MAML1-induced HPV E6 oncoprotein stability is required for cellular proliferation and migration of cervical tumor-derived cells

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Abstract

While a small proportion of high-risk (HR) alpha (α) Human Papillomaviruses (HPVs) is associated with numerous human malignancies, of which cervical cancer is the most prevalent, beta (β) HPVs predominantly act as co-factors in skin carcinogenesis. A characteristic feature of both α- and β-E6 oncoproteins is the presence of the LXXLL binding motif, which α-E6s utilize to form a complex with E6AP and which enables β-E6s to interact with MAML1. Here we show that multiple α-E6 oncoproteins bind to MAML1 via the LXXLL binding motif and that this results in increased protein stability. Moreover, β-E6 oncoprotein stability is also dependent on the interaction with MAML1. Additionally, in the absence of MAML1, endogenous HPV-8 E6 and HPV-18 E6 are rapidly degraded at the proteasome. Ablation of both E6AP and MAML1 leads to an even more profound down-regulation of α-E6 protein expression, whereas this is not observed with β-E6. This highly suggests that there is one cellular pool for most of β-E6 that interacts solely with MAML1, whereas there are two cellular pools of HR α-E6, one forming a complex with MAML1 and the other interacting with E6AP. Furthermore, MAML1 induces HPV-8 E6 shuttling from the nucleus to the cytosolic fraction, while MAML1 interaction with HR E6 induces a drastic nuclear and membrane upregulation of E6. Interestingly, the HR α-E6 /MAML1 complex does not affect targeting of some of the known HR E6 cellular substrates such as p53 and DLG1. However, MAML1 and E6AP joint co-expression with HR α-E6 leads to a significant increase in cellular proliferation, whereas silencing MAML1 decreases wound closure in HeLa cells. These results demonstrate that HR α-E6 interaction with MAML1 results in a stable form of E6, which likely modulates MAML1's normal cellular activities, one consequence of which being an increased proliferative capacity of HPV-transformed cancer cells. Thus, this study shows a novel function of the α-E6 oncoprotein and how it's activity might affect HPV-induced pathogenesis.

42 ## Introduction

43 Human papillomaviruses (HPVs) are a family of small DNA viruses that infect epithelial cells at
44 various anatomical sites of the human body. It is a large and diverse group of viruses, divided into five
45 genera (alpha (α), beta (β), gamma (γ), mu (μ) and nu(ν)) based on the open reading frame sequences
46 coding for the L1 capsid protein. Thus far, more than 200 different HPVs have been discovered and
47 recognized by the International Human Papillomavirus Reference Center [1]. Of the five genera, α and β
48 have been most extensively studied, mostly due to their ability to cause pathological conditions in humans.
49 Based on their ability to promote carcinogenesis, α-HPVs can be classified as either high-risk (HR) or low-
50 risk (LR), with no such classification existing for β-HPVs [1].

51 Almost all HPV types classified as cancer-causing reside within the genus α-papillomavirus. Cancers
52 caused by these types are predominantly anogenital, the most important of which being cervical cancer,
53 but also include a subset of head and neck cancers [1,2]. Of all HR HPVs, HPV-16 and HPV-18 account for
54 the vast majority of HPV-associated malignancies –approximately 70% world-wide cervical cancer cases,
55 with the remaining 30% being caused by other HR HPVs [3]. By contrast, infections with LR α-HPV types ,
56 such as HPV-6 and HPV-11, merely lead to self-limiting benign warts [1,4]. The oncogenic potential of HR
57 HPVs is attributed to the E6 and E7 oncoproteins and their interaction with a diverse range of cellular
58 substrates, the most pivotal of which being p53 and pRB, respectively [5,6]. The E7 oncoprotein binds to
59 pRB as well as other members of the pocket protein family and targets them for destruction by the
60 ubiquitin-proteasome system [7]. Thus, E7 effectively disables the G1 cell cycle checkpoint, promotes cell
61 proliferation and, as an unintended consequence, contributes to genomic instability [8]. HPV E6 forms a
62 tertiary complex with the E6AP (E6-associated protein) ubiquitin ligase and p53, which leads to p53
63 polyubiquitination and degradation by the proteasome, ensuring the resistance of infected cells to
64 apoptosis, and cell cycle progression [9,10]. Furthermore, analyses of the association between E6 and E6AP
65 have revealed that the protein stability of HR E6 oncoproteins was dependent on the presence of E6AP.

66 Moreover, ablation of E6AP from HPV-18 positive HeLa cells resulted in a dramatic increase in E6
67 oncoprotein turnover at the proteasome [11]. In addition to p53, HR E6 oncoproteins also target a number
68 of other cellular substrates for a proteasomal degradation in both E6AP-dependent and -independent
69 manners, resulting in the modulation of various cellular pathways. Some of the more comprehensively
70 described E6 targets include DLG1, SCRIB and the MAGI protein family members, which are all PDZ domain-
71 containing proteins involved in several important cellular processes ranging from cell growth, polarity and
72 adhesion to cell differentiation, proliferation, apoptosis, migration and intracellular trafficking [12,13].

73 Unlike α-HPVs, HPVs of the genus β are not considered carcinogenic by themselves, but can
74 contribute to the development of skin cancer in the presence of certain underlying health conditions, such
75 as in patients with the genetic disorder Epidermodysplasia verruciformis (EV) or in otherwise
76 immunocompromised individuals [14,15]. β-HPV types, such as HPV-5 and HPV-8, have been found in EV
77 patient skin lesions and squamous cell carcinoma samples, however, subsequent analyses have shown that
78 β-HPVs do not cause the cancer but facilitate carcinogenesis by inhibiting apoptosis in response to UV
79 damage [16]. Like their α-HPV counterparts, β-HPVs also employ E6 and E7 proteins, yet with some
80 significant differences. For instance, while β-E7s bind to pRB tumor suppressor, they do so with much
81 weaker affinity than α-E7s, and thus this binding does not induce pRB degradation. Unlike α-HPV E6 β-E6
82 oncoproteins do not target p53 for proteasome-mediated degradation, but inhibit its stabilization in
83 response to genome-destabilizing events, thus allowing for the accumulation of DNA damage [17]. Of note,
84 the β-genus HPV-38 E7 oncoprotein additionally stabilizes p53 [8,18]. Interestingly, a recent study has
85 demonstrated that, while α-E6s form a complex with E6AP and thus modulate its ubiquitin ligase activity,
86 E6 oncoproteins of the other four genera, including β-E6s, preferentially bind MAML1 (Mastermind Like
87 Transcriptional Coactivator 1) [19]. Nevertheless, it is important to note that, while a distinction between
88 the preferred binding partners of different genera exists, this binding preference is by no means mutually

89 exclusive, as some cutaneous HPV E6s have been shown to also interact with E6AP, which contributes to
90 E6 protein stabilization [20].

91 MAML1 is a transcriptional co-activator of the Notch signaling pathway. Activated Notch signaling
92 has different effects depending on different cells and tissue types, and this complexity is notably profound
93 in the context of HPV infection, where Notch activation has been found to be both positively and negatively
94 involved in HR HPV infection and progression of infected tissues from precursor lesions towards cervical
95 cancers [21]. During HPV infections of cutaneous epithelia β-HPV E6 oncoproteins interact with MAML1 to
96 prevent the formation of the Notch-activating complex and, thus preventing differentiation and cell cycle
97 arrest, and in this way enabling the viruses' survival and stability in skin keratinocytes [22]. It is currently a
98 well-established fact that the binding of α-E6s to the LXXLL motif on E6AP is crucial for their interaction and
99 for the subsequent cellular functions of these E6 oncoproteins [11,23,24]. β-E6 and MAML1 interact via
100 the same binding motif present on both proteins, and the effects of these interactions on the Notch
101 signaling pathway have been characterized in numerous reports as well. However, to the best of our
102 knowledge, no detailed research has been done on investigating the effects of MAML1 binding on E6
103 protein stability and how this might affect E6 functionality [25–27]. Therefore, we wanted to investigate
104 whether the binding of both α- and β-HPV E6 to MAML1 would have an effect on E6 oncoprotein stability
105 and, if so, how this might influence E6 molecular activities and their potential impact on cell proliferation
106 and migration.

107 In the present manuscript, we show that MAML1 binding to E6 leads to increased protein stability,
108 not only in the case of β-E6s, but also for E6 proteins of the genus α. MAML1 binds to a defined cellular
109 pool of HR E6 oncoproteins, but does not influence its capacity to target tumor suppressors p53 and DLG1
110 for proteasome-mediated degradation. However, MAML1 decreases the rate of protein turnover of this
111 distinct pool of HR E6 oncoprotein as well as of HPV-8 E6 oncoprotein, and this appears to be E6AP-

112 independent. Furthermore, E6AP and MAML1 increase cellular proliferation by synergistically stabilizing HR
113 E6 protein levels, which is likely to have a profound role on HPV-driven oncogenesis.

114 Materials and methods

115 *Cell lines and culture, transfections*

116 HEK-293 (human embryonic kidney), CRISPR/Cas9 gRNA-mediated E6AP-null HEK-293 cells [24] ,
117 HeLa (cervical carcinoma, HPV-18 positive), CaSki (cervical carcinoma, HPV-16 positive), HT1080 8 E6
118 (fibrosarcoma epithelial cells, stably expressing HPV-8 E6) [17] and C33A (cervical carcinoma, HPV-negative)
119 cells were used in the experiments. All cell lines were grown in Dulbecco's modified Eagle's medium
120 (DMEM) (Gibco, Thermo Fisher Scientific, USA) with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher
121 Scientific, USA) and penicillin/streptomycin (100 U ml^{-1}) (Gibco, Thermo Fisher Scientific, USA) and L-
122 glutamine (300 µg ml^{-1}) (Gibco, Thermo Fisher Scientific, USA) at 37°C in a humidified air incubator
123 containing 10 % CO_2. Cells were transfected by calcium phosphate precipitation method [28] and analyzed
124 or harvested 24hrs or 48hrs post transfection. Cells transfected with β-galactosidase and a suitable amount
125 of empty pcDNA3 vector were used as controls for transfection efficiency.

126 *Plasmids*

127 HA-tagged HPV-8, -11, -12, -14, -16, -18, -24, -33 and -38 E6, as well as pcDNA3 FLAG-E6AP,
128 pCDNA3-FLAG-p53 [20,29,30] and pCDNA3 plasmid expressing HA-DLG1, cloned into pcDNA3 expression
129 vectors were used and have been described previously [31]. Other plasmids included a FLAG-tagged
130 MAML1 cloned into pCMV2-FLAG plasmid, pCMV2-FLAG MAML LHHLL mutant [32], pCS2 MYC-MAML1
131 [33], pCIG-MYC E6AP [34], the empty vector pXJ41-FLAG or pXJ41-16E6-FLAG coding for FLAG-tagged
132 HPV16 E6 [35]. pGEX-2T:16E6, pGEX-2T:18E6, pGEX-2T:E6 and pGEX-2T:11E6 plasmids expressing the GST-
133 E6 fusion proteins have been previously described [11,30,36].

134 *Antibodies*

135 The following antibodies were used: anti-MAML1 (clones D3E9 and D3K7B) (Cell Signaling
136 Technology, USA), anti-E6AP (UBE3A; clone 13/UBE3A) (BD Bioscience, USA), anti-p53 (clone DO-1) (Santa
137 Cruz Biotechnology, USA), anti-β-actin-HRP (clone B-7) (Sigma-Aldrich, DE), anti-HPV-18 E6 (clone G-7)
138 (Santa Cruz Biotechnology, USA), anti-HA-peroxidase (clone HA-7) (Sigma-Aldrich, DE), anti-FLAG (clone
139 M5, Sigma-Aldrich, DE), anti-FLAG (H-5) (Santa Cruz Biotechnology, USA), anti-c-myc (9E10) (Santa Cruz
140 Biotechnology, USA), anti-β-galactosidase (Promega, USA), anti-DLG1 (SAP-97, clone 2D11) (Santa Cruz
141 Biotechnology, USA), anti-MCM7 antibody (clone 141.2) (Santa Cruz Biotechnology, USA), anti-GAPDH
142 antibody (Abcam, UK), anti-histone H3 antibody (clone D1H2) (Cell Signaling Technology, USA) and anti-
143 Vimentin antibody (clone E5) (Santa Cruz Biotechnology, USA), GAPDH-Alexa Fluor 680 (G9, Santa Cruz
144 Biotechnology, USA); and mouse and rabbit secondary antibodies conjugated to horseradish peroxidase
145 (HRP) (Dako, Agilent, USA), IRDye-coupled secondary antibody and rhodamine red or Alexa Fluor 488
146 (Invitrogen, Thermo Fisher Scientific, USA).

147 *Inhibitors*

148 The inhibitors were dissolved in dimethyl sulfoxide (DMSO) and used at the indicated
149 concentrations. The proteasome inhibitor bortezomib (BTZ; 10 µM) (Sigma-Aldrich, DE) was used at the
150 concentration 1:10000. Cycloheximide (Sigma-Aldrich, DE) was used at the concentration 1:1000 in order
151 to block protein synthesis in half-life experiments. The lysis buffers were supplemented with protease
152 inhibitor, 1 × Cocktail Protease Inhibitors (Roche Diagnostics, DE).

153 *Western blot*

154 Following treatment, cells were harvested by scraping, centrifuged and pellets resuspended in SDS
155 buffer (100 mM Tris HCl pH=6,8, 200 mM DTT, 4% SDS, 20% glycerol and bromophenol blue). Samples were
156 then subjected to sonification (1'') and heated at 95°C for 10'. Proteins were separated on either 7,5% or

157 10% sodium dodecyl sulfate - polyacrylamide gels (SDS-PAGE) and transferred by tank blotting to a
158 nitrocellulose membrane with a pore size of 0,2 µm. Membranes were blocked with 10% dry milk in PBS or
159 TBS containing 0,1% Tween-20 (PBST/TBST) for 30' at 37°C, after which they were incubated with indicated
160 antibodies diluted in 10% milk in PBST or TBST (2h at RT or overnight at 4°C). Membranes were washed in
161 PBST or TBST (3 x 10') and then incubated for 1h with HRP-linked secondary antibodies (anti-rabbit 1:1000
162 or anti-mouse 1:1000), after which they were washed again. Signals were developed either with
163 SuperSignal™ West Pico PLUS Chemiluminiscent Substrate (Thermo Fisher Scientific, USA) or Amersham
164 ECL western blotting detection reagents (Cytiva, USA) detected by Uvitec Cambridge – Alliance 4.7 and
165 Alliance Q9 imaging systems (Cleaver Scientific, UK) or a LI-COR Odyssey Fc imager (LI-COR, USA), and
166 quantified by ImageJ [37]. If needed, antibodies were detached from membrane using with 0.2 M NaOH
167 for 20' after which membranes were re-probed. E6 band intensity was first normalized with the image
168 background after which it was divided with that of normalized β galactosidase (LacZ) which served as
169 transfection control. The average normalized relative expression of E6 of at least four experiments where
170 E6 was transfected alone, was taken as control. All the normalized relative expressions of E6 (transfected
171 alone or with MAML1 or E6AP) were expressed as fold changes in regards to the control E6. The LOG2 of
172 these fold changes was calculated to better depict the increase or decrease in protein amounts. The same
173 procedure was applied in experiments with p53 and DLG1 overexpression.

