senescence, and apoptosis (25-30). In HPV
86 infection, the 150 amino acid E6 oncoprotein interferes with the transcriptional activity of tumor
87 suppressor p53, as well as induces its degradation (7, 9, 31). Direct degradation is initiated by
88 the formation of a complex with p53 and the host partner protein E6AP, which is an ubiquitin
89 ligase. E6 directs the ligase activity of E6AP to p53, promoting its degradation via the proteasome
90 (7, 31-33). This is in direct contrast with many HPV-negative cancers where p53 often becomes
91 mutationally inactivated (34, 35). The degradation of p53 by HPV is also regulated by alternative
92 splicing of high-risk E6 proteins, resulting in a short modulatory isoforms of the oncoprotein such
93 as E6* and E6*I which do not bind to p53 and can inhibit E6-E6AP-p53 complex formation
94 preventing p53 degradation in a cell-cycle specific manner (36-38). We, and others, have reported
95 that expression of alternatively spliced forms of E6 are more dominant compared to full-length E6
96 in HPV-positive head and neck cancer, and ectopic expression of these isoforms have anti-
97 tumorigenic effects (38-41). The disruption of the E6-E6AP-p53 degradation complex by E6* and
98 E6*I, allowing for p53 expression, suggests that p53 expression may be important in HPV
99 immortalized cells.

100 Another reported viral interactor with p53 is E2. E2 proteins from high-risk HPVs can bind to p53
101 and this interaction can trigger apoptosis in cervical cancer cells (42, 43). Additionally, E2
102 replication function is regulated by this interaction with p53 as overexpression of p53 reduces viral
103 replication (44). This led us to hypothesize that in HPV16 immortalized cells, residual p53 is
104 necessary to maintain a healthy viral life cycle, perhaps via an interaction with E2.

105 Here, we demonstrate that p53 expression is clearly detectable in a variety of cell lines and tumors
106 immortalized by HPV16 (45, 46). Depletion of residual p53 by overexpression of full length E6
107 protein led to a significant reduction in cellular proliferation in keratinocytes immortalized by wild-
108 type HPV16. Expression of a mutant E6 protein lacking p53 binding had no effect on cell growth.

109 To determine whether an interaction between E2 and p53 was important for cellular proliferation
110 of HPV16 positive cells, we generated an HPV16 genome with point mutations in the E2 gene
111 eliminating p53 binding (42, 43). Keratinocytes were then immortalized by this mutant.
112 Remarkably, compared to wild type HPV16, E2^{-p53} mutant cells, following an initial burst in
113 proliferation, stopped growing and had increased levels of senescence, and accumulated DNA
114 breaks as evidence by single-cell gel electrophoresis assay (COMET). When subjected to
115 differentiation via organotypic raft culturing, these mutant cells had reduced proliferation leading
116 to marked reduction in raft thickness. There was also a reduction in viral replication markers in
117 the mutant cells. These results suggest that although p53 is downregulated by E6 in high-risk
118 HPV infection, p53 is still necessary to permit HPV induced proliferation and that the interaction
119 with E2 plays an important role in the requirement for p53 expression.

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121 **Results:**

122 **Tumor Suppressor p53 is expressed in HPV16 immortalized cells and is critical for their** 123 **optimal growth.**

124 Previous studies demonstrated that alternative splice variants (E6*) are the dominant E6
125 transcripts in HPV associated head and neck cancer, preventing E6-E6AP-p53 complex formation
126 and inhibiting p53 degradation (36-41). We confirmed the presence of p53 in a series of HPV16
127 positive cell lines (Fig. 1). Expression of the entire HPV16 full genome in N/Tert-1 cells results in
128 partial reduction in p53 compared to near complete abrogation by expression of HPV16 E6 and
129 E7 (Fig. 1A compare lanes 2 and 4 to lane 1). Moreover, human tonsil cells immortalized by
130 HPV16 retain p53 expression near N/Tert-1 control cells (compare lane 1 to 3). To further
131 investigate these findings, we studied two independent donors of human foreskin keratinocytes
132 (HFK) immortalized with HPV16. In both donor lines, p53 levels were much less reduced

133 compared to HFK immortalized by HPV16 E6 and E7 overexpression (Lanes 5-8). To determine
134 whether this expression is affected by tumor microenvironment, we surveyed p53 expression in
135 8 patient derived xenografts (PDX) from oropharyngeal and oral cavity carcinomas (four HPV16
136 positive and four negative) (45, 46). All HPV16 positive PDX samples and 3 out of 4 HPV negative
137 retained detectable p53 expression illustrating no clear association between HPV status and p53
138 expression (Fig. 1B). Platinum based DNA-damaging agents such as cisplatin are critical in the
139 treatment of late stage systemic head and neck cancers (47-50). Because DNA-damage is known
140 to stabilize and activate p53, and p53 is most often wild-type in HPV-positive cancers, we
141 predicted that in HPV+ head and neck cancer cell lines the expression of active wild-type p53 can
142 be promoted by cisplatin treatment. We confirmed dose-dependent cisplatin induced p53
143 expression in SCC-47 and SCC-104 cells (Fig. 1C). These results indicate that p53 expression is
144 conserved in many cell lines immortalized by the HPV16 genome and can be induced by
145 treatment with DNA-damage. This suggests that although E6 degrades p53 to help promote cell
146 immortalization and carcinogenesis, p53 often retains expression under a variety of conditions,
147 indicating that it still may play an important role in the HPV16 life-cycle.

148 To determine whether reduction of p53 would compromise the growth of HPV16 immortalized
149 cells we introduced full length E6 (using a retroviral delivery of the E6 gene which does not allow
150 alternative splicing) into N/Tert-1 (foreskin keratinocytes immortalized by telomerase) and
151 HFK+HPV16 cells. Fig. 2A demonstrates that the additional expression of E6 in N/Tert-1 cells
152 results in significantly increased cellular proliferation as has been described (51). However,
153 introduction of E6 into HFK+HPV16 resulted in an attenuation of cell growth (Fig. 2B). Because
154 E6 possesses a variety of mechanisms for regulating cellular proliferation independent from p53
155 degradation, we attempted to isolate these other mechanisms by expressing an E6 mutant unable
156 to promote degradation of p53 but retains all other known functions (52). This mutant did not have
157 a deleterious effect on cell growth indicating that it is the E6 targeting of p53 that results in the

158 attenuation of cellular proliferation. Additionally, we found that these proliferation rates were
159 inversely correlated with senescence levels (Fig. 2C and 2D). In the HFK+HPV16+E6 cells, we
160 noticed that over time the cells began proliferating once again. To determine whether the
161 recovered cells had a restoration of p53 protein levels we carried out western blots of
162 HFK+HPV16+E6 cells at different stages following E6 introduction (Fig. 2E). Lane 4 demonstrates
163 that there is an initial reduction in p53 protein levels in these cells immediately following selection
164 compared with control cells (compare lane 4 with lane 3). However, following 13 days of culturing
165 (when we noticed proliferation begin to restore to that of the control cells) there is a restoration of
166 p53 protein expression (compare lane 7 with lane 4). These results suggest that reduction of p53
167 protein may lead to growth attenuation and enhanced senescence of HFK+HPV16 cells. They
168 also suggest that to begin to proliferate again, restoration of p53 likely helps promote growth in
169 the HFK+HPV16+E6 cells. We monitored the exogenous E6 RNA levels (Fig 2F). There is a clear
170 reduction in the E6 RNA expressed from the exogenous vector between days 0 and 13 correlating
171 with the restoration of p53 protein expression and cellular proliferation. When we analyzed for E6
172 protein expression via western blot, while we did not notice an appreciable change in E6 protein
173 levels, we found that there was a significant increase of E6 Δ p53 expression compared to wild-
174 type E6 at both time points (Fig. 2G). This supports our claim that expression of E6 is more
175 deleterious to growth than E6 Δ p53 in HFK+HPV16 and this is most likely due to p53 degradation.
176 Overall, these results demonstrate that p53 is expressed in HPV16 immortalized cells, and that
177 this expression may be critical for continuing proliferation of these cells. We next moved on to
178 investigate possible reasons for the requirement of p53 expression in HFK+HPV16 cells.