174 *Immunoprecipitation*

175 HEK-239 cells were transfected with an empty plasmid, HA-tagged HPV-16 E6 and HPV-8 E6 alone,
176 or in combination with FLAG-MAML1, FLAG-MAML1 (LHHLL) or myc-MAML1 (LHHLL). For
177 immunoprecipitation experiments, transfected cells were harvested by scraping and centrifuged. Cell
178 pellets were resuspended on ice in modified E1A lysis buffer (0.25% NP-40, 1 mM EDTA, 50 mM HEPES, pH
179 7.4, 150 mM NaCl). Samples were subjected to sonification (2 x 30'') and left on ice for 20'. After
180 centrifugation supernatants were collected and incubated with anti-HA beads (Sigma-Aldrich, DE) for 2hrs

181 on a rotating wheel at RT. The beads were extensively washed and subjected to western blot analysis with
182 anti-FLAG or anti-myc antibodies. C33A cells were transfected with the empty vector pXJ41-FLAG or pXJ41-
183 16E6-FLAG coding for FLAG-tagged HPV16 E6. Cell extracts were generated 48hrs post transfection using
184 low salt dilution buffer (LSDB) (50 mM Tris/HCl (pH 8), 20 % Glycerol, 100 mM KCl, 0,1% NP40, 1 mM DTT,
185 50 mM NaF, 1 mM orthovanadate, 1 mM PMSF), followed by incubation with anti-FLAG antibody coupled
186 to magnetic beads (Thermo Fisher Scientific, USA) for 2hrs at room temperature, followed by three washes
187 with LSDB. Co-precipitated cellular proteins were detected by western blotting with anti-MAML1 and anti-
188 FLAG.

189 *Fusion protein purification and GST pull-down assay*

190 DH5α *Escherichia coli* competent cells were transformed with GST-E6 constructs (GST-16 E6, -18
191 E6, -11 E6 and -8 E6) and GST fusion protein synthesis and protein purification were performed as
192 previously described [38]. GST- pull-down assays using cellular extracts were performed by incubating equal
193 amounts of GST-tagged proteins immobilized on glutathione agarose, with pre-cleared whole cell lysates
194 of HEK-293 cells (previously transfected with FLAG-tagged MAML1) in modified E1A buffer (0.25% NP-40,
195 1 mM EDTA, 50 mM HEPES, pH 7.4, 150 mM NaCl) for 2hrs on a rotating wheel at 4°C. After extensive
196 washing with E1A buffer, the bound proteins were detected using SDS-PAGE and western blotting with
197 anti-FLAG antibody.

198 *siRNA silencing*

199 The cells were seeded on 60 mm tissue culture dishes and transfected the following day with ON-
200 TARGETplus human MAML1 siRNA (siMAML1, smartpool, Horizon discovery, UK) and ON-TARGETplus
201 human UBE3A siRNA (siE6AP, individual, Horizon discovery, UK) according to the manufacturer's
202 instructions and harvested 72hrs after silencing. siRNA against luciferase (siLuc, individual, Horizon
203 discovery, UK) was used as control.

204 *Immunofluorescence*

205 HeLa and HT1080 8 E6 cells were grown overnight on coverslips and transfected the following day.
206 Seventy-two hrs post transfection cells were fixed with 4.7% paraformaldehyde for 10' at RT and
207 permeabilized in PBS containing 0.1% Triton X-100. The staining was performed by incubating samples with
208 indicated antibodies diluted in PBS overnight at 4°C in a humidified chamber. After washing, the coverslips
209 were incubated with fluorescently conjugated mouse or rabbit secondary antibodies. Nuclei were labelled
210 with DAPI [36]. Samples were monitored on a laser scanning microscope Leica TCS SP8 X, equipped with a
211 HC PL APO CS2 63×/1.40 oil objective (Leica Microsystems, DE).

212 *Half-life experiments*

213 HeLa cells and HT1080 HPV-8 E6 expressing cells were transfected with siRNA against luciferase
214 (siLuc – control), siMAML1 and siE6AP alone or in combination. Seventy-two hrs later, prior to harvesting,
215 cells were treated with cycloheximide (Sigma-Aldrich, DE), which was added in fresh medium. The cells
216 were harvested at time points indicated and analyzed by western blotting. siRNA against luciferase was
217 used as control.

218 *Cell fractionation assays*

219 For cell fractionation analyses, HEK-293 cells were seeded on 100 mm tissue culture dishes at a
220 density of approximately 7 x 10^5 prior to transfections. Twenty-four hrs post transfection the cells were
221 collected by scraping and fractionated into cytoplasmic, membrane, nuclear and cytoskeletal fractions
222 using the ProteoExtract Cell Fractionation Kit (Calbiochem, Merck, USA) according to the manufacturer's
223 instructions. The fractions were analyzed by SDS-PAGE followed by western Blotting using anti-HA antibody
224 (Sigma-Aldrich, DE), anti-MCM7 antibody (clone 141.2) (Santa Cruz Biotechnology, USA), anti-GAPDH
225 antibody (Abcam, UK), anti-histone H3 antibody (clone D1H2) (Cell Signaling Technology, USA) and anti-
226 Vimentin antibody (clone E5) (Santa Cruz Biotechnology, USA) as indicated.

227 *Cell proliferation assay*

228 The assay was performed as described previously [39]. In brief, HEK-293 cells (1.2×10^5) were
229 seeded on 60 mm tissue culture dishes, cultured overnight and transfected by calcium phosphate
230 precipitation method [28], using a total of 9.0 µg of plasmid DNA. The cells were transfected with pcDNA3
231 plasmids expressing HPV-16 E6 and HPV-8 E6, alone or in combination with MAML1, E6AP or both as
232 indicated. In order to monitor transfection efficiency, cells were co-transfected with β-galactosidase (LacZ)
233 and verified by western blotting using the appropriate antibodies. Twenty-four hrs post transfection, the
234 cells were detached, counted, and seeded at 0.5×10^4 cells per well to a final volume of 100 µl in a 96-well
235 plate and incubated for a further 48hrs. Cell proliferation was monitored using Uptiblue reagent (Interchim)
236 as previously described [39]. Uptiblue reagent (5% [vol/vol]) was added to the culture medium 72hrs after
237 transfection, and absorbance was measured at 575 nm and 590 nm on a Labystems Multiskan MS 352
238 multiwell plate reader with accompanying Ascent Software (Thermo Fischer Scientific, USA). The results are
239 expressed as a percentage of the reduced Uptiblue reagent with the indicated standard error of the mean
240 (SEM).

241 *Wound healing/scratch assay*

242 A monolayer scratch/wound healing assay was done as described previously [40]. HeLa and HT1080
243 8 E6 cells were transfected with a control siLuc, siMAML1 and siE6AP as indicated. After 48hrs, a scratch
244 wound was generated in the confluent cells with a sterile Artline p2 pipette tip (Thermo Fisher Scientific,
245 USA). The wounds were photographed under a microscope using a Dino-Eye digital eyepiece camera
246 (AM7023(R4); IDCP B.V., NE) that was connected to a computer and DinoCapture 2.0 microscope imaging
247 software. After an additional 24hrs, the wounds were photographed again, and wound closure was
248 calculated; the images were saved in TIFF format, and gap areas were measured using the MRI wound
249 healing tool macro for ImageJ software (NIH)[37] (http://dev.mri.cnrs.fr/projects/imagej-
250 macros/wiki/Wound_Healing_Tool). The cells were then harvested by scraping, centrifuged and pellets

251 resuspended in SDS buffer. MAML1, E6AP and E6 protein levels were analyzed by western blotting. β-actin
252 was used as a loading control.

253 *Statistical analysis*

254 Data were analyzed by GraphPad Prism (GraphPad Inc. USA). The differences and their statistical
255 significance between groups were determined by one-way ANOVA or student's t-test. P values of under
256 0.05 were considered significant (*), under 0.01 very significant (**) and under 0.001 extremely statistically
257 significant (***). All values are averages of at least three independent experiments and the standard error
258 of the mean is depicted as error bars.

259 **Results**

260 *HPV E6 oncoproteins from α and β types interact with MAML1*

261 Several proteomic analyses have demonstrated an interaction between a number of β-HPV E6
262 oncoproteins and MAML1, a co-activator of the Notch signaling pathway [25,26]. One of these studies also
263 showed an association between MAML1 and HPV-16 E6 in co-immunoprecipitation assays, although the
264 association described there was only minimal [25]. To verify that MAML1 is indeed a binding partner of
265 both α- and β-HPV E6 oncoproteins we first performed co-immunoprecipitation analyses using HEK-293
266 cells, which had been transfected with either an empty plasmid, or plasmids expressing HA-tagged HPV-16
267 E6 and HPV-8 E6, alone or in combination with FLAG-tagged MAML1. Twenty-four hrs post transfection the
268 cells were harvested and lysed, and the cell extracts were incubated with anti-HA beads. The protein
269 complexes immunoprecipitated in this manner were analyzed by western blot. The results shown in Figure
270 1A demonstrate an interaction between HPV-8 E6 and MAML1, as well as a somewhat weaker, but still very
271 evident interaction between HPV-16 E6 and MAML1. To additionally verify these observations, we
272 expanded our analysis and conducted co-immunoprecipitation assays between ectopically expressed 16 E6
273 and endogenous MAML1 in the HPV-negative cervical tumor derived cell line C33A. The cells were

274 transfected either with an empty plasmid or plasmid expressing FLAG-tagged 16 E6. Cell extracts were
275 generated 48hrs post transfection, incubated with anti-FLAG-antibody coupled to magnetic beads and the
276 immunoprecipitated complexes were further subjected to western blot analysis. As indicated in Figure 1B,
277 16 E6 also interacted with endogenous MAML1 in this experimental setting, further supporting the
278 observation that these two proteins form a complex. These results corroborate those previously published,
279 which state that, even though there is a distinct preference across different β-HPV types for MAML1, HPV
280 genera cannot be exclusively divided based on this interaction [19]. Next, we were interested in
281 investigating whether MAML1 could complex with other α-type E6 oncoproteins. To establish this, we
282 performed a series of glutathione S-transferase (GST) pull-down assays using GST-16 E6, GST-18 E6, GST-
283 11 E6, GST-8 E6 fusion proteins, or GST alone as a control. Bound proteins were detected by SDS-PAGE and
284 western blotting, and the results are shown in Figure 1B. In this setting, we observed that E6 proteins from
285 all the HPV types tested bind to MAML1. HPV-8 E6 interaction was decisively the strongest, followed by 18
286 E6, with a weaker interaction with 16 E6, while the interaction with 11 E6 was the weakest (Figure 1B).
287 Taken together these results suggest that, even though E6 oncoproteins from α- and β-HPV types can
288 complex with MAML1, the preferred interacting partners are E6 oncoproteins from the β genus.

289 *MAML1 stabilizes E6 oncoproteins from α- and β-HPV types*

290 After confirming that both α- and β-HPVs interact with MAML1, we sought to establish whether
291 these interactions have any impact on E6 oncoprotein stability. HEK-293 cells were transfected with a panel
292 of plasmids expressing HA-tagged E6 proteins from HPV types -8, -12, -14, -16, -18, -24, -33 and -38, either
293 alone or in combination with FLAG- or Myc-tagged MAML1 or E6AP. After overnight incubation, the
294 transfected cells were harvested and proteins isolated, separated and analyzed by means of western
295 blotting. E6 protein band intensities from HEK-293 cells transfected with the panel of E6s alone were
296 compared with those co-transfected with MAML1 or E6AP. Considering that the stabilizing effect of E6AP
297 on HR HPV E6 oncoproteins is well defined [11], we chose to compare the impact of MAML1 on E6 stability

298 with that of E6AP. As shown in Figure 2A (Supplementary Figure 1, left panel), E6 oncoproteins of all HPV
299 α- and β-types analyzed were stabilized by MAML1, showing that HPV proteins cannot be separated based
300 on MAML1 and E6AP association, as well as demonstrating a previously unknown role for MAML1 in E6
301 stabilization. As expected, E6AP stabilized α-type HPV E6s, but, interestingly, also β-types 24 E6 and 38 E6,
302 which were previously reported to bind E6AP [20].

303 As HEK-293 cells endogenously express the functional E6AP ubiquitin ligase, it was necessary to
304 confirm that the upregulation of E6 observed in HEK-293 cells was MAML1 specific and not dependent on
305 the effects of the endogenous E6AP. For this reason, HEK-293 E6AP knock-out cells (E6AP KO) [24] were
306 transfected with plasmids expressing HA-tagged E6 proteins from HPV types -8, -12, -14, -16, -18, -24, -33
307 and -38 E6, either alone or together with MAML1 or E6AP, and analyzed in the manner described above.
308 E6 proteins of all HPV types tested were again stabilized by MAML1 (Figure 2B and Supplementary Figure
309 1, right panel), supporting the initial observations and confirming that the stabilizing effect is indeed due
310 to MAML1, and is independent of E6AP. Transfecting E6AP KO cells with plasmid expressing HPV-24 E6,
311 alone or in combination, again provided the same results, as did the transfection with plasmid expressing
312 HPV-38 E6 protein, confirming the observation that both MAML1 and E6AP stabilize those cutaneous types.
313 Interestingly, whilst E6 protein stabilization by E6AP and MAML1 was evident in both cellular backgrounds,
314 the actual degree of stabilization varied among different E6 types examined. This is likely to be attributed
315 to the differences in strength of the interactions between MAML1 and E6 proteins among distinct HPV
316 types, as evident from the GST pull-down assays.

317 *E6 protein stabilization is LXXLL dependent*

318 Both MAML1 and E6AP share a common structural feature - the LXXLL motif, through which they
319 interact with E6 oncoproteins. α-HPV E6 oncoproteins associate with E6AP by binding to a conserved LXXLL
320 motif on E6AP, forming a stable complex which targets a number of cellular substrates for proteasome-

321 mediated degradation [9,41]. This interaction is also responsible for E6 oncoprotein stability, which is
322 further reflected by the effects E6 has on global transcription [11,23]. The C-terminus of MAML1 carries an
323 acidic domain containing the LXXLL motif responsible for interacting with β-HPV E6 [25,26]. Therefore, we
324 aimed at further elucidating whether an intact, wild type LDDLL motif might be necessary for MAML1 to
325 form a complex with E6 and consequently induce E6 protein stabilization. In order to investigate this, we
326 transfected HEK-293 cells with plasmids expressing HPV-16, -18, and -8 E6 oncoproteins either alone or
327 together with a wild type MAML1 or a MAML1 mutant (MAML1 LHHLL). The mutant in this experiment had
328 the two aspartate residues of the LDDLL motif, which have previously been described to be indispensable
329 for the interaction, mutated to histidines (LHHLL) [32]. Regardless of the mutated LDDLL domain, we
330 observed that both α- and β-HPV E6 oncoproteins were again stabilized. HPV -8 and -18 E6s were stabilized
331 by both wt MAML1 and the mutant, but the mutant stabilized E6 to a lesser degree than the wt MAML1
332 (Figure 3A, top panel and Supplementary Figure 2A, left panel). Interestingly, HPV-16 E6 was not stabilized
333 by the MAML1 mutant, possibly because its initial interaction was weaker and thus completely abrogated
334 by mutating the residues (Figure 3A, top panel). Again, the findings were confirmed by repeating the same
335 experiment in HEK-293 E6AP KO cells, resulting in the same effect on E6 oncoproteins (Figure 3A bottom
336 panel and Supplementary Figure 2A, right panel). We sought to further explore these findings by
337 performing co-immunoprecipitaton experiments of HPV-8 E6 oncoproteins with wt and mutated MAML1.
338 Here we found that, indeed, the mutation did not fully abrogate the interaction (Figure 3B and
339 Supplementary Figure 2B). Therefore, it is likely that all 5 amino acids or even other amino acids upstream
340 or downstream of the LXXLL motif are involved in the association with E6s, since the mutant still interacted
341 with HPV-8 E6, although to a lesser extent, which was then reflected by a reduction of E6 protein
342 stabilization.

343 *HPV E6 protein stability in CaSki, HeLa and 8 E6 expressing HT1080 cells is MAML1-*
344 *dependent*

345 Having established that ectopic co-expression of MAML1 and E6 oncoproteins from α- and β-HPV
346 types leads to stabilization of E6 protein levels in HEK-293 cells and HEK-293 E6AP KO cells, we wanted to
347 further examine this effect in HPV-positive cell lines, which endogenously express E6. In order to achieve
348 our goal we used cervical cancer-derived HeLa cells, which contain HPV-18 E6/E7 DNA sequences; CaSki
349 cells, which contain HPV-16 E6/E7 DNA sequences; and HT1080 cells, stably expressing FLAG-tagged HPV-
350 8 E6 [17]. To assess whether the stabilization indeed occurs at the protein level, MAML1 and E6AP were
351 silenced in these cell lines by transfection with appropriate siRNAs, while the proteasome activity was
352 abrogated using the proteasomal inhibitor Bortezomib (BTZ). The effects of silencing on the E6 oncoprotein
353 expression in HeLa and HPV-8 E6 expressing HT1080 cells were assessed using primary antibodies, with the
354 effect on the E6 protein levels in CaSki cells being extrapolated from the p53 levels, as multiple previous
355 studies had confirmed that p53 is degraded in the presence of HR E6 and cellular E6AP [6,9,42].

356 Silencing MAML1 in HeLa cells induced a notable decline in HPV-18 E6 protein levels, with inhibition
357 of proteasomal degradation nullifying this effect, which demonstrates that this process is proteasome-
358 dependent. Interestingly, silencing MAML1 had no effect on p53 protein levels, even though it would have
359 been expected that a decrease in the amount of E6 would also result in a reduction of p53 degradation
360 (Figure 4A) [9,43]. However, this effect was observed when E6 was downregulated as a result of E6AP
361 silencing, which is consistent with previously published results (Figure 4A) [11]. When the same experiment
362 was performed in CaSki cells, the expression profile of p53 was similar to the one observed in HeLa cells.
363 Silencing E6AP resulted in the depletion of E6 and subsequent accumulation of p53 (Figure 4B).
364 Furthermore, silencing MAML1 in HPV-8 E6 expressing HT1080 cells also resulted in downregulation of E6.
365 This effect was nullified when BTZ was added, suggesting that MAML1 also stabilizes HPV-8 E6 in a
366 proteasome-dependent manner (Figure 4C). Conversely, silencing E6AP had no discernable effect on HPV-

367 8 E6 levels (Figure 4C). These results are in accordance with those depicted in Figure 2, where
368 overexpression of E6AP with HPV-8 E6 does not lead to its increased protein stabilization. Even though
369 E6AP has been shown to interact with some E6 oncoproteins of β-HPV types and stabilize them, no such
370 studies, thus far, have been performed for HPV-8 E6 [20].

371 Having established via western blot that MAML1 has an impact on HPV-16, -18 and -8 E6 protein
372 stability, we aimed to further confirm these results by investigating how MAML1 knockdown influences
373 cellular localization of HPV-18 and -8 E6 cellular pools. To assess this, we again performed siRNA silencing
374 of E6AP and MAML1 in HeLa cells and HPV-8 E6-expressing HT1080 cells. Seventy-two hrs post transfection,
375 cells were fixed and immunolabeled, and the localizations of both HPV-8 E6 and p53 (serving as a control
376 for E6 expression in HeLa cells) proteins were monitored by laser confocal microscopy. As expected, E6AP
377 ablation in HeLa cells led to p53 recovery in the nucleus, likely due to the downregulation of HPV-18 E6 in
378 the absence of E6AP (Figure 5A). By contrast, there was no discernible effect on HPV-8 E6 protein levels
379 under the same experimental conditions (Figure 5C). Ablation of MAML1 in HeLa cells did not have any
380 effect on nuclear accumulation of p53 (Figure 5B), while MAML1 silencing in HPV-8 E6-expressing HT1080
381 cells did result in the downregulation of nuclear HPV-8 E6 (Figure 5D). These results are in line with
382 observations from our western blot analyses (Figure 4), suggesting that silencing MAML1 does not affect
383 the HPV-18 E6 pool involved in p53 degradation. Furthermore, the fact that E6AP silencing did not have
384 any effect on HPV-8 E6 protein stability additionally confirmed the aforementioned results (Figure 4C),
385 indicating that there is likely only one pool of HPV-8 E6, which solely interacts with MAML1.

386 *p53 and DLG1 degradation is not E6/MAML1 complex dependent*

387 Following the observed effect on endogenous p53 in HPV-18 positive background, we sought to
388 confirm the results by ectopically expressing the proteins in question in HEK293 E6AP KO cells. We wanted
389 to see whether the opposite effect on p53 could be observed when overexpressing MAML1 or E6AP with

390 HPV-16 E6. As depicted in Figure 6A, MAML1 overexpression with HPV-16 E6 does not induce additional
391 degradation of p53, even though it increases E6 protein levels. By contrast, overexpression of E6AP leads
392 to both higher level of E6 and lower level of p53 (Figure 6A), as previously published [34].

393 Apart from p53, E6 targets a number of other cellular proteins for proteasome-mediated
394 degradation and this process is considered to play an essential role in later stages of HPV-induced
395 tumorigenesis. Some of these protein targets include PDZ domain-containing proteins, for example, the
396 tumor suppressor DLG1 [41]. It was previously shown that catalytically active E6AP is essential for the ability
397 of E6 to degrade p53, while DLG1 and the MAGI family proteins were shown to be degraded by E6 quite
398 effectively without requiring E6AP, indicating that E6 targets these cellular substrates in an E6AP-
399 independent manner [34]. Therefore, we proceeded by investigating whether MAML1 stabilization of HR
400 E6 might have any impact on targeting DLG1. As with p53, we co-transfected HEK-293 E6AP KO cells with
401 plasmids expressing DLG1 in the presence of HPV-16 E6, together with either wild type E6AP or MAML1.
402 Twenty-four hrs later, the protein extracts were separated by electrophoresis and changes in DLG1 protein
403 levels were assessed by immunoblotting. As can be seen in Figure 6B, DLG1 was degraded very effectively
404 by HPV-16 E6 both in the absence and presence of the functional E6AP, as expected [34]. Ectopic co-
405 expression of MAML1 and HPV-16 E6 did not result in any additional degradation of DLG1, when compared
406 to 16 E6 alone. All of this suggests that the E6/MAML1 complex is likely to be involved in other cellular
407 activities which do not involve degradation of some of the well-characterized E6 substrates. Taken together
408 with our previous results, these observations suggest that, while both E6AP and MAML1 stabilize HPV-16
409 and -18 E6, they do not promote stability of the same cellular pools of E6. When MAML1 is silenced, the
410 remaining pool of E6 is still in complex with E6AP and retains its catalytic activities. However, when MAML1
411 is overexpressed together with HR E6, the stability of the E6 pool bound to MAML1 is increased, yet it
412 appears that this cellular pool of E6 is not catalytically active in targeting cellular proteins such as p53 or
413 DLG1.

414 *HPV E6 protein turnover is regulated by MAML1*

415 Since we demonstrated that MAML1 silencing downregulates E6 steady state levels, we further
416 wanted to examine the effects on E6 protein turnover. We again used siRNAs to silence MAML1, E6AP or
417 both, in HeLa and HPV-8 E6-expressing HT1080 cells, also including siRNA against luciferase as a control.
418 Seventy-two hrs post transfection protein synthesis was blocked by cycloheximide in order to determine
419 the rate of E6 protein turnover under these conditions. After blocking protein synthesis, the cells were
420 collected and proteins isolated at indicated time points, separated on SDS-PAGE and detected by western
421 blot analysis. As previously reported, the half-life of HPV-18 E6 in control HeLa cells treated with siRNA
422 luciferase was around 120' [11,44]. Silencing either MAML1 or E6AP led to a shorter E6 half-life; when
423 MAML1 was ablated, the half-life of HPV-18 E6 was around 90', whereas silencing E6AP caused a shortening
424 of half-life to around 80'. Simultaneous silencing of both MAML1 and E6AP induced a further decrease to
425 approximately 60', demonstrating a synergistic effect on E6 half-life (Figure 7A). HPV-8 E6 stably expressed
426 in HT1080 had a half-life longer than the 120' in this experimental setting. When MAML1 was silenced, the
427 half-life decreased to between 90 and 120', while, as expected, silencing E6AP had no impact on HPV-8 E6
428 half-life (Figure 7B). Silencing both MAML1 and E6AP did not lead to any further decrease of HPV-8 E6 half-
429 life. These results further suggest that MAML1 has a direct role in HPV E6 protein turnover of both α- and
430 β-HPVs. Additionally, the exclusive impact of MAML1 on HPV-8 E6 protein turnover regulation was further
431 confirmed, as E6AP was again shown to have no direct impact on HPV-8 E6 stability.

432 *E6 cellular distribution is altered by MAML1*

433 Since both MAML1 and E6AP stabilize E6, we next aimed at determining whether these interactions
434 may also influence subcellular distribution of E6. For this reason, we transfected HEK-293 cells with either
435 HPV-16 or -8 E6, either alone or in combination with MAML1 or E6AP. Cellular proteins were extracted
436 using the "ProteoExtract® Subcellular Proteome Extraction Kit" which allowed for further separation into
437 cytosolic (C), membrane (M), nucleic (N) and microtubule (T) fractions. The fractionated proteins were

438 separated on SDS-PAGE and detected via western blotting. When transfected alone 16 E6 primarily
439 localized in the cytosolic fraction. Co-transfection with E6AP led to a primary stabilization of cytosolic E6,
440 but also to an increase in E6 in other fractions. When co-transfected with MAML1, these proportions
441 changed, and there was a notable increase in the amounts of E6 detected in the nucleic and membrane
442 fractions, which was also accompanied with MAML1 accumulation in these fractions (Figure 8). When
443 examining the effect of MAML1 and E6AP on HPV-8 E6 localization, a different distribution of E6 was
444 observed. E6AP had no discernible effects on HPV-8 E6 localization, while MAML1 caused an increase in
445 the amount of E6 in the cytosol, where MAML1 was predominantly detected (Figure 8). The increase in the
446 cytosolic E6, as well as the stabilization of the nucleic E6, further underpinned the immunofluorescence
447 results (Figure 5D). Based on these results, as well as those from the gene silencing experiments (Figure 4),
448 we further confirmed that MAML1 and E6AP have an impact on two distinct pools of HR HPV E6. This
449 observation highly suggests that the proportion of E6 proteins stabilized by MAML1 likely has distinct
450 cellular functions, and possibly different interacting partners compared with E6 stabilized by E6AP and, as
451 a direct consequence of this, it may therefore be distributed in different cellular compartments.

452 *E6/MAML1 complex impacts cell proliferation and migration*

453 Aforementioned experiments have shown that the ablation of MAML1 has a direct impact on the
454 stability, distribution and turnover of E6. Thus, we wanted to assess whether these changes in E6 have an
455 effect on the behavior of the cell, that is, whether the decrease in E6 levels leads to an alteration of the cell
456 proliferation potential. To test this, we ectopically expressed HPV-18 or -16 E6, together with MAML1, E6AP
457 or both, in HEK-293 E6AP KO cells. Five thousand transfected cells were transferred from dishes to 96 well
458 plates in quadruplicates, and Uptiblue reagent was added in the log phase of growth to measure cell
459 proliferation, 48hrs after the transfer. Absorbance was detected 5hrs later and the percentage of reagent
460 reduction calculated. Compared with HPV-16 E6 transfected alone, transfection with E6AP led to an
461 increase in the proliferation of E6AP KO cells, whereas transfection in combination with MAML1 did not

462 change the rate of proliferation. In cells co-transfected with all of the expression plasmids proliferation was
463 significantly elevated, demonstrating a synergistic effect of MAML1 and E6AP (Figure 9). HPV-18 E6 showed
464 a similar trend compared to that of HPV-16 E6. HPV-18 E6 alone induced an increase in cellular
465 proliferation, which became even more pronounced with the addition of E6AP. Addition of both E6AP and
466 MAML1 resulted in a further increase in proliferation, strongly hinting at a synergistic effect, despite
467 MAML1 not having an individual impact. Very little is known about the interactions between α-HPVs and
468 MAML1, but our hypothesis of two distinct pools of HR E6 could potentially explain the effects described
469 here. E6 promotes cell proliferation in a variety of ways, but its effects are limited in the absence of E6AP.
470 Transfecting cells with both E6 and E6AP allows the viral oncoprotein to exert its typical functions, while
471 transfection with MAML1 does not have the same effect, even though it does increase the overall stability
472 of E6.