179 **Disruption of p53 interaction with the HPV16 E2 protein attenuates cell growth and blocks** 180 **the viral life cycle**

181 A known and relatively understudied interaction of p53 with HPV16 is the direct physical
182 interaction with E2 (43). We generated an E2 mutant predicted to not interact with p53 (E2^{-p53})

183 and generated stable N/Tert-1 cell lines expressing this mutant, as we have done for wild type E2
184 (43). There was robust, stable expression of E2 and E2^{-p53} in N/Tert-1 cells (Fig. 3A, lane 3).
185 Immunoprecipitation with a p53 antibody brought down p53, and E2 wild type co-
186 immunoprecipitated with p53 while E2^{-p53} did not (Fig. 3B, compare lanes 2 and 3). To
187 demonstrate that E2^{-p53} was functional we carried out transcriptional studies in N/Tert-1 cells.
188 Because the binding of p53 takes place in the DNA-binding domain (DBD) of E2, we confirmed
189 that the mutant E2 retained DNA-binding function. Both E2 wild type and E2^{-p53} were able to
190 repress transcription from the HPV16 long control region (LCR) efficiently and comparatively (Fig.
191 3C). We also measured that transcriptional activation function of E2 wild type and E2^{-p53} (Fig. 3D).
192 While E2^{-p53} is able to activate transcription it was compromised when compared with E2 wild type
193 (compare lanes 5-7 with lanes 2-4). We conclude from these experiments that E2^{-p53} is nuclear
194 and able to bind to its DNA target sequences but that its transcriptional activation property (but
195 not repression) is attenuated.

196 Having confirmed that the E2^{-p53} mutant was functional, we introduced the mutations that abrogate
197 the E2-p53 interaction into the entire HPV16 genome (HPV16^{-p53}). We introduced the wild type
198 and mutant HPV16 genomes into 2 independent primary human foreskin cell populations that we
199 recently used to investigate the role of the E2-TopBP1 interaction in the viral life cycle (53). Both
200 the wild type and mutant genomes efficiently immortalized both HFK donor cells. We carried out
201 Southern blotting on *SphI* cut DNA (a single cutter for the HPV16 genome) (Fig. S1A). To further
202 characterize the status of the genomes in these cells we used TV exonuclease assays (this assay
203 is based on the fact that episomal HPV16 genomes are resistant to exonuclease digestion) (54,
204 55). This assay demonstrated that the viral DNA in the immortalized donor cell lines retained a
205 predominantly episomal status, irrespective of whether the viral genomes were wild type or
206 HPV16^{-p53} (Fig. S1B).

207 Next, we investigated the expression of markers relevant to HPV infection in HFKs. Fig. 4A
208 demonstrates that p53 levels are similarly reduced in HFK+HPV16 and HFK+HPV16^{-p53} cells
209 when compared with N/Tert-1 cells (compare lanes 2-5 with lane 1) (Fig. 4B). For comparison,
210 cells immortalized with an E6/E7 expression vector had almost no p53 expression (lane 6), likely
211 due to the inability of the E6 to be spliced to E6* variants with this expression vector. To further
212 characterize these cell lines, we investigated whether the DNA damage response is turned on as
213 HPV infections activate both the ATR and ATM pathways. We investigated the phosphorylation
214 status of CHK1 and CHK2 as surrogate markers for activation of these DNA damage response
215 kinases (Fig. 4D). Compared with N/Tert-1 cells there is an overall increase of CHK1 and CHK2
216 levels in cells immortalized with HFK+HPV16, HFK+HPV16^{-p53} or E6/E7 expression. CHK1 and
217 CHK2 phosphorylation is also elevated in the presence of all of the HPV16 positive cells when
218 compared with N/Tert-1 cells. It is important to note that E6 and E7 immortalization of HFK
219 induced phosphorylation of CHK1 but not CHK2 when compared with the entire genome (Lane
220 6). This is likely due to the ATM pathway being largely activated by viral replication rather than by
221 the viral oncogenes E6 and E7 which we have previously reported (56). Overall, these results
222 suggest that markers of HPV16 infection are activated in HFK cells immortalized with HPV16
223 irrespective of the ability of p53 to bind E2.

224 Even though the HFK+HPV16^{-p53} cells had markers indicative of HPV16 immortalization, we
225 noticed an aberrant growth phenotype in both foreskin donor cells (Fig. 5A). There was an initial
226 enhanced proliferation of the HFK+HPV16^{-p53} cells when compared with HFK+HPV16. However,
227 around the 3-4 week mark, the HFK+HPV16^{-p53} cells began to slow their growth and eventually
228 stopped proliferating. To determine the mechanism of the attenuation of cell growth we
229 investigated senescence in N/Tert-1, HFK+HPV16 and HFK+HPV16^{-p53} cells by staining for beta-
230 galactosidase (Fig. 5B). There was a significantly increased number of senescent cells with the
231 p53 mutant cells and this was quantitated (Fig. 5C). Senescence can be induced by increased

232 DNA damage, particularly double strand breaks (DSB) (25, 26). Because CHK1 and CHK2
233 pathway activation was not noticeably different between HFK+HPV16 and HFK+HPV16^{p53}, we
234 decided to look at DSBs more directly using single-cell gel electrophoresis (COMET assay). As
235 expected, the expression of wild type or mutant HPV16 genomes in HFKs led to increased
236 formation of DSBs as indicated by olive tail moment (OTM) when compared to HPV negative
237 N/Tert-1 cells (57) (Fig. 6D). However, the mutant HFKs consistently exhibited larger OTM values
238 compared to HFK+HPV16 (Fig. 5D and 5E). As the expression of full-length E6 from a
239 heterologous vector attenuates the growth of HFK+HPV16 wild type cells (Fig. 2) we rationalized
240 that expression of E6 should not alter the growth of HFK+HPV16^{p53} cells. Stable expression of
241 exogenous full length E6 or the E6Δp53 mutant had no additional effect on the proliferation of
242 HFK+HPV16^{p53} cells, illustrating that the drastic differences in proliferation are potentially due to
243 the E2-p53 interaction (Fig. 5F).

244 **HFK+HPV16^{p53} cells have an aberrant life cycle in differentiating epithelium.**

245 We organotypically rafted HFK+HPV16 and HFK+HPV16^{p53}. Both lines were placed on collagen
246 plugs at early passage when the HFK+HPV16^{p53} cells retained proliferative capacity. Due to the
247 large difference in growth rates between the wild type and mutant cells, the original plating was
248 performed with both 1×10^6 and 2×10^6 cells to promote production of a monolayer on the collagen
249 plugs prior to lifting to the liquid-air interface for differentiation. Fig 6A demonstrates an aberrant
250 differentiation process with the HFK+HPV16^{p53} cells when compared with HFK+HPV16 cells at
251 both cell densities. It is noticeable that at the lower cell density (1×10^6) there was a failure to form
252 a monolayer prior to induction of differentiation (as evidenced by gaps between keratinocyte cell
253 clusters on the collagen plug). Using a seeding density of 2×10^6 eliminated the formation of gaps
254 but did not improve the proliferation. A representative of two independent donors is shown, both
255 donors had identical phenotypes. Fig. 6B quantitates the results from two independent rafts from
256 two independent donors; the mutant genomes have dramatically lower raft area when compared

257 with wild-type genomes. To investigate whether differentiation has occurred in these cells we
258 stained with Involucrin and Keratin 10 (Fig. 6C). The mutant genome cells stained positive for
259 both differentiation markers demonstrating that, even though raft growth is markedly attenuated,
260 differentiation still occurs. We also stained for viral replication using the DNA-damage marker γ -
261 H2AX. Recently we reported that an E2 mutant that failed to interact with TopBP1 results in
262 degradation of E2 during organotypic rafting; this degradation would block viral replication and
263 indeed these cells had no γ -H2AX staining (53). This demonstrates that the γ -H2AX staining
264 indicates the occurrence of viral replication. Fig. 6D demonstrates that there is abundant nuclear
265 γ -H2AX staining throughout HFK+HPV16 cells, indicating replication is occurring. The
266 HFK+HPV16^{-p53} cells also support viral replication although there is a reduction in the number of
267 rafted cells stain positively for γ -H2AX (Fig. 6D).

268 **Discussion:**

269 The HPV E2 protein is essential for viral genome replication, segregation of viral episomes into
270 daughter cells following cell division, and can transcriptionally regulate both virus and host
271 genomes (12, 19, 20). E2 interacts with a variety of host factors to promote progression of the
272 viral life cycle, many of which are essential such as interactions with TopBP1 and BRD4 (12, 19,
273 23, 53, 58). In this report, we propose that E2 binding to p53 is also an essential interaction as
274 abrogation of the interaction leads to catastrophic failure of the viral life cycle.