473 Despite the fact that we saw no significant effects of the interaction of MAML1 and E6 on cell
474 proliferation in the overexpression experiments, it is well known that Notch signaling is involved in
475 proliferation as well as cellular migration, and it could therefore very well be possible for E6 and MAML1
476 to influence cell behavior in this manner. For this reason, we silenced either MAML1 or E6AP (or both) in
477 HeLa cells with designated siRNAs, and also included siRNA against luciferase as a control. Forty-eight hrs
478 after silencing the confluent cell monolayer was scratched with a pipette tip and photographed (Figure 10,
479 left panel). Eighteen hrs later, the cells were photographed again, and the surface area of the wound was
480 determined with the MRI wound-healing tool macro for the ImageJ software. By creating a gap in the cell
481 monolayer, we were able to observe changes in the migratory/proliferation potential of cells at the edge
482 of the gap depending on the silencing. Silencing E6AP led to a decrease in wound closure from 73% to 57%,
483 most likely due to the consequential diminished amount of E6 driving proliferation and deregulating cell
484 polarity (Figure 10, right panel). Surprisingly, considering that overexpressing MAML1 alone did not impact
485 proliferation, silencing MAML1 decreased HeLa migratory/proliferation capacity to a similar degree as

486 silencing E6AP – i.e. from 73% to 64% (Figure 10, right panel). This could be due to the decline in the total
487 amounts of E6 protein. Alternatively, this could also be an effect resulting from the loss of Notch signaling.
488 It was a quite curious observation that, in contrast to what had been observed in our proliferation
489 experiments, in this experimental setup E6AP and MAML1 did not act synergistically since the rate of
490 migration/proliferation remained the same as with individually silenced E6AP and MAML1, i.e. migratory
491 activity decreased from the 73% in controls to 64% in both treated samples (Figure 10, right panel).

492 ## Discussion

493 α-HPVs have been in the focus of HPV research for many years, mostly due to their impact on
494 human health and the disease development, while β-HPVs have, thus far, been considered auxiliary factors
495 in skin carcinogenesis, with the exception of HPV-38 [15]. Species from both α and β genera use E6 and E7
496 oncoproteins to remodel the cellular environment to suit viral needs, but in distinct and genus-specific
497 ways. A recent manuscript emphasized these differences by demonstrating a clear preference of α-E6
498 oncoproteins to bind to E6AP, while E6 oncoproteins from the remaining four genera complex with MAML1
499 [19]. Indeed, it is well-described that β-HPV E6s' binding to MAML1 has an inhibitory effect on Notch
500 signaling and, subsequently, on keratinocyte differentiation. This effect is extremely important for the
501 maintenance of a productive viral life cycle, considering that Notch signaling in keratinocytes regulates cell
502 cycle progression [22,25]. Interestingly, α-HPV-16 E6 was also found to bind to MAML1, but to a much
503 lesser degree [19]. As the effect of Notch signaling in α-HPV infections and carcinogenesis is poorly
504 described, and furthermore, considering that MAML1 interaction was thought to be exclusive for β-HPVs,
505 the biochemical basis of this potential interplay has not been explored in much detail up to present.

506 Our experiments now confirm the previously reported interaction between MAML1 and HPV-16
507 E6, which is evidently weaker than the interaction between HPV-8 E6 and MAML1. This observation
508 encouraged us to further investigate if other α-E6s could also associate with MAML1. Using GST pull-down

509 assays, we demonstrated that, in addition to HPV-16 and HPV-8 E6s, -18 E6 and -11 E6 also interact with
510 MAML1. We chose to examine these species because HPV-16 and HPV-18 are the most prominent HR HPVs,
511 and HPV-11 is a common representative of LR mucosal type [1]. As expected, HPV-8 E6 was found to be
512 the strongest interactor of MAML1, followed by HPV-18 E6 and -16 E6, and finally HPV-11 E6 which had
513 the weakest overall indicating that both HR and LR mucosal HPVs bind to MAML1. Because of this very
514 weak interaction between HPV-11 E6 and MAML1, we chose not to pursue this avenue further.

515 The main interaction partners of E6 serve to adapt the cellular milieu to viral replication,
516 irrespective of the genera the E6 oncoproteins belong to. HR E6s hijack E6AP and form a stable complex
517 with it, resulting in E6 protein stabilization. The stable complex is then directed by E6 towards a number of
518 cellular targets important for the maintenance of a virus-optimized environment, ensuring a regulated
519 turnover at the proteasome [41]. β-PVs interact with MAML1 in order to block cell differentiation and cell-
520 cycle arrest of target cells as completion of the viral life cycle is highly dependent on the host cell
521 differentiation status [22,25]. Interestingly, both α- and β-HPV E6 proteins utilize the same LXXLL motif to
522 bind to E6AP and MAML1, respectively, and, as the impact of E6AP on E6 protein turnover is well defined,
523 we sought to determine if MAML1 would induce a similar effect. By co-expressing MAML1, or E6AP as a
524 control, together with the E6 oncoproteins of different α- and β-HPVs, we demonstrated that MAML1 had
525 an upregulatory effect on E6 protein expression levels, irrespective of the HPV genus. E6AP demonstrated
526 positive effects on the stability on all α-PVs examined, but had no effect on the protein stability of the
527 analyzed β-PV E6, with the exception of HPV-24 E6 and HPV-38 E6. Previous reports have noted that these
528 types interact with both MAML1 and E6AP, making it likely that, like α-HPVs, they might also possess two
529 pools of E6 proteins. [20]. The initial analyses were repeated in an E6AP-null background and these
530 experiments yielded similar results. This allowed for the conclusion that the effects of MAML1 in inducing
531 E6 oncoprotein stability were indeed mostly E6AP-independent. The stabilization effect of MAML1 on β-E6
532 oncoproteins makes perfect sense, considering MAML1 utilization for Notch inhibition by β-HPV E6s, as

533 well as the absence of stabilization by E6AP, but the reasons behind α-HPV E6 stabilization appear to be
534 much more complex. One possible reason could be that α-HPVs, like β-HPVs, use MAML1 to block the
535 formation of the Notch ternary complex and thus consequently block the activation of Notch and
536 keratinocyte differentiation. HR E6 oncoproteins could have evolved to use the same conserved interacting
537 motif to maintain both their primary strong interaction with E6AP and the weaker secondary interaction
538 with MAML1 in order to maximize their overall protein stability and the potential for viral propagation. It is
539 also likely that each of these two interacting complexes might be required for establishing an optimal
540 cellular environment for the virus to successfully complete its life cycle.

541 The LXXLL motif is exceedingly important for the binding of E6 to many cellular targets, including
542 E6AP and MAML1, and disrupting the binding of E6 to this peptide leads to the inhibition of E6 downstream
543 effects [45]. With some E6 oncoproteins, auxiliary interactions in the carboxyl-terminal HECT domain or a
544 region in the amino terminus are required for E6-E6AP binding, and we do not know whether similar
545 accessory regions are required for E6-MAML1 interactions [46]. That being said, not all E6 mutations
546 completely inhibit the binding of E6 to its targets, and there is a certain degree of flexibility in respect to
547 amino acid residues included, as far as E6 interaction capabilities with its targets are concerned. For this
548 reason, we wanted to further examine whether E6 stabilization by MAML1 might be exclusively dependent
549 on the LXXLL motif or if it might also require binding to additional regions of MAML1, and to determine
550 how susceptible these interactions might be to mutations in the LXXLL motif [45,46] . Previous research has
551 shown that mutating the aspartate residues of the MAML1 LXXLL motif to histidine decreases the binding
552 of HPV-8 E6 to MAML1 and, consequently, the rate of Notch inhibition [32]. We assessed whether mutating
553 the same residues (LDDLL to LHHLL) would lead to a decreased stability of HPV-8, - 16 and – 18 E6 proteins,
554 or have an impact on binding to MAML1. Interestingly, HPV-8 and -18 E6s were still stabilized by the
555 mutated MAML1, albeit to a lesser degree, while HPV-16 E6 was not. The MAML1 LHHLL was found to
556 retain a weaker affinity for HPV-8 E6 and HPV-18 E6, indicating that binding to the motif is indeed required

557 for the interaction with E6, but it opens a possibility that broader peptide domains or specific neighboring
558 amino acids may be involved in achieving the complete interaction. These results are in accordance with
559 those of the GST pull-down assay, where in the comparison with HPV-18 E6, HPV-16 E6 demonstrated a
560 weaker interaction with MAML1. It is therefore possible that their binding is somewhat different, as it is
561 the case with E6AP [42], and that mutating the two aforementioned amino acid residues might be sufficient
562 to completely disable the binding capacity of HPV-16 E6 to MAML1. Most surprisingly, HPV-16 E6 and HPV-
563 18 E6 have shown opposing results, even though their biochemical behavior has thus far been considered
564 identical. This again demonstrates that the interactions of different E6 oncoproteins, even of those of the
565 same genus, cannot not be generalized and could have potentially different impacts on HPV-mediated
566 pathogenesis.

567 Ectopic expression assays demonstrated that E6 binds to MAML1, and that this binding results in
568 E6 protein stabilization. However, since overexpression experiments do not always translate entirely into
569 naturally occurring systems, we aimed at determining whether the same effect can be observed in the cell
570 lines that express E6 endogenously. Silencing MAML1 in an HPV-8 E6 expressing HT1080 fibrosarcoma cell
571 line [17] decreased the amount of detectable 8 E6, while silencing E6AP had no effect. These results were
572 expected, as it was previously described that E6AP does not interact with or impact a vast majority of β-
573 HPV E6s [20]. Silencing endogenous MAML1 expression in HPV-18 positive HeLa cells resulted in the
574 downregulation of E6 protein levels. Interestingly, this did not have any effect on some of the main E6
575 functions, such as regulation of p53 protein turnover. E6 was stabilized at the protein level, as blocking the
576 proteasomal turnover nullified the effect. We also confirmed that this effect was not HPV-18 E6 specific,
577 since it was also seen in HPV-16 E6-positive CaSki cells. These results led us to believe that there are two
578 distinct cellular pools of HR E6. One of these E6 pools binds to MAML1, resulting in E6 stabilization, but this
579 complex appears not to be involved in some of the known E6/E6AP dependent functions. The remaining
580 cellular E6 is E6AP-bound and plays a role in some of the previously characterized E6 oncogenic functions,

581 as demonstrated by our immunofluorescence experiments and the observed restoration of p53 levels
582 following E6AP silencing. Likewise, we observed a downregulation of endogenous p53 protein level in the
583 presence of ectopically expressed HPV-16 E6 alone or with E6AP, while overexpressing MAML1 with HPV-
584 16 E6 led to restoration of initial p53 levels. This observation strongly indicates that overabundantly present
585 MAML1 may occupy all available E6 molecules within the cell and prevent E6 from interacting with E6AP,
586 and as such marking of p53 for proteasomal degradation. Furthermore, we wanted to expand our analysis
587 by examining another well-defined E6 target, such as PDZ domain-containing protein DLG1. Unlike p53,
588 which was previously found to be degraded exclusively in the presence of E6AP, an additional, currently
589 unknown, E6AP-independent mechanism is involved in E6-mediated DLG1 degradation [34]. As expected,
590 DLG1 was degraded in the presence of 16 E6, alone or in combination with E6AP, while no additional change
591 in DLG1 level was detected when 16 E6 was overexpressed with MAML1, indicating that the E6 pool
592 stabilized by MAML1 is not involved in regulation of DLG1 turnover.

593 Having established that silencing MAML1 affects E6 protein levels irrespective of the HPV genus,
594 and that this regulation is proteasome-dependent, we wanted to further verify these results by determining
595 the half-life of E6 in the presence or absence of MAML1. Ablating MAML1 caused a notable decrease in the
596 half-life of both HPV-8 and -18 E6s, while silencing E6AP only decreased HPV-18 E6 half-life, confirming that
597 there is only one cellular pool of HPV-8 E6, exclusively regulated by binding to MAML1. With HPV-18 E6,
598 the synergistic impact of MAML1 and E6AP was also visible when both were silenced, as this silencing led
599 to a further decrease in protein half-life, when compared with cells with individually silenced genes. These
600 findings corroborate our two-pools hypothesis; when either MAML1 or E6AP are depleted HPV-18 E6 half-
601 life is noticeably shorter than when both are present (Figure 11).

602 The binding of proteins to their interacting partners can lead to the blocking of their active or
603 binding sites, or can induce shuttling of the entire complex to a new subcellular location, leading to
604 modulations in protein functions. Consequently, we went on to determine whether E6 localization and the

605 area of accumulation might be changed by interacting with MAML1 in comparison with E6AP. HPV-16 and
606 -18 E6 have previously been shown to be localized mostly in the nucleus and the ribosomal areas of the
607 cytoplasm [36,47]. This localization may just be a snapshot of a more dynamic state of E6 protein
608 localization within a cell, as there are reports indicating that the formation of a tertiary complex of HPV-18
609 E6, p53 and E6AP causes protein shuttling from the nucleus to the cytoplasm and that the majority of p53
610 is degraded in the cytoplasm [48]. MAML1 and E6AP are both usually found in the nucleus, but can be
611 found in the cytoplasm as well. Interestingly, stabilizing HPV-16 E6 by MAML1 leads to a drastic increase of
612 16 E6 localized in the nucleus and membranes, while stabilizing it by E6AP increases the E6 cytosolic
613 fraction, indicating that the two different pools localize in different cellular compartments [20,42]. It is clear
614 that MAML1 and E6AP do not stabilize HPV-16 E6 in the same manner. The increase of HPV-16 E6 in the
615 nuclear fraction may impact E6 functions, as previously shown for HPV-18 E6, by exposing it to different
616 binding partners [48]. On the other hand, considering the subcellular location of MAML1 and the lack of
617 evidence for active protein shuttling, the likely explanation could be that MAML1 might simply increase the
618 amount of protein present in the nuclear fraction because it stabilizes this particular pool and thus increases
619 its total amount and activity in the nucleus. On the other hand, E6AP is used as a molecular tool by E6 for
620 performing distinct cellular functions, mostly protein degradation in the cytoplasm, so the E6/E6AP
621 complex is primarily found in that part of the cell.

622 Like HPV-16 E6, HPV-8 E6 has been previously found to be localized predominately in the nucleus
623 and to a lesser extent, in the cytoplasm [47]. When examining HPV-8 E6 overexpressed in HEK-293 cells,
624 we noticed again that the distribution of HPV-8 E6 changes depending on the presence of MAML1. When
625 expressed alone, HPV-8 E6 is mostly found in the nucleus, as when expressed with E6AP which, in this case,
626 served as a negative control for HPV-8 E6 distribution and confirmed that E6AP does not influence 8 E6
627 cellular shuttling. When co-expressed with MAML1, we found HPV-8 E6 to be distributed in the cytosolic
628 and nuclear fractions. This nuclear localization of HPV-8 E6 is most evident in immunofluorescence, where

629 cytoplasmic localization is less noticeable and silencing MAML1 leads to a much dimmer HPV-8 E6 signal,
630 pointing towards the importance of MAML1 in HPV-8 E6 turnover. Considering that HPV-8 E6 interacts
631 mostly with proteins located in the nucleus and cytoplasm, it would be of interest to see whether this
632 binding has an effect on the binding of HPV-8 E6 to its other partners, and whether the presence or absence
633 of MAML1 impacts the profile of the interacting partners of HPV-8 E6.

634 MAML1 binding clearly has a profound effect on E6 oncoprotein stability and localization of both
635 α- and β- HPVs, but whether and how this interaction translates into biological functions is of great interest.
636 Considering that the interaction of HPV-8 E6 with MAML1 has well-established biological consequences on
637 Notch signaling, cell proliferation and keratinocyte differentiation, the focuses of this research were to
638 elucidate biochemical and cellular effects of HR α-HPVs complexing with MAML1 [25]. We overexpressed
639 HPV-16 E6 and -18 E6 alone or in combination with MAML1, E6AP or both, in E6AP KO cells, in order to
640 ensure that E6 stabilization would not be promoted by endogenous E6AP. We found that, when
641 overexpressed in a cellular system, E6AP-mediated E6 stabilization leads to a statistically significant
642 increase of cell proliferation, as we and others have previously described [39]. MAML1-mediated
643 stabilization of E6 had no discernible effects on cell proliferation, when compared with the effects of HR
644 HPV E6 proteins alone. Remarkably, when HPV-16 E6 or -18 E6 were co-expressed with both MAML1 and
645 E6AP, the effect on cell proliferation was much stronger than that of E6AP alone. This effect could be due
646 to MAML1-mediated increase in E6 stability which might, in turn, increase the amount of E6 molecules
647 present within a given cell and, as a long-term consequence, increase the amount of E6 available for
648 interaction with E6AP. When this concept is transferred to a naturally occurring cell line, such as Hela,
649 silencing E6AP by RNA interference leads to decreased wound healing, probably due to growth suppression
650 by p53, whose protein level within the cell increases as a consequence of decreased E6 presence [49]. In
651 the same system, inhibition of MAML1 leads to a decrease in wound healing, comparable to that of
652 abrogated E6AP. Interestingly, the same effect has previously been reported and associated with inhibition

653 of Notch signaling in HeLa cells, but the authors offered no further explanation as to how this came to be
654 [50]. It is possible that maintaining E6 stability with MAML1 is indispensable for sustaining HeLa cell viability,
655 but it is likely that completely abrogating Notch signaling by silencing MAML1 leads to a growth arrest, as
656 Notch signaling is very finely tuned and both under- and over-modulation can be detrimental for cell
657 proliferation [50,51]. Interestingly, in the same experimental setting, MAML1 and E6AP do not exhibit
658 synergistic effects, as silencing both does not lead to an additional decrease in the percentage of the wound
659 healed. MAML1 could therefore contribute to an increase in absolute amounts of E6 within the cell. In this
660 context, MAML1 silencing would influence proliferation only through decreasing the availability of E6 for
661 binding to E6AP, through which it exerts most of its functions. HeLa proliferation is only partially dependent
662 on the effects of E6, which also means that reducing the amount of E6 by any means will only influence the
663 cellular phenotype to a limited degree. Considering that E7 is considered to be the main driver of
664 proliferation combined with the fact that it is still abundantly present in HeLa cells could be the explanation
665 for the observed effects [52].

666 Our research focused on the previously uninvestigated effects that E6s interacting partners have
667 on its stability and function. Here, we show that a well-defined interaction of β-HPVs with MAML1 serves
668 to increase E6 protein stability, in addition to inhibiting Notch signaling and abrogating keratinocyte
669 differentiation. Moreover, we show for the first time that a formerly overlooked, LXXLL motif-dependent
670 interaction of HR α-HPVs with MAML1 has a stabilizing effect on E6s. The increased stability of E6 does not
671 translate to an increase in E6-mediated degradation efficiencies for some of its most common targets.
672 However, importantly, it seemingly has an impact on cell migration and proliferation, presumably via a still
673 undiscovered mechanism. Taken together, and as summarized in Figure 11, our results imply the existence
674 of two distinct pools of HR E6 oncoproteins; E6/E6AP being well-characterized, and E6/MAML1, whose
675 functions are currently being investigated further.

Author Contributions

Conceived and designed the experiments: JS, AĐ, BA, VT; Performed the experiments: JS, AĐ, MH, VF; Analyzed the data: JS, AĐ, VF, BA, VT; Contributed reagents/materials/analysis tools: MH, BA, MT, LB; Wrote the paper: JS, AĐ, VT. All authors read and approved the final version of the manuscript.

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Conflicts of interest

Authors declare no conflict of interest.

Data availability statement

All relevant data are within the manuscript and its Supporting Information files.

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843

844 Figure legends

845 **Fig. 1. α- and β- HPV E6 oncoproteins bind to MAML1**. A) HEK-293 cells were transfected with HA-
846 tagged HPV-16 E6 or HPV-8 E6 alone or in combination with FLAG-tagged MAML1. Transfected cells were
847 harvested, resuspended in lysis buffer and incubated with anti-HA beads on a rotating wheel at RT. The
848 beads were extensively washed and co-immunoprecipitated complexes were subjected to western blot
849 analysis with anti-FLAG antibody and compared with the amount of MAML1 present in 10% of the input.
850 Dotted line divides two different exposures of the same membrane. B) Extracts from C33A cells, transiently
851 transfected with either empty vector or plasmids coding for FLAG-16E6 were incubated with anti-FLAG
852 beads. Co-immunoprecipitated MAML1 and HPV-16 E6 and 10% of the input extracts were subjected to
853 western blots with specific antibodies. Equal protein loading was confirmed by immunoblotting for GAPDH.
854 * immunoglobulin light chain. C) HEK-293 cells were transfected with FLAG-tagged MAML1. After 24hrs,
855 the cells were harvested, and the cell lysates were incubated with 16 E6, 18 E6, 8 E6 and 11 E6 GST fusion
856 proteins. GST alone was included as a control. After extensive washing, bound MAML1 was detected by
857 western blotting using anti-FLAG antibody and compared to the amount of MAML1 present in 10% of the
858 input. The lower gel shows the positions of purified GST proteins used in the pulldowns visualized by
859 Ponceau staining.

860 **Fig. 2. MAML1 stabilizes E6 oncoproteins from both α- and β- HPV types.** A) HEK-293 cells and B)
861 HEK-293 E6AP KO cells were transfected with HA-tagged HPV-8, -12, -14, -16, -18, -24, -33 and -38 E6 alone
862 or in combination with either FLAG- or Myc-tagged MAML1 or E6AP. After 24hrs cells were harvested and
863 isolated proteins from complete cell lysates separated on SDS-page, detected by anti-FLAG, anti-Myc and
864 anti-HA antibodies and visualized by chemiluminescence. B-galactosidase (LacZ) was used as the control to
865 monitor transfection efficiency and loading. Densitometric analysis was performed with ImageJ software.
866 The band densities were first normalized for background for each of the samples after which relative
867 expression was calculated by dividing E6 normalized density with LacZ normalized density. Average relative

868 expressions of control E6s were calculated and all other relative E6 expressions were compared to it in
869 order to obtain E6 fold change. LOG2 of that fold change is depicted on the bar chart so that the increase
870 in intensity is seen as a positive value and the decrease as a negative value. Each experiment was repeated
871 at least three times.

872 **Fig. 3. E6 protein stabilization is MAML1 LXXLL motif dependent**. A) HEK-293 and HEK-293 E6AP KO
873 cells were transfected with HA-tagged HPV-8, -11, -16 and -18 E6 alone or in combination with FLAG-tagged
874 MAML1 or FLAG-tagged MAML1 mutant (MAML1 LHHLL). After 24hrs cells were harvested and isolated
875 proteins from complete cell lysates were separated on SDS-page and analyzed by western Blotting. E6,
876 MAML1 and MAML1 LHHLL were detected by anti-HA and anti-FLAG antibodies. B-galactosidase (LacZ) was
877 used as the control for the transfection efficiency and loading. B) HEK-293 cells were transfected with HA-
878 tagged HPV-8 E6 alone or in combination with FLAG-tagged MAML1 or FLAG-tagged MAML1 mutant.
879 Transfected cells were harvested, resuspended in lysis buffer and incubated with anti-HA beads on a
880 rotating wheel at RT. The beads were extensively washed and co-immunoprecipitated complexes subjected
881 to western blot analysis with anti-FLAG antibody and compared to the amount of MAML1 or MAML1 LHHLL
882 present in 10% of the input.

883 **Fig. 4. HPV E6 protein stability in CaSki, HeLa and HPV-8 E6 expressing HT1080 cells is MAML1**
884 **dependent**. A) HeLa cells, B) CaSki and C) HT1080 HPV-8 E6 cells were transfected with individual siRNA
885 against Luciferase (siLuc), or a pool of siRNA against MAML1 (siMAML1) or siRNA against E6AP (siE6AP).
886 Forty-eight hrs later, proteasome inhibitor Bortezomib (BTZ) was added as indicated and the cells were
887 then incubated for a further 24hrs after which they were harvested, separated on SDS-page, and detected
888 by western blotting with anti-MAML1, anti-E6AP, anti-HPV-18 E6 (B) and anti-FLAG antibodies (C) followed
889 by HRP-coupled anti-mouse and anti-rabbit antibodies and ECL detection. β-actin was used as internal
890 control to monitor protein loading.

891 **Fig. 5. MAML1 stabilizes a distinctive cellular pool of HPV-18 E6 and an exclusive single cellular pool**
892 **of HPV-8 E6.** (A and B) HeLa cells and (C and D) HT1080 8 E6 cells were transfected with indicated siRNAs.
893 Seventy-two hrs post transfection the cells were fixed and stained with antibodies against MAML1 and
894 E6AP to determine the silencing efficiency; p53 as readout for 18 E6 ablation; and FLAG (HPV-8 E6). Anti-
895 mouse and rabbit secondary antibodies conjugated to Alexa Fluor 488 or rhodamine red were used to
896 detect the primary antibodies, and nuclei were visualized with DAPI. Scale bars 10 µm; the white arrows
897 point towards cells of interest.

898 **Fig. 6. E6/MAML1 complex does not induce p53 and DLG1 degradation**. HEK-293 E6AP KO cells
899 were transfected with A) p53 or B) HA-tagged DLG1, in combination with HA-tagged 16 E6 alone or in
900 combination with either FLAG-tagged MAML1 or E6AP. After 24hrs the cells were harvested and isolated
901 proteins from complete cell lysates were separated on SDS-page, detected by HRP-linked anti-HA or anti-
902 p53 antibodies with an appropriate secondary antibody, after which the proteins were visualized by
903 chemiluminescence. β-galactosidase (LacZ) was used as control to monitor transfection efficiency and
904 loading. Densitometric analysis was performed with ImageJ software. Band densities were first normalized
905 for background for each of the samples, after which relative expression was calculated by dividing p53 or
906 DLG1 normalized density with LacZ normalized density. p53 or DLG1 average normalized relative expression
907 was calculated for control samples, and all normalized relative expressions were compared to it. Fold
908 change was obtained by dividing the relative expression of p53 or DLG1 in co-transfected samples with
909 average relative expression of samples transfected alone. Each experiment was repeated at least four
910 times.

911 **Fig. 7**. **HPV E6 protein turnover is regulated by MAML1.** (A) HeLa cells and (B) HT1080 8 E6 cells
912 were transfected with siRNA against Luciferase (siLuc), MAML1 (siMAML1) and E6AP (siE6AP) alone or in
913 combination. Seventy-two hrs later the cells were treated with Cycloheximide prior to harvesting at the
914 time points as indicated. E6 protein expression levels were analyzed by western blotting with anti-MAML1,

915 anti-E6AP, anti-18 E6 (A) and anti-FLAG antibodies (B) followed by HRP-coupled anti-mouse and anti-rabbit
916 antibodies and ECL detection. β-actin was used as internal control to monitor protein loading.
917 Densitometric analysis was performed with ImageJ software. Band intensities were first normalized for
918 background for each of the samples after which relative expression was calculated by dividing E6
919 normalized density with β-actin normalized density. The initial band intensity of E6 was taken as 100%, and
920 the density at other time points were calculated as percentage of the starting amount. The experiment was
921 repeated at least three times. The results obtained are depicted in the graph (lower panel).

922 **Fig. 8. MAML1 causes changes in 16 E6 and 8 E6 cellular accumulation**. HEK-293 cells were
923 transfected with HA-tagged HPV-16 E6 or HPV-8 E6 alone or in combination with either FLAG-tagged or
924 myc-tagged MAML1 or E6AP. After 24hrs, the protein lysates were separated into the cytosolic (C),
925 membrane (M), nucleic (N) and microtubule (T) cellular fractions by ProteoExtract® Subcellular Proteome
926 Extraction Kit. The fractionated proteins were separated on SDS-PAGE and E6 was detected by western
927 blotting with HRP-linked anti-HA antibody, while MAML1 was detected with anti-FLAG or anti-myc
928 antibodies. The protein fraction purity was determined by incubation with specific antibodies (MCM7 for
929 cytoplasm, GAPDH for membrane, histone H3 for nucleus and vimentin for cytoskeleton).