275 In the classical high-risk HPV model, upon initial infection, the viral oncogenes E6 and E7 inhibit
276 and degrade tumor suppressor proteins p53 and pRb respectively, promoting hyperproliferation,
277 unregulated DNA replication, mutation accumulation and potentially eventual carcinogenesis.
278 Therefore, immortalization of cell lines can be achieved with overexpression vectors of E6 and
279 E7 (Fig. 1A, Lanes 4 and 8). Previous studies suggest that E6 splice variants and their action on
280 E6-E6AP-p53 complex disruption is cell-cycle dependent (38). In HPV18 cell lines, E6*1 shows
281 marked upregulation during and p53 restoration during G2/M (38). We have previously illustrated

282 that E2 is stabilized during mitosis which is important for its association with TopBP1 and its role
283 as a segregation factor (53). It is entirely possible that the cell-cycle mediated p53 restoration
284 corresponds with E2 stabilization allowing these proteins to interact, and may also play an
285 important role in genome segregation.

286 E2 can regulate host transcription in a multitude of ways. We recently reported that E2 can
287 epigenetically repress the TWIST1 at the histone level, inhibiting EMT and promoting a less
288 aggressive cellular phenotype (20). E2 can also promote the recruitment of DNA
289 methyltransferase 1 to interferon response genes, resulting in DNA base methylation and global
290 innate immunity downregulation (21). It is currently unclear how E2 recruits epigenetic modifiers
291 to these genes and p53 may play an important role. DNA methyltransferases (DNMTs) are often
292 part of large multimeric complexes and use transcription regulatory proteins to help target specific
293 genes undergoing epigenetic silencing (59, 60). p53 is known to also interact with DNMT1
294 resulting in the methylation of antiapoptotic genes (61). It is possible that the interaction between
295 E2 and p53 is important for the rerouting of DNMTs to different genes whose regulation is
296 important for a healthy viral life cycle. It is also noticeable that E2-p53 has an attenuated ability to
297 activate transcription (Fig. 3), indicating that regulation of host gene transcription by E2 may
298 require co-operation with p53 in some cases.

299 The results from Fig. 5D suggest that additional double strand breaks play a role in the enhanced
300 damage and proliferation rate of HFK+HPV16-p53 mutant cells compared to wild type
301 immortalized HFKs. HPV uses homologous recombination (HR) factors to assist in viral replication
302 (62-64). Conversely, p53 binds to replication protein A (rpA) resulting in repression of HR,
303 reducing DSB repair and promoting apoptosis during catastrophic genome instability (27, 29). It
304 is possible that E2 helps regulate this activity of p53 and inability to do so results in accumulation
305 of DSBs as seen in Fig. 5D.

306 In conclusion, this report indicates that p53 expression is retained in HPV16 positive cell lines and
307 tumor samples under a variety of conditions. Depletion of this residual p53 by full length E6 results
308 in significant reduction in proliferation and enhanced senescence in cells immortalized with
309 HPV16 which we attribute to loss of E2 interaction with p53. Human foreskin cells immortalized
310 by HPV16 where E2 can no longer bind to p53 exhibit aberrant phenotypes including dysregulated
311 proliferation, enhanced levels of DSBs and overall failure of the viral life cycle during organotypic
312 raft culturing. Due to the importance of p53 in the context of HPV related cancers as well as the
313 profound phenotypes demonstrated in this report, further investigation on the interaction between
314 E2 and p53 is warranted.

315

316 **Methods:**

317 **Cell culture.** N/Tert-1 cells and head-and-neck cancer lines UMSCC47 and UMSCC104 were
318 cultured as previously described (20, 21, 65-67). Immortalization and culturing of human foreskin
319 keratinocytes with HPV16 are described below. Cells were incubated at 37°C and 5% CO₂ with
320 media changed every 3 days. For cisplatin treatment, cells were incubated with indicated
321 concentrations of drug dissolved in DMF or DMF vehicle control for 24-hours

322 **Immortalization of human foreskin keratinocytes (HFK).** HPV16 mutant genome (HPV16-p53,
323 which contained an E2 unable to bind p53) was generated and sequenced by Genscript (42, 44,
324 68). Residues Aspartic acid 388, Tryptophan 341 and Aspartic acid 344 were all mutated to
325 alanine resulting in inability for E2 to interact with p53. The HPV16 genome was removed from
326 the parental plasmid using *SphI*, and the viral genomes isolated and then re-circularized using T4
327 ligase (NEB) and transfected into early passage HFK from three donor backgrounds (Lifeline
328 technology), alongside a G418 resistance plasmid, pcDNA. Cells underwent selection in 200
329 µg/mL G418 (Sigma-Aldrich) for 14 days and were cultured on a layer of J2 3T3 fibroblast feeders

330 (NIH), which had been pre-treated with 8 µg/ml mitomycin C (Roche). Throughout the
331 immortalization process, HFK were cultured in Dermalife-K complete media (Lifeline Technology).

332 **Western blotting.** Protein from cell pellets was extracted with 2x pellet volume protein lysis buffer
333 (0.5% Nonidet P-40, 50mM Tris [pH 7.8], and 150mM NaCl) supplemented with protease inhibitor
334 (Roche Molecular Biochemicals) and phosphatase inhibitor cocktail (Sigma). Protein extraction
335 from patient derived xenografts was performed as previously described.(45, 46) The cells were
336 lysed on ice for 30 min followed by centrifugation at 18,000 rcf (relative centrifugal force) for 20
337 min at 4°C. Protein concentration was estimated colorimetrically using a Bio-Rad protein assay
338 and 25 µg of protein with equal volume of 2X Laemmli sample buffer (Bio-Rad) was denatured at
339 70°C for 10 min. The samples were run on a Novex™ WedgeWell™ 4 to 12% Tris-glycine gel
340 (Invitrogen) and transferred onto a nitrocellulose membrane (Bio-Rad) using the wet-blot method,
341 at 30 V overnight. The membrane was blocked with *Li-Cor* Odyssey® blocking buffer (PBS)
342 diluted 1:1 v/v with PBS for 1 hour at room temperature and then incubated with specified primary
343 antibody in *Li-Cor* Odyssey® blocking buffer (PBS) diluted 1:1 with PBS. Afterwards, the
344 membrane was washed with PBS supplemented with 0.1% Tween20 and further probed with the
345 Odyssey secondary antibodies (IRDye® 680RD Goat anti-Rabbit IgG (H + L), 0.1 mg or IRDye®
346 800CW Goat anti-Mouse IgG (H + L), 0.1 mg) in *Li-Cor* Odyssey® blocking buffer (PBS) diluted
347 1:1 with PBS at 1:10,000 for 1 hour at room temperature where applicable. After washing with
348 PBS-tween, the membrane was imaged using the Odyssey® CLx Imaging System and ImageJ
349 was used for quantification. Primary antibodies used for western blotting studies are as follows:

350 p53, 1:1000 (Santa Cruz; cat. no. sc-47698) HPV16 E2, 1:1000 (TVG261) (Abcam; cat. no.
351 ab17185) phospho-CHK1, 1:1000 (Ser345) (Cell Signaling; cat. No. 2341S), phosphor CHK2
352 (Thr68), 1:1000 (Cell Signaling; cat. No. 2661S), CHK1, 1:1000(Cell Signaling; cat. No. 2360),
353 CHK2, 1:1000 (Abcam; cat. No. ab47443), GAPDH, 1:250 (Santa Cruz; cat. no. sc-47724).

354 **Plasmids.** The following plasmids were used the completion of these studies: pMSCV-N-FLAG-
355 HA-GFP, pMSCV-N-FLAG-HA-HPV16E6, pMSCV-IP-N-FLAG-HA-16E6 8S9A10T (“E6Δp53”).
356 Wild-type 16E2 or 16E2-p53 (Mutated residues W341A, D344A, D338A) were cloned into pCDNA
357 vector for confirmation of p53 interaction in N/Tert-1 cells. pCDNA was used for empty vector
358 control.

359 **Real-time qPCR.** RNA was isolated using the SV Total RNA isolation system (Promega)
360 according to manufacturer’s instructions. 2 μg of RNA was reverse transcribed into cDNA using
361 the high-capacity reverse transcription kit (Applied Biosystems). The PowerUp SYBR green
362 master mix (Applied Biosystems) was used along with cDNA and gene specific primers and real-
363 time PCR was performed using a 7500 Fast real-time PCR system as previously described. (20,
364 21, 65) Expression was quantified as relative quantity over GAPDH using the $2^{-\Delta\Delta C_T}$ method.
365 Primers used are as follows. FLAG-HA Tag fwd 5'- GACTACAAGGATGACGATG- 3', FLAG-HA
366 Tag rev 5'- GCGTAATCTGGAACATCG -3'.