930 **Fig. 9. MAML1 and E6AP cooperate in increasing E6-dependent cellular proliferation.** HEK-293
931 E6AP KO cells were transfected with HA-tagged HPV-16 E6 or HPV-18 E6, alone or in combination with
932 FLAG-tagged MAML1, E6AP or both. The cells were also transfected with pCDNA3, used as a negative
933 control, and β-galactosidase (LacZ) as an internal standard to monitor transfection efficiency and loading.
934 Twenty-four hrs after transfection, the cells were detached, counted and seeded in quadruplicates in a 96
935 well plate (5 x 10^4 cells per well), after which they were grown for a further 48hrs. Uptiblue was then added
936 to the wells and the absorbance at 570 nm and 595 nm was measured 5hrs later. The percentage of
937 Uptiblue reduction was calculated following manufacturer's instructions and it responds to the rate of
938 proliferation. The average of at least three experiments is depicted on the graph, along with standard

939 deviations. Significance was determined by student's t-test, with $p<0.05$ taken as significant, and marked
940 with * above the bar ($p<0.01$ marked with **).

941 **Fig. 10. Silencing MAML1 impacts cell migration in HPV-18 positive cells.** HeLa cells were
942 transfected with individual siRNAs either against Luciferase (siLuc), against MAML1 (siMAML1) or against
943 E6AP (siE6AP). Forty-eight hrs later, a wound scratch was made in the cell monolayer, the cells were washed
944 and photographed (left panel). Eighteen hrs after scratching, the cells were photographed again (left
945 panel). The surface area of the wounds was calculated with the MRI wound healing tool macro for ImageJ
946 software, and the significance in the surface area difference between treatments was determined by
947 student's t-test, with $p<0.05$ taken as significant and marked with * ($p<0.01$ marked with **) (right panel).
948 All experiments were repeated at least four times.

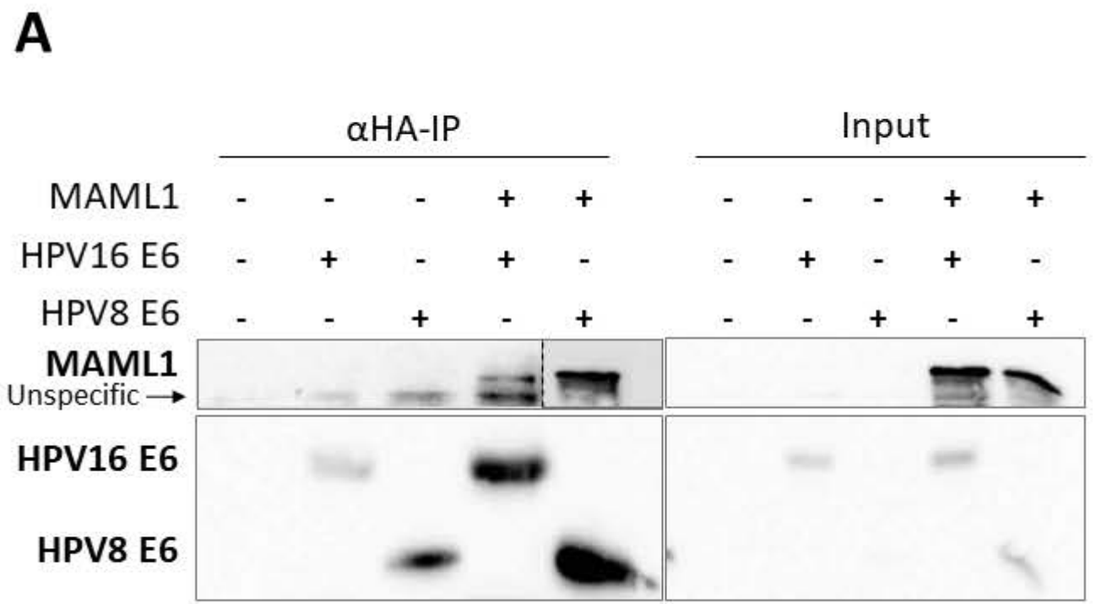
949 **Fig. 11. Working model for α-HPV E6 and β-HPV E6 interplay with MAML1**. While majority of β-E6
950 oncoproteins bind exclusively to MAML1 (right), α-E6 oncoproteins bind either MAML1 or E6AP (left).
951 MAML1 binding increases both mucosal and cutaneous E6 protein stability, impacts cell migration in HPV-
952 18 positive background, but it does not influence HR mucosal E6 target degradation.

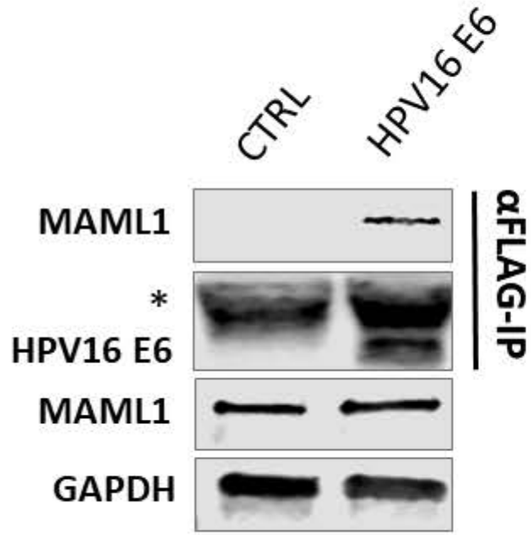
953 **Supp. Fig. 1. Negative controls for Figure 2.** HEK-293 cells (left panel) and HEK-293 E6AP KO cells
954 (right panel) were transfected with either FLAG- or Myc-tagged MAML1 or E6AP, alone or in combination
955 with HA-tagged HPV-16 E6 or HPV-8 E6. After 24hrs cells were harvested and isolated proteins from
956 complete cell lysates separated on SDS-page, detected by anti-HA, anti-FLAG and anti-Myc antibodies and
957 visualized by chemiluminescence. B-galactosidase (LacZ) was used as the control to monitor transfection
958 efficiency and loading.

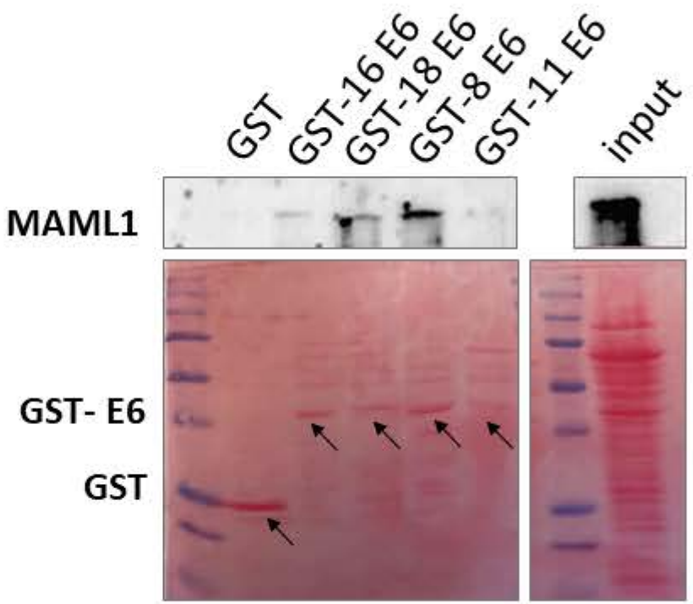
959 **Supp. Fig. 2. Negative controls for Figure 3.** (A) HEK-293 (left panel) and HEK-293 E6AP KO cells
960 (right panel) were transfected with Myc-tagged MAML1 or Myc-tagged MAML1 mutant (MAML1 LHHLL),
961 alone or in combination with HA-tagged HPV18 E6 or HPV-8 E6. After 24hrs cells were harvested and

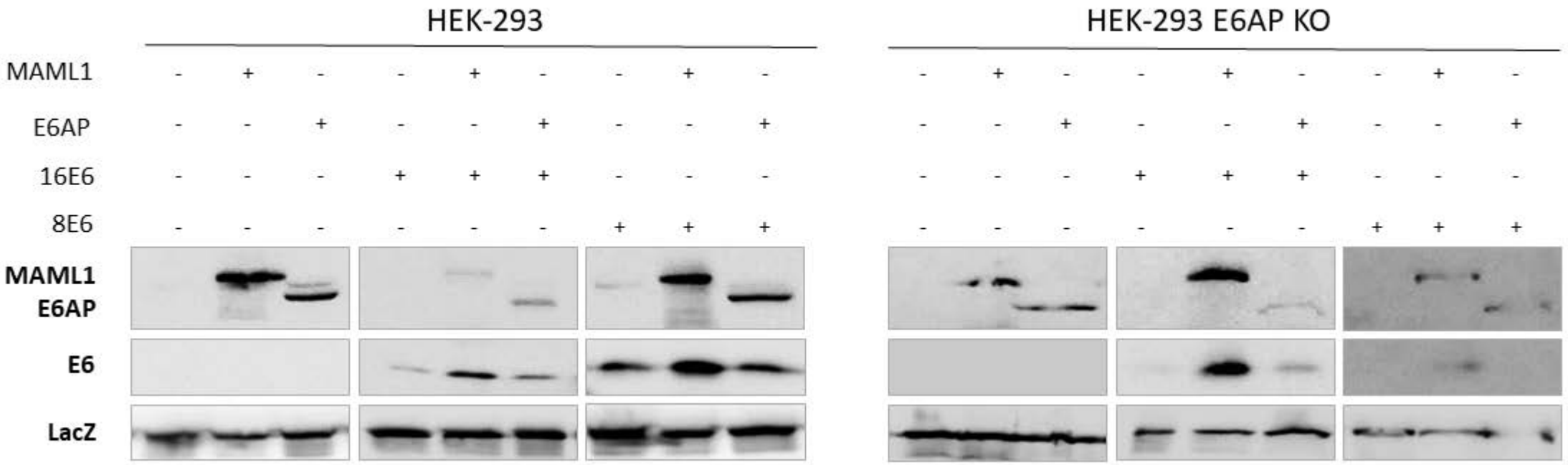
962 isolated proteins from complete cell lysates were separated on SDS-page and analyzed by western Blotting.
963 MAML1 and MAML1 LHHLL were detected by anti-Myc antibodies, while HPV-8 E6 was detected with an
964 anti-HA antibody. B-galactosidase (LacZ) was used as the control for the transfection efficiency and loading.
965 (B) HEK-293 cells were transfected with either an empty plasmid, Myc-tagged MAML1, FLAG-tagged
966 MAML1 LHHLL mutant and HA-tagged HPV-8 E6 alone or in combination. Twenty-four hrs post transfection
967 the cells were harvested, resuspended in lysis buffer and incubated with anti-HA beads on a rotating wheel
968 overnight at 4°C. The beads were extensively washed and co-immunoprecipitated complexes subjected to
969 western blot analysis with anti-myc, anti-FLAG anti-HA-HRP antibodies and compared to the amount of
970 MAML1, MAML1 LHHLL or 8 E6 present in 10% of the input.