367 **p53 Immunoprecipitation.** Primary polyclonal antibody against p53 (Invitrogen; PA5-27822) or
368 a HA-tag antibody (used as a negative control) was incubated in 200 μg of cell lysate (prepared
369 as described above), made up to a total volume of 500 μl with lysis buffer (0.5% Nonidet P-40,
370 50mM Tris [pH 7.8], and 150mM NaCl), supplemented with protease inhibitor (Roche Molecular
371 Biochemicals) and phosphatase inhibitor cocktail (Sigma) and rotated at 4°C overnight. The
372 following day, 50 μl of pre-washed protein A-sepharose beads per sample was added to the
373 lysate/antibody solution and rotated for 4 hours at 4°C. The samples were gently washed with
374 500 μl lysis buffer by centrifugation at 1,000 rcf for 2-3 min. This wash was repeated 4 times. The
375 bead pellet was resuspended in 4X Laemmli sample buffer (Bio-Rad), heat denatured and
376 centrifuged at 1,000 rcf for 2-3 min. Proteins were separated using an SDS-PAGE system and
377 transferred onto a nitrocellulose membrane before probing for the presence of E2 or p53, as per
378 western blotting protocol.

379 **Transcription and LCR Repression assays.** A pTK6E2-Luciferase reporter plasmid was
380 utilized to analyze transcriptional activation of Wild-type HPV16 E2 and E2 Δ p53 proteins as
381 previously described (23). 5×10^5 N/Tert-1 cells were seeded onto 100mm² plate and transfected
382 24 hours later with 0 ng, 10 ng, 100 ng or 1000 ng of E2 WT or E2-p⁵³ plasmid DNA along with
383 1000ng of pTK6E2-Luciferase reporter plasmid using Lipofectamine 2000 according to the
384 manufacturer's instructions (ThermoFisher Scientific). Cells were harvested the next day using
385 Promega luciferase assay system. For LCR repression activity, a pHPV16-LCR-Luciferase
386 reporter was used in place of the pTK6E2-Luciferase plasmid.(65)

387 **Southern blotting.** Total cellular DNA was extracted by proteinase K-sodium dodecyl sulfate
388 digestion followed by a phenol-chloroform extraction method. 5 μ g of total cellular DNA was
389 digested with either SphI (to linearize the HPV16 genome) or HindIII (which fails to cut HPV16
390 genome). All digestions included DpnI to ensure that all input DNA was digested. All restriction
391 enzymes were purchased from NEB and utilized as per manufacturer's instructions. Digested
392 DNA was separated by electrophoresis of a 0.8% agarose gel, transferred to a nitrocellulose
393 membrane, and probed with radiolabeled (32-P) HPV16 genome as previously described.(67)
394 This was then visualized by exposure to film for 1 to 24 hours. Images were captured from an
395 overnight-exposed phosphor screen by GE Typhoon 9410 and quantified using ImageJ.

396 **Exonuclease V assay.** PCR based analysis of viral genome status was performed using methods
397 described by Myers et al (2019). Briefly, 20 ng genomic DNA was either treated with exonuclease
398 V (RecBCD, NEB), in a total volume of 30 μ l, or left untreated for 1 hour at 37°C followed by heat
399 inactivation at 95°C for 10 minutes. 2 ng of digested/undigested DNA was then quantified by real
400 time PCR using a 7500 FAST Applied Biosystems thermocycler with SYBR Green PCR Master
401 Mix (Applied Biosystems) and 100 nM of primer in a 20 μ l reaction. Nuclease free water was used
402 in place of the template for a negative control. The following cycling conditions were used: 50°C
403 for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and a dissociation stage of

404 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds, and 60°C for 15 seconds. Separate
405 PCR reactions were performed to amplify HPV16 E6 F: 5'- TTGCTTTTCGGGATTTATGC-3' R:
406 5'- CAGGACACAGTGGCTTTTGA-3', HPV16 E2 F: 5'- TGGAAGTGCAGTTTGATGGA-3' R: 5'-
407 CCGCATGAACTTCCCATACT-3', human mitochondrial DNA F: 5'-
408 CAGGAGTAGGAGAGAGGGAGGTAAG-3' R: 5'-TACCCATCATAATCGGAGGCTTTGG -3', and
409 human GAPDH DNA F: 5'- GGAGCGAGATCCCTCCAAAAT-3' R: 5'-
410 GGCTGTTGTCATACTTCTCATGG-3'

411 **Senescence Staining.** 7.5×10^4 cells were seeded in 6-well plates. The following day, cells were
412 stained for senescence using the Cell Signal Senescence β -Galactosidase Staining kit according
413 to manufacturer's instructions (9860). Randomly selected images were taken using the Keyence
414 imaging system at 10X. Positively stained cells were counted by a blinded observer and average
415 number of positively stained cells per field were calculated.

416 **Single-cell gel electrophoresis (COMET) Assay.** 1×10^4 cells were plated in 24-well plate with
417 1mL media one day prior to harvest. The next day, cells were trypsinized and resuspended in a
418 mixture 0.5% w/v Low molecular weight agarose (Lonza, cat. No. #50101) and PBS at a ratio of
419 10:1. Suspension was immediately pipetted onto Trivegen COMET Slides™ (4250-004-03) and
420 allowed to dry for 30 min at 4°C. Slides underwent lysis for 90 min at 4°C in the dark (Lysis buffer:
421 10mM Tris, 100mM EDTA, 2.5M NaCl, 1% TritonX100, 10%DMSO titrated to pH 10.0).
422 Afterwards slides were placed in Alkaline buffer for 25 min at at 4°C in the dark (Alkaline buffer:
423 1mM EDTA, 200mM NaOH, pH >13.0). Slides were transferred to an agarose gel electrophoresis
424 box filled with additional alkaline buffer. Electrophoresis was performed at 25V for 20min at room
425 temperature in the dark. Slides were then washed 2x in dd (double distilled) H₂O for 5 min at RT
426 and then placed in neutralization buffer for 20min at RT in dark (Neutralization buffer: 400mM
427 Tris-HCl titrated to pH 7.5). Neutralized slides were then left to dry at 37°C in the dark. Dried
428 slides were stained with DAPI (1:10,000 in dd H₂O) for 15 min at RT then washed 2x with dd H₂O
429 for 5 min. Stained and rinsed slides were left to dry overnight. Slides were imaged using the

430 Keyence imaging system at 20x with >5 images taken per replicate. Quantization of olive tail
431 moments (OTM) was achieved using the CASPLab COMET Assay imaging software by Końca K
432 et al, 2003 (69).

433

434 **Organotypic raft culture.** Keratinocytes were differentiated via organotypic raft culture as
435 described previously.(21, 67, 70) Briefly, cells were seeded onto type 1 collagen matrices
436 containing J2 3T3 fibroblast feeder cells. Cells were cultured to confluency atop the collagen
437 plugs, lifted onto wire grids and cultured in cell culture dishes at the air-liquid interface. Media was
438 replaced on alternating days. Following 14 days of culture, rafted samples were fixed with
439 formaldehyde (4% v/v) and embedded in paraffin. Multiple 4µm sections were cut from each
440 sample. Sections were stained with hematoxylin and eosin (H&E) and others prepared for
441 immunofluorescent staining via HIER. Fixing and embedding services in support of the research
442 project were generated by the VCU Massey Cancer Center Cancer Mouse Model Shared
443 Resource, supported, in part, with funding from NIH-NCI Cancer Center Support Grant P30
444 CA016059. Fixed sections were antigen retrieved in citrate buffer, and probed with the following
445 antibodies for immunofluorescent analysis: phospho-γH2AX 1/500 (Cell Signaling Technology;
446 9718), Involucrin 1/1000 (abcam; ab27495), Keratin 10 1/1000 (SigmaAldrich; SAB4501656), and
447 HPV16 E2. (monoclonal B9)(71) Cellular DNA was stained with 4',6-diamidino-2-phenylindole
448 (DAPI, Santa Cruz sc-3598). Microscopy was performed using the Keyence imaging system,

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454 **Acknowledgements:**

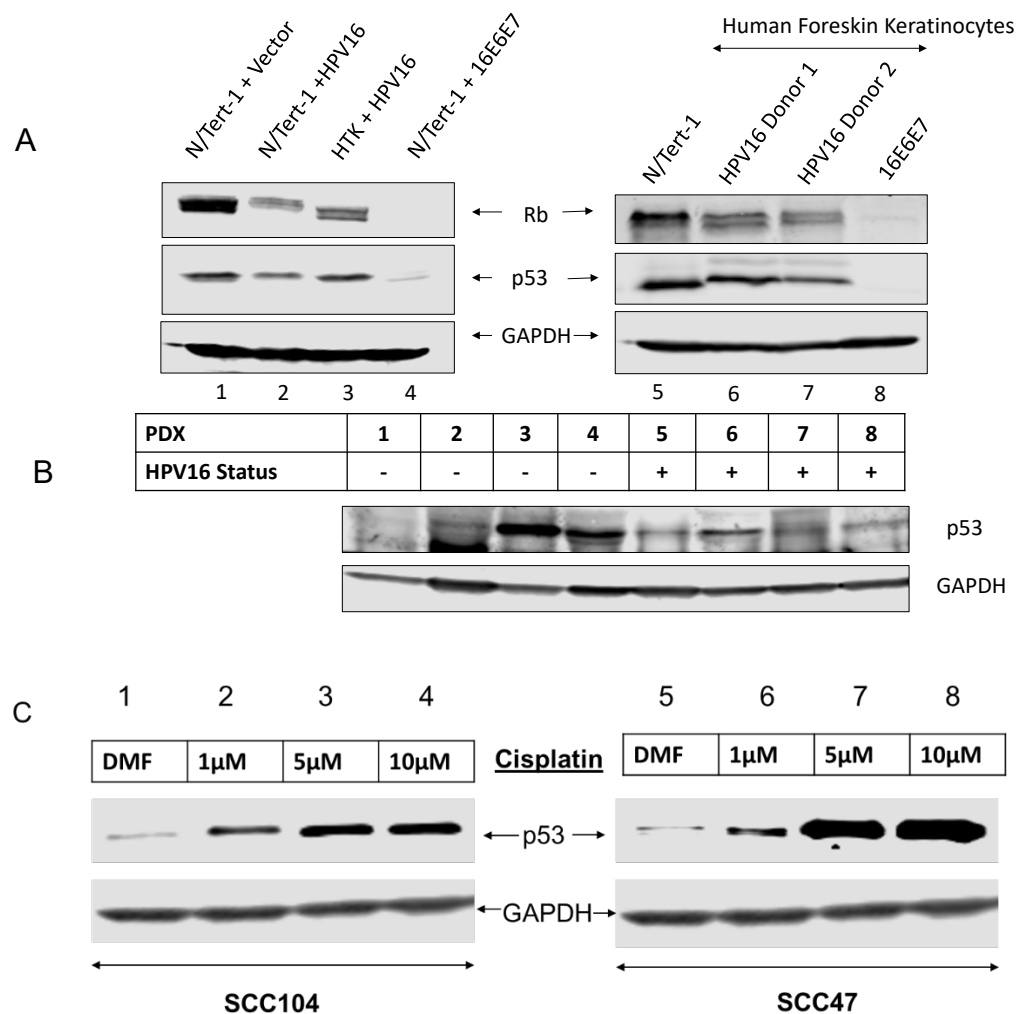
455 This work was supported by VCU Philips Institute for Oral Health Research and the National
456 Cancer Institute designated Massey Cancer Center grant P30 CA016059 (IMM). DB is supported
457 by R01DE027185, a grant from the National Institute of Dental and Craniofacial Research.

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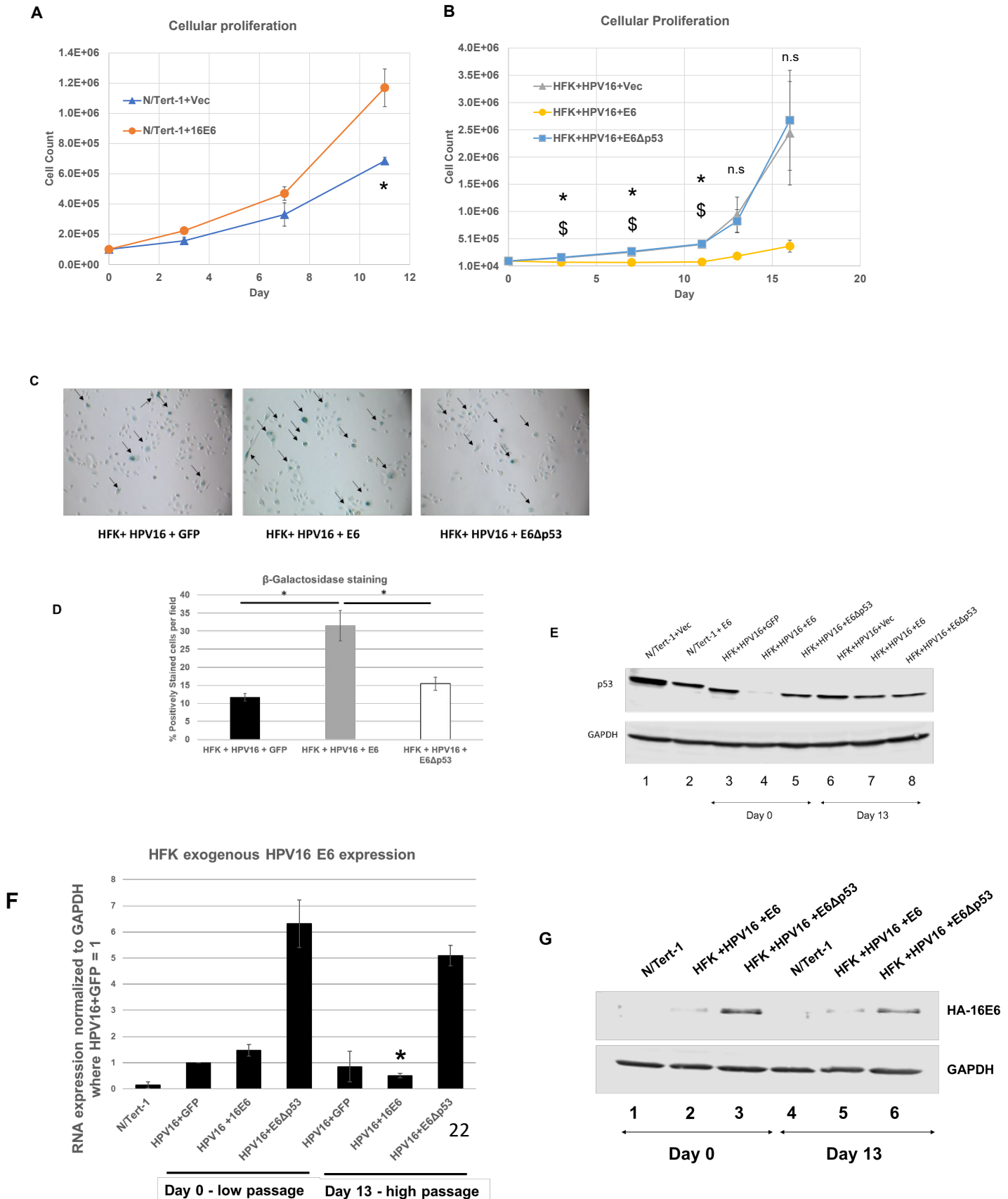
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464 **Figure 1. Tumor suppressor p53 expression is conserved in HPV16 immortalized cell lines**
 465 **and patient derived xenografts** (A) Western blot analysis of p53 and pRb in N/tert-1 cells stably
 466 expressing the HPV16 genome (lane 2), HPV16 E6+E7 (lane 4) or empty vector (lane 1)
 467 compared to Human tonsillar keratinocytes immortalized with HPV16 (lane 3). Two independent
 468 human foreskin keratinocyte (HFK) donors were immortalized with wild-type HPV16 (lanes 6 and
 469 7) or overexpression of HPV16 E6 and E7 oncogenes (lane 8, cells from donor 1). (B) Western
 470 blot analysis of p53 expression in 4 HPV-negative and 4 HPV-positive patient derived xenografts.
 471 (C). p53 expression following 24-hour cisplatin treatment of primary HPV-positive head and neck