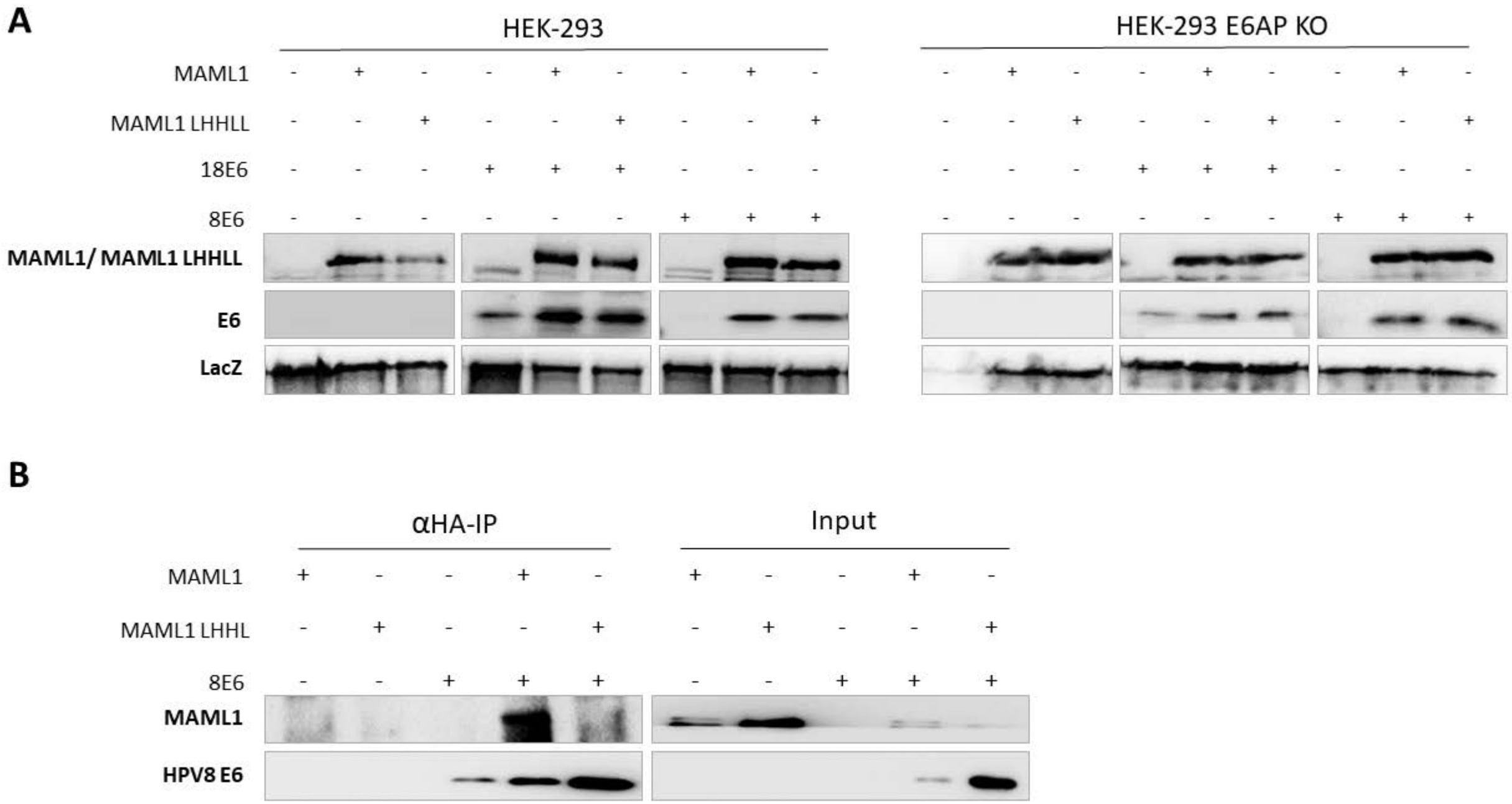
971

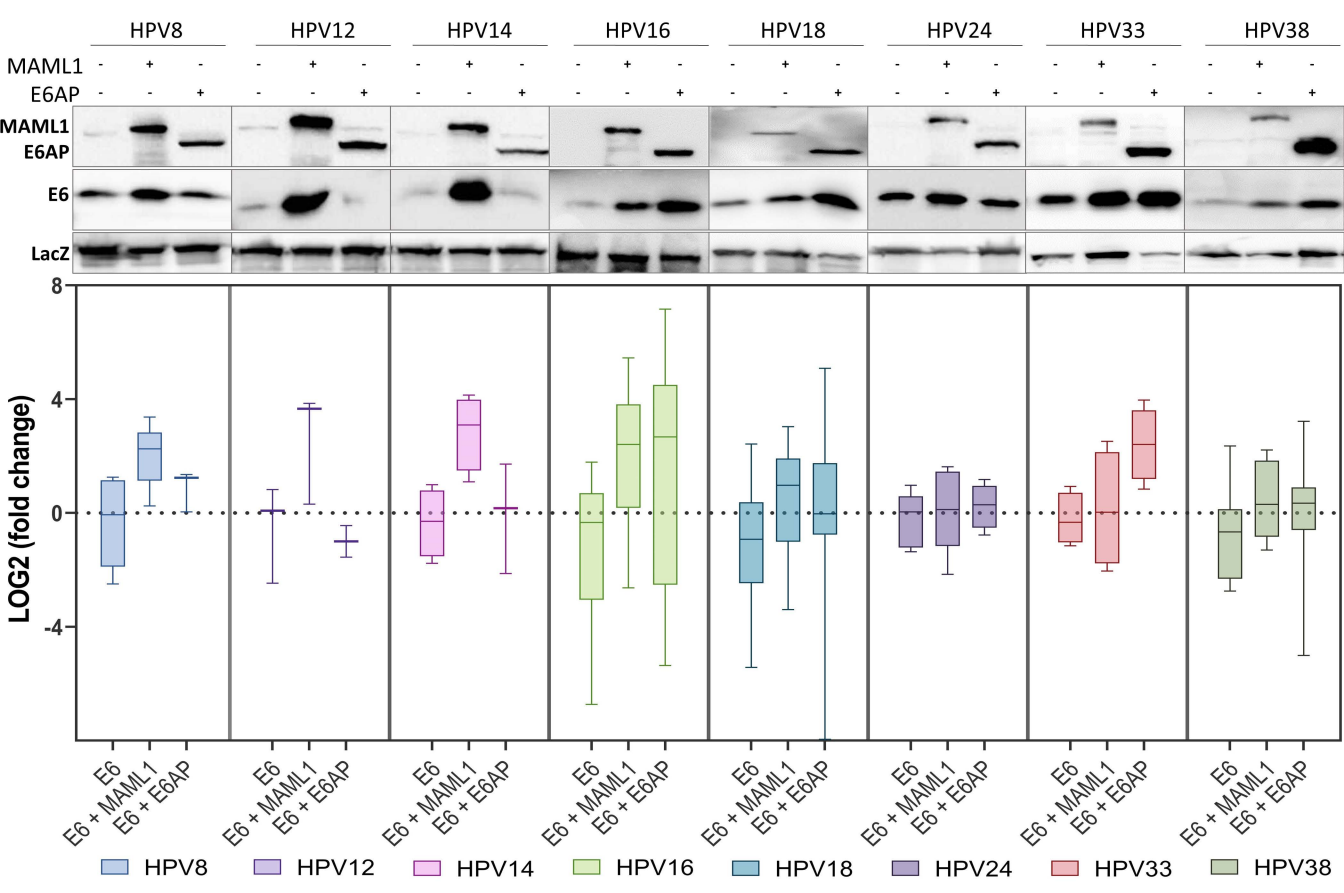

A
αHA-IP
Input
MAML1 - - - + + - - - + +
HPV16 E6 - + - + - - + - + -
HPV8 E6 - - + - + - - + - +
MAML1
Unspecific
HPV16 E6
HPV8 E6