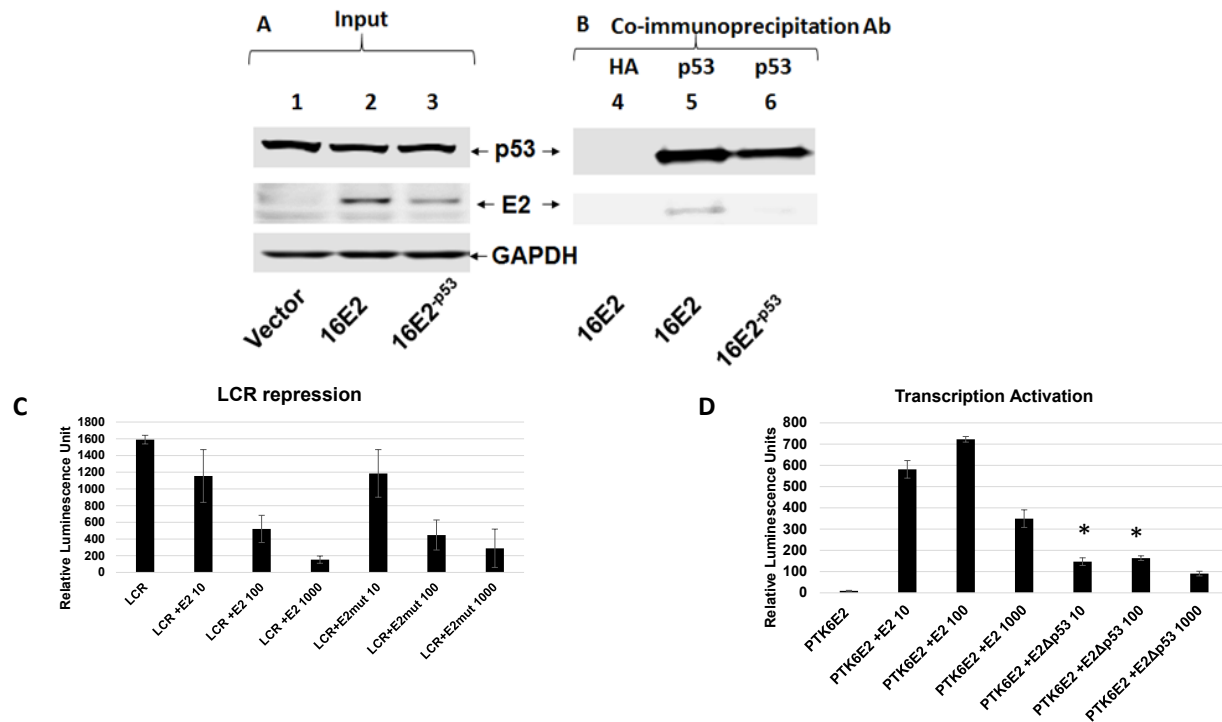
472 cancer cell lines (SCC47 and SCC104) DMF solvent only was used as drug-free control. All
 473 western blots utilized GAPDH as an internal loading control.



478 **Figure 2. p53 reduction in via introduction of full length HPV16 E6 reduces cellular**
479 **proliferation in HPV16 immortalized foreskin keratinocytes.** (A) 11-day growth curve of
480 N/Tert-1 cells expressing exogenous HPV16 E6 compared to empty vector. (B) 13-day growth
481 curve of human foreskin keratinocytes immortalized by HPV16 and stably expressing
482 exogenous full length E6, mutant E6 that does not bind and degrade p53 (E6 Δ p53) or GFP
483 control vector. (C) Senescence staining of cells in B at day 11. Arrows indicate positively
484 staining cells. (D) Quantification of senescence staining in C. Western blot analysis of p53
485 expression following transfection of E6 plasmids (day 0) and after growth rate recovery of
486 HFK+HPV16+E6 (day 13). (E) Western blot analysis of exogenous E6 and E6 Δ p53 expression
487 at day 0 and day 13 using primary antibody against HA tag. HFK+GFP samples were omitted
488 due to signal oversaturation and bleed over into neighboring wells. GAPDH was used as
489 internal loading control. (F) RT-qPCR analysis of exogenous GFP, E6 and E6 Δ p53 expression
490 at day 0 and day 13 using primers against FLAG-HA tag. Relative quantity calculated by the
491 $\Delta\Delta C_T$ method using GAPDH as an internal control. Bonferroni correction utilized when
492 applicable. (G) Western blotting of the indicated extracts using FLAG antibody (the E6 is double
493 tagged with HA and FLAG).

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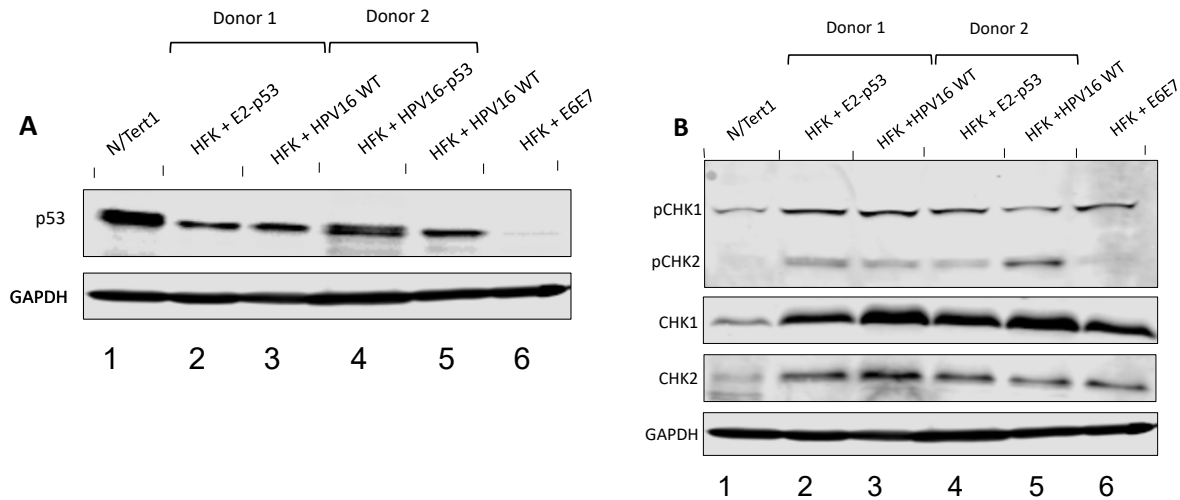


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497 **Figure 3. Generation and characterization of p53 binding mutant of HPV16 E2 (E2-p53) in**
 498 **N/Tert-1 cells.** (A) Input western blot of stably expressing E2 and E2^{p53} in N/Tert-1 Cells. For E2^{p53},
 499 residues W341, D344 and D338 were mutated to alanine as previously described (42, 43).
 500 (B) Co-immunoprecipitation pull down of E2 using polyclonal antibody against p53. (C) HPV16
 501 long control region repression assay of wild-type E2 and E2^{p53}. N/Tert-1 cells were transiently
 502 transfected with 1 µg pHPV16-LCR-Luciferase reporter plasmid along with 10ng, 100ng, or
 503 1000ng of E2 or E2-p53 plasmid. (D) E2 transcriptional activity assay of wild-type E2 and E2-p53.
 504 Similar to LCR repression assay, N/Tert-1 cells were transiently transfected with 1 µg pTK6E2-
 505 Luciferase reporter plasmid along with increasing amounts of E2 wild-type and E2-p53 plasmids.
 506 For (C) and (D), relative luminescence units were calculated by normalizing absolute
 507 luminescence readouts to input protein concentration.

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513 **Figure 4. Generation and characterization of HPV16-p53 immortalized human foreskin**

514 **keratinocytes (HFKs).** (A) p53 protein expression in two independent HFK donors immortalized

515 by wild-type HPV16 (Lanes 3 and 5) and HPV-p53 (Lanes 2 and 4). N/Tert-1 and HFK

516 immortalized by E6 and E7 are provided for reference (lanes 1 and 6 respectively). (B) Activation

517 of the ATR and ATM DNA-damage pathways in immortalized HFKs. ATR and ATM activation by

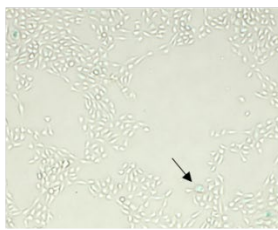
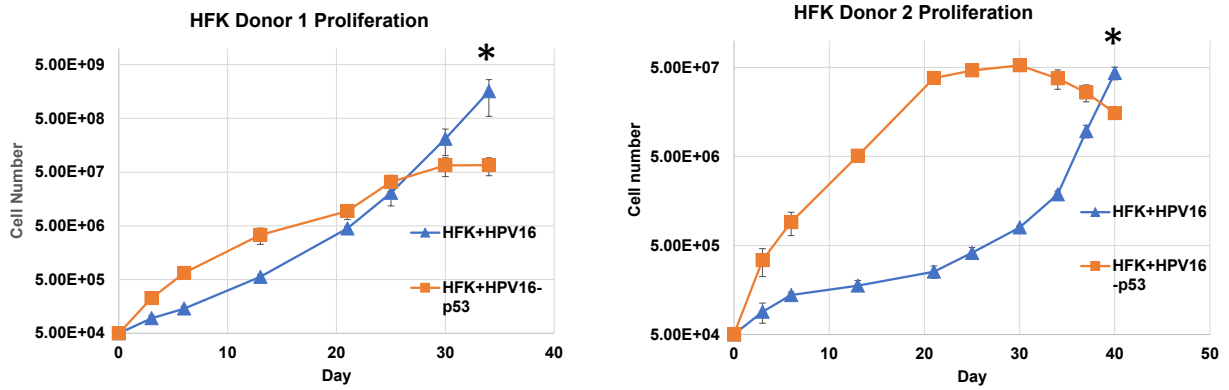
518 HPV16 leads to phosphorylation of Checkpoint kinases 1 and 2 respectively and serve as markers

519 for HPV infection and replication.

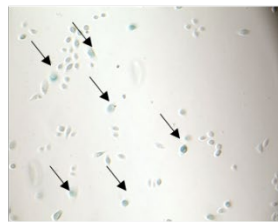
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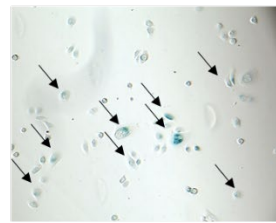
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N/Tert-1

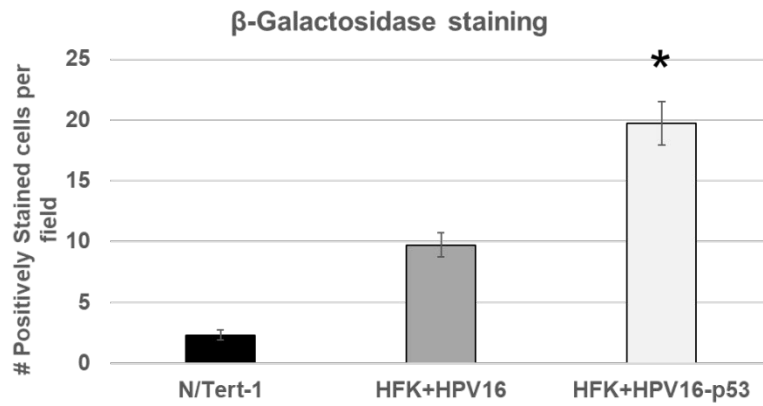


HFK + HPV16



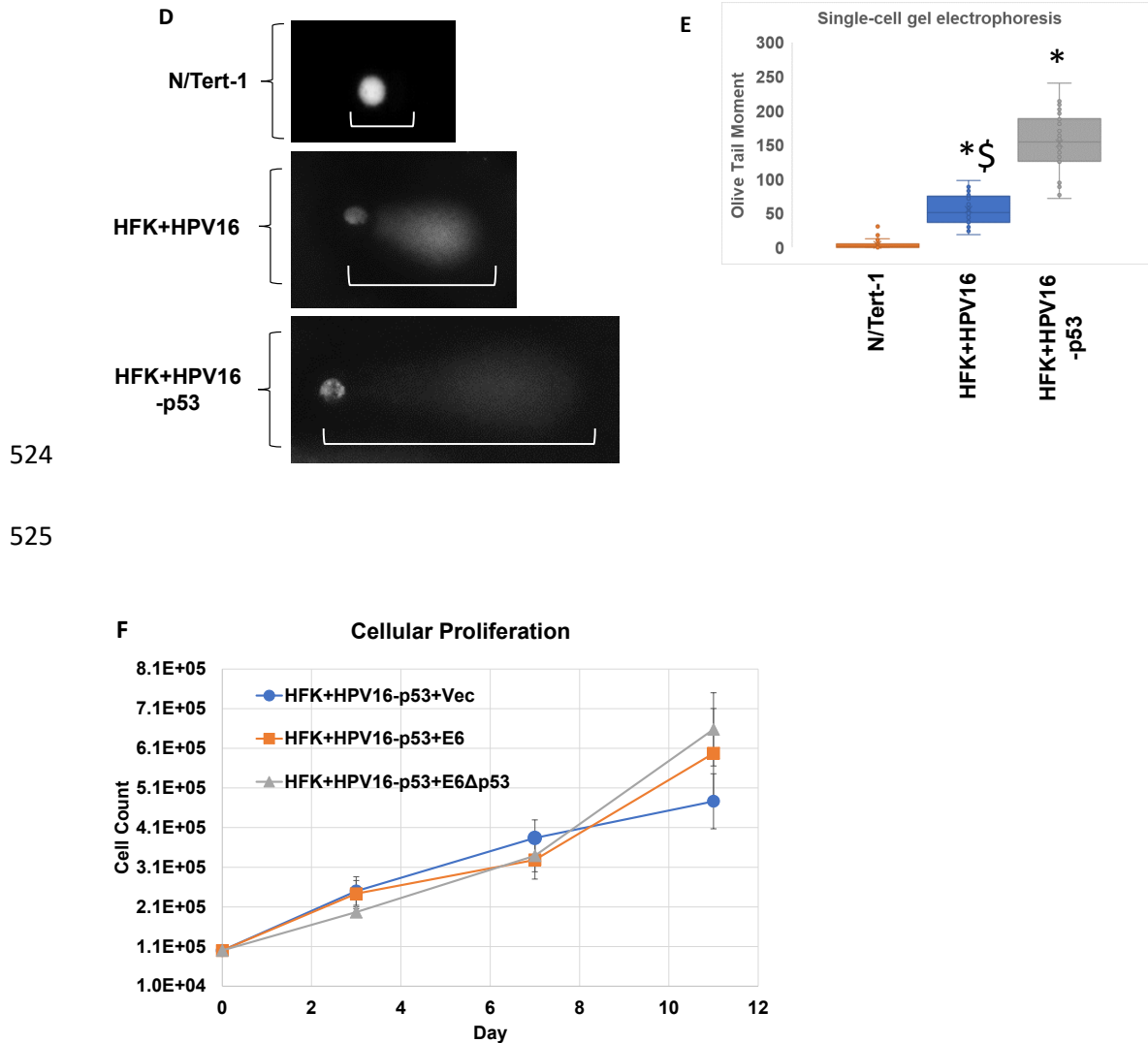
HFK + HPV16-p53

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527 **Figure 5. HFKs immortalized by HPV16-p53 exhibit aberrant growth phenotype, elevated**
 528 **levels of senescence and increased DNA damage and fragmentation.** (A) Extended growth
 529 curve on HFK's immortalized by wild-type HPV16 and HPV16-p53. Cells were grown over a
 530 period of 34-40 days depending on HFK donor cell line. In general, donor 1 proliferated quicker
 531 than donor 2 regardless of HPV genome status. (B). β -galactosidase staining as a marker of
 532 senescence for proliferating HFK+HPV16 and HFK+HPV16-p53 cells compared to N/Tert-1 cells.
 533 Images taken at 10X. Five random fields were imaged per replicate per cell line. Representative
 534 image presented with positively stained cells marked by arrows. (C) Quantification of β -

535 galactosidase staining. Average number of positively stained cells per high power field were
536 calculated by a blinded observer +/- SEM. (D) Single-cell gel electrophoresis (COMET) Assay.
537 Cells were grown in 24-well plate for 24 hours then trypsinized, washed, resuspended in 0.5%
538 low molecular weight agarose, and subjected to single cell gel electrophoresis. DNA was stained
539 with DAPI. Five randomly selected fields were imaged at 20x per replicate per cell line.
540 Representative comets are presented with white bars highlighting comet tails. (E) The olive-tail
541 moments (OTMs) of all non-overlapping comets in each high-power field were were quantified
542 using CaspLab COMET assay software. Average OTM +/- SEM. *p<0.05 for HFK+HPV16 vs
543 HFK+HPV16-p53. \$p<0.05 for HFK+HPV16 vs N/Tert-1. Bonferoni correction used where
544 applicable. (F). Eleven-day growth curve on HFK+HPV16-p53 stably expressing exogenous E6,
545 E6Δp53 or GFP control.

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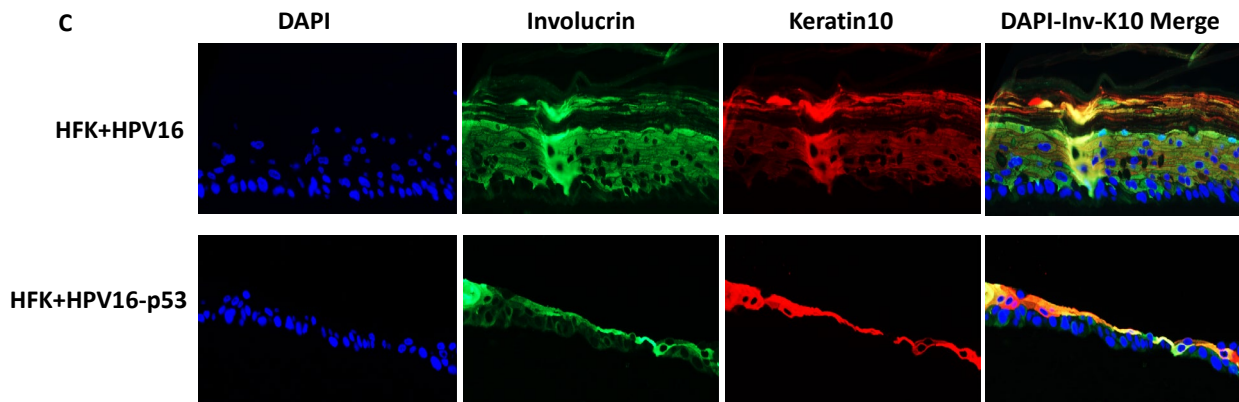
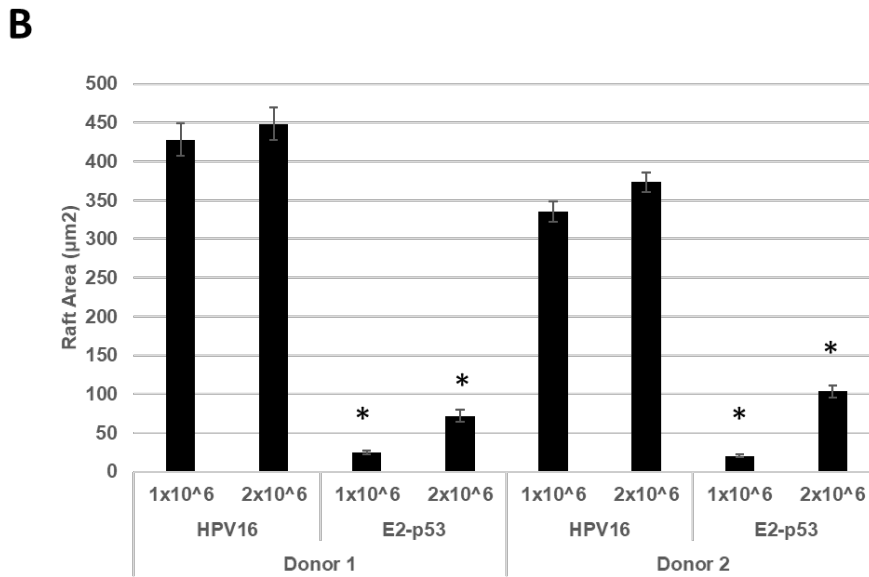
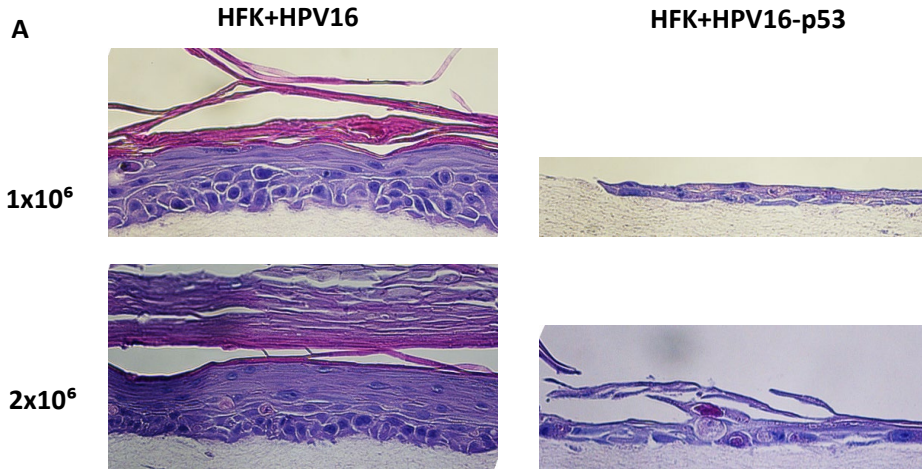
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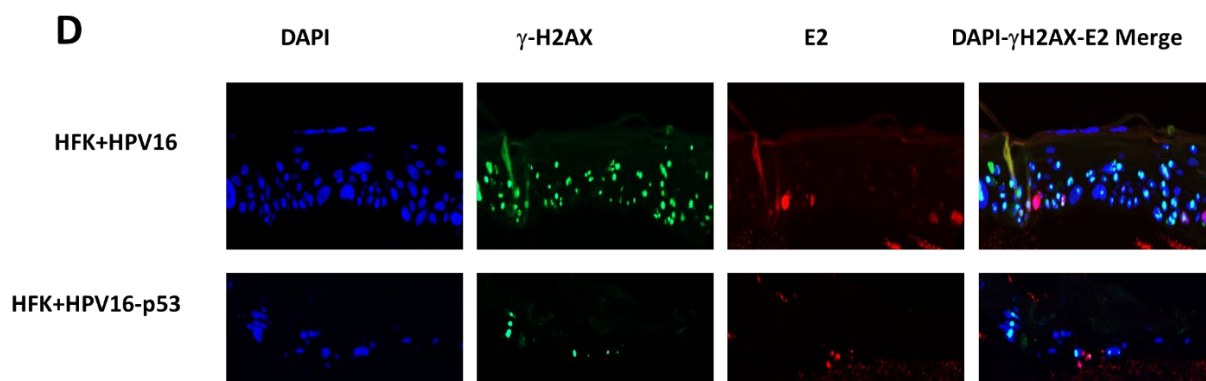
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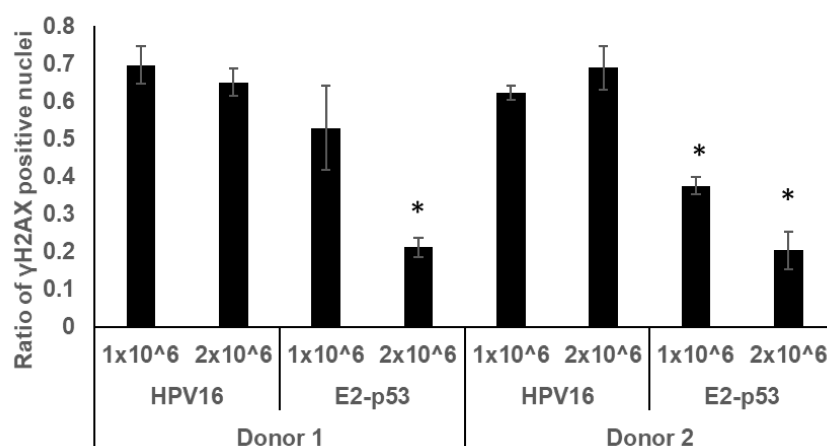
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561 **Figure 6. Organotypically rafted HFKs immortalized by HPV16-p53 exhibit aberrant life**
 562 **cycle with dysregulated differentiation, lower markers of viral replication and overall**
 563 **reduced raft proliferation.** (A) Organotypic raft cultures and H&E staining of samples from figure
 564 5. HFKs were seeded onto collagen matrices at densities of 1×10^6 (upper panels) and 2×10^6
 565 (lower panels). (B). The experiment in A was repeated in a second independent HFK donor and
 566 average raft areas were calculated for each donor using a Keyence imaging system. (C) HFK
 567 rafts stained using indicated antibodies as markers of keratinocyte differentiation. (D) DNA
 568 damage and viral replication marker γ -H2AX was stained for in HPV16 and HPV16-p53 HFK rafts.

569 (E) γ -H2AX staining was repeated in a second HFK donor and quantified using a Keyence imaging
570 system.

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