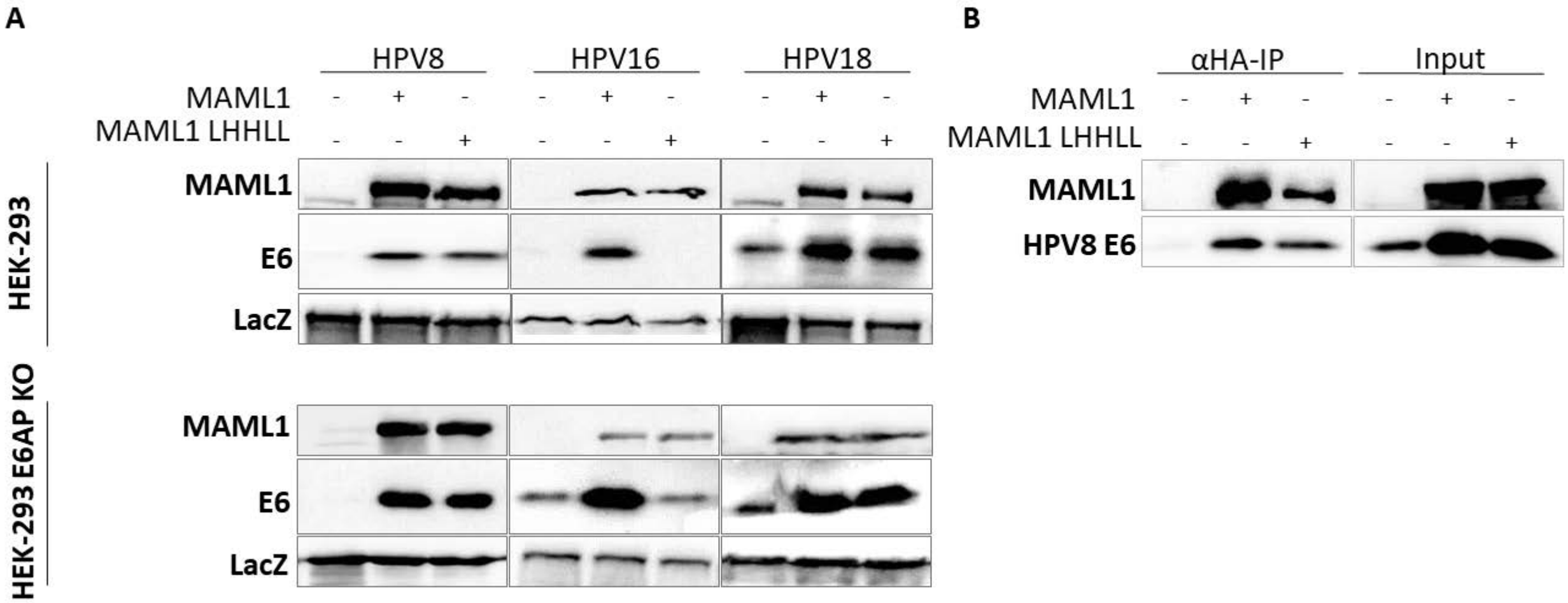

B
CTRL
HPV16 E6
MAML1
*
HPV16 E6
MAML1
GAPDH
αFLAG-IP

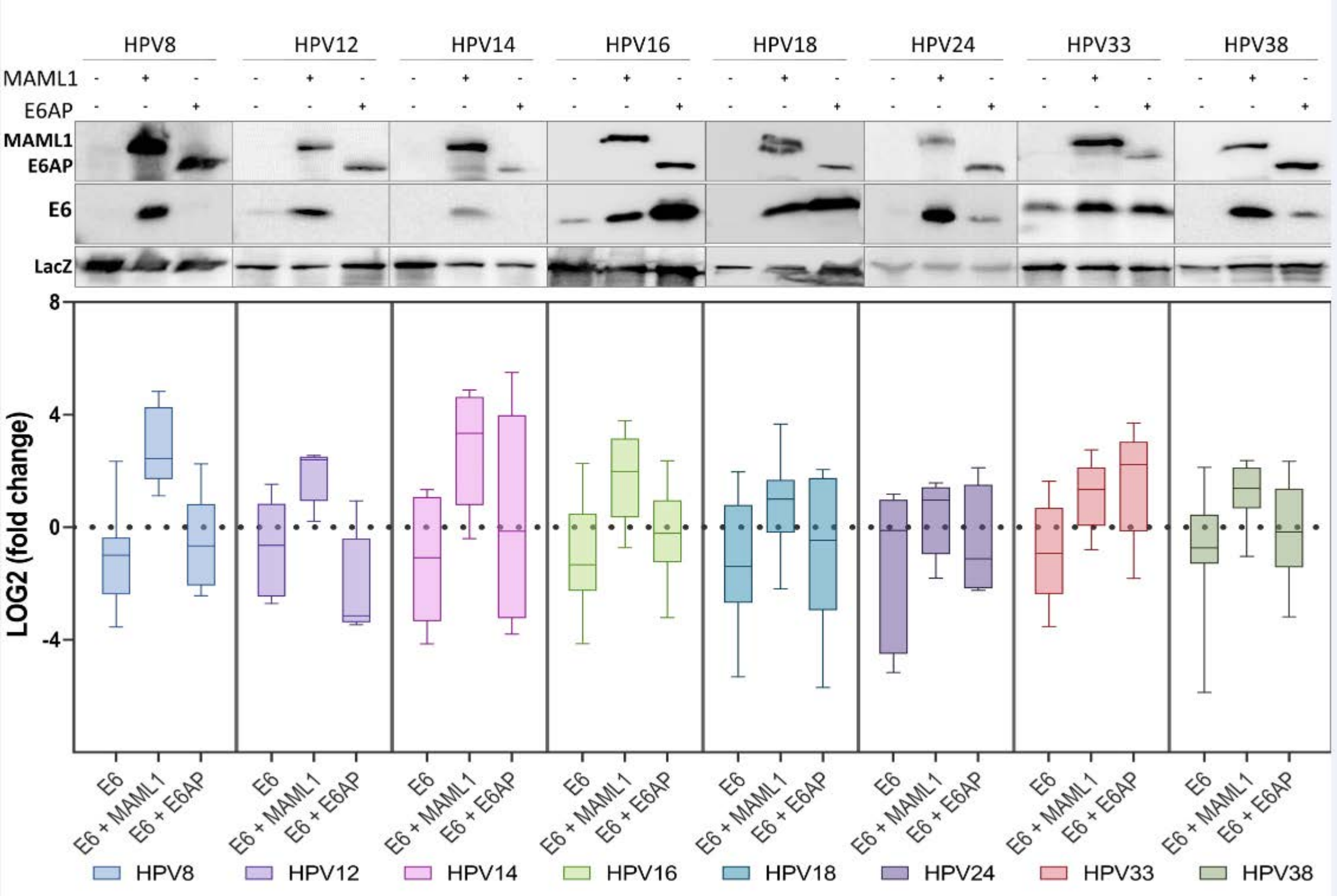

C
GST
GST-16 E6
GST-18 E6
GST-8 E6
GST-11 E6
input
MAML1
GST- E6
GST

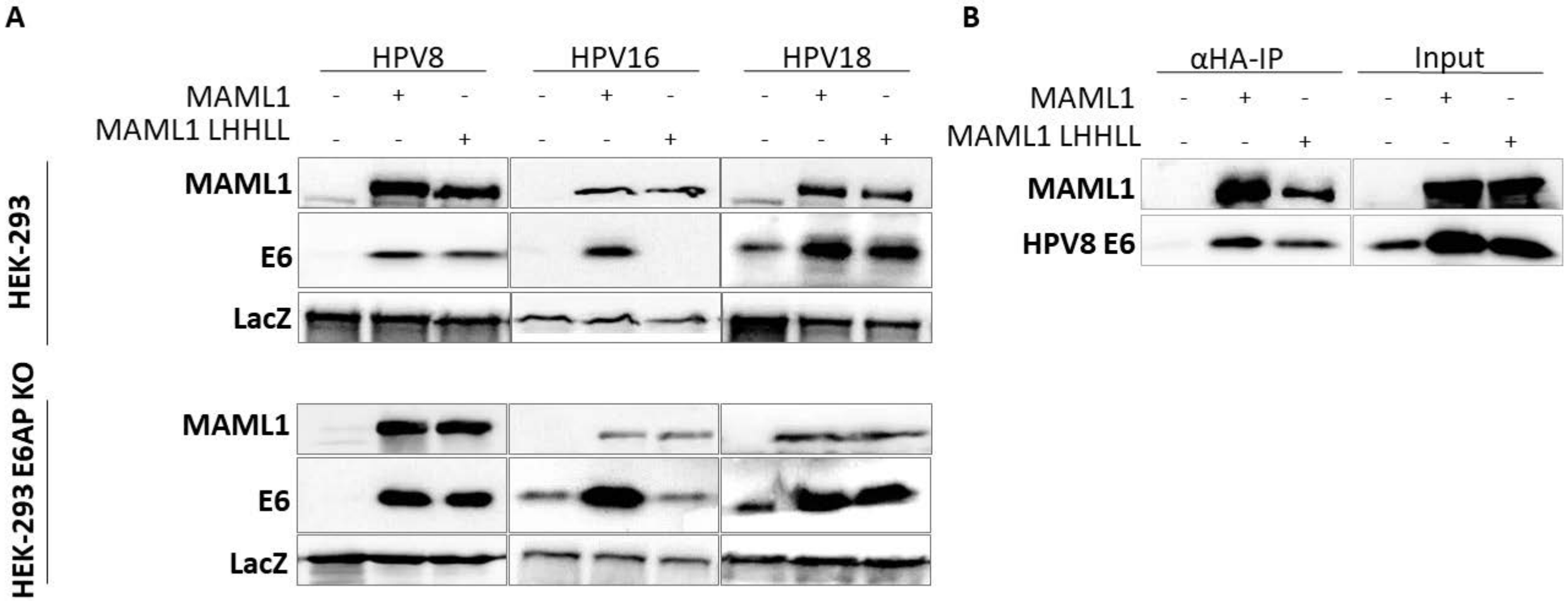

HEK-293
HEK-293 E6AP KO
MAML1
E6AP
16E6
8E6
MAML1
E6AP
E6
LacZ

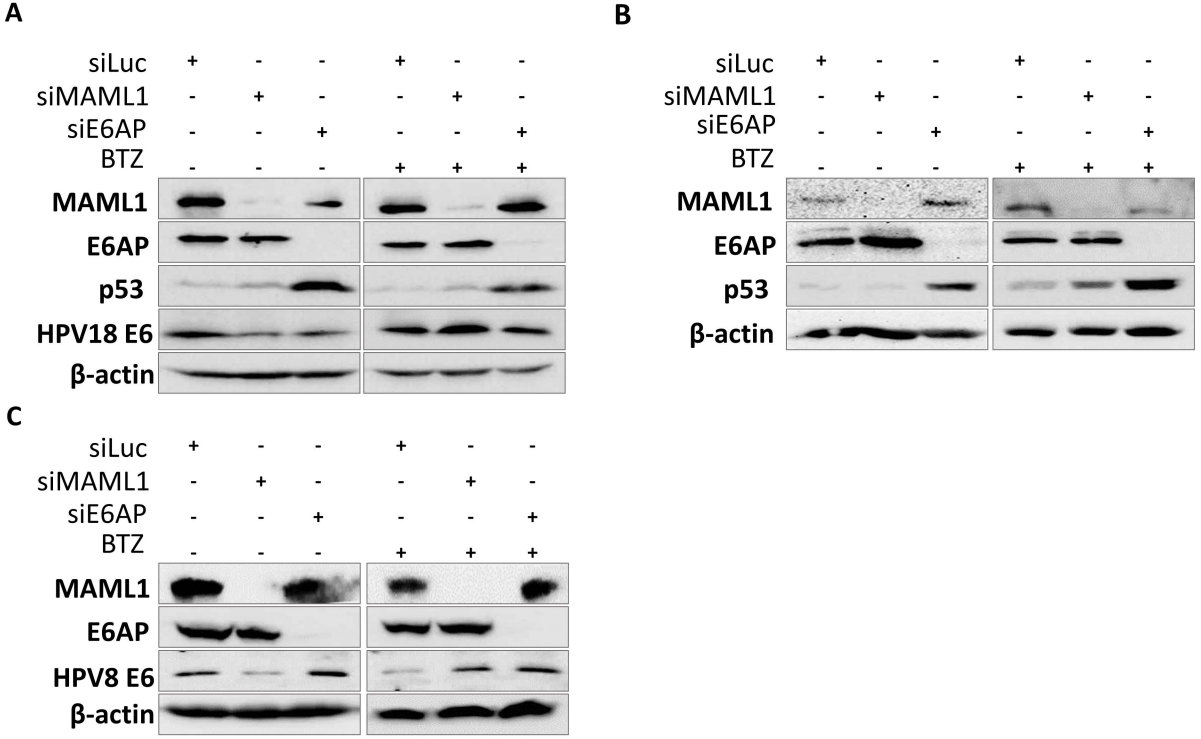

A
HEK-293
HEK-293 E6AP KO
MAML1
MAML1 LHHLL
18E6
8E6
MAML1/ MAML1 LHHLL
E6
LacZ
B
αHA-IP
Input
MAML1
MAML1 LHHL
8E6
MAML1
HPV8 E6

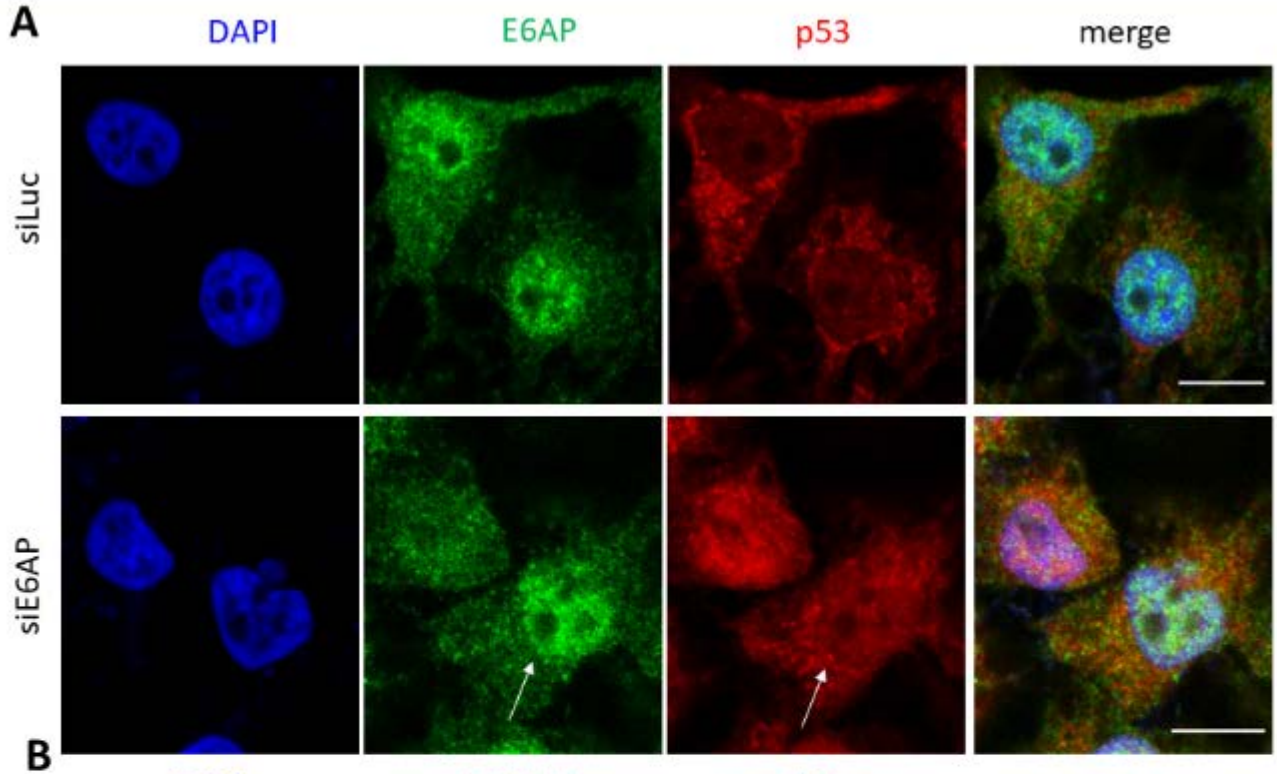

HPV8
HPV12
HPV14
HPV16
HPV18
HPV24
HPV33
HPV38
MAML1
E6AP
MAML1
E6AP
E6
LacZ
LOG2 (fold change)
8
4
0
-4
E6
E6 + MAML1
E6 + E6AP
HPV8
HPV12
HPV14
HPV16
HPV18
HPV24
HPV33
HPV38

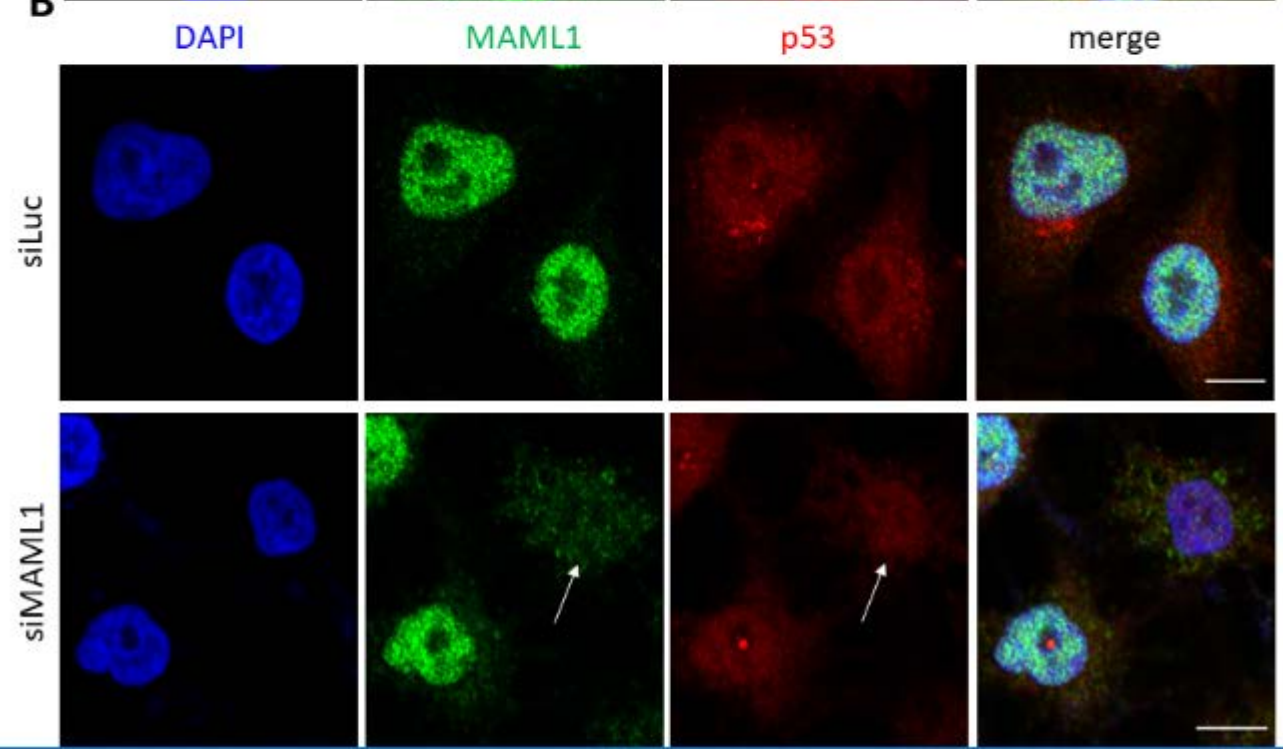

A
HPV8
HPV16
HPV18
MAML1
MAML1 LHHLL
HEK-293
MAML1
E6
LacZ
HEK-293 E6AP KO
MAML1
E6
LacZ
B
αHA-IP
Input
MAML1
MAML1 LHHLL
MAML1
HPV8 E6

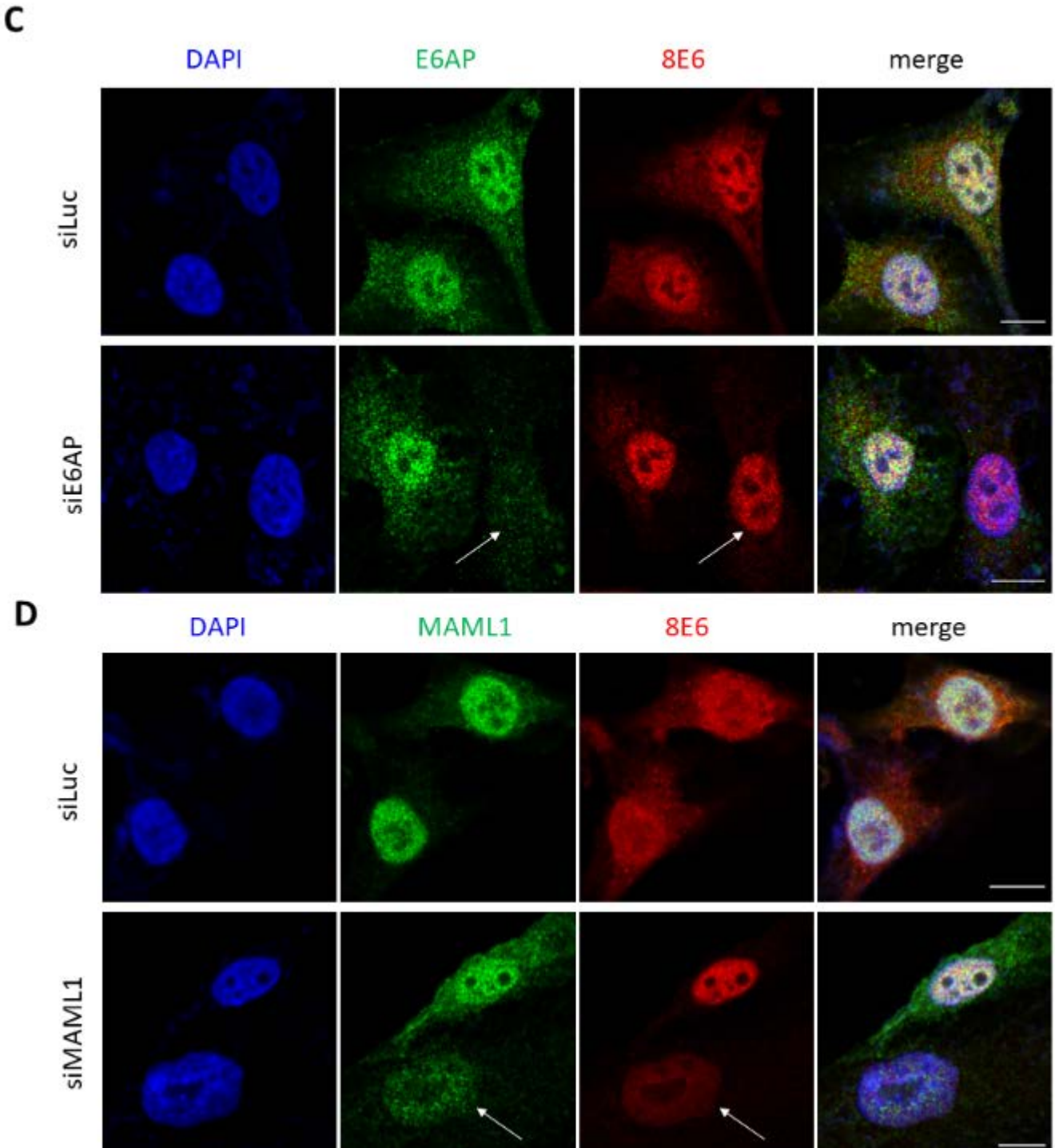

HPV8
HPV12
HPV14
HPV16
HPV18
HPV24
HPV33
HPV38
MAML1
E6AP
MAML1
E6AP
E6
LacZ
LOG2 (fold change)
8
4
0
-4
E6
E6 + MAML1
E6 + E6AP
HPV8
HPV12
HPV14
HPV16
HPV18
HPV24
HPV33
HPV38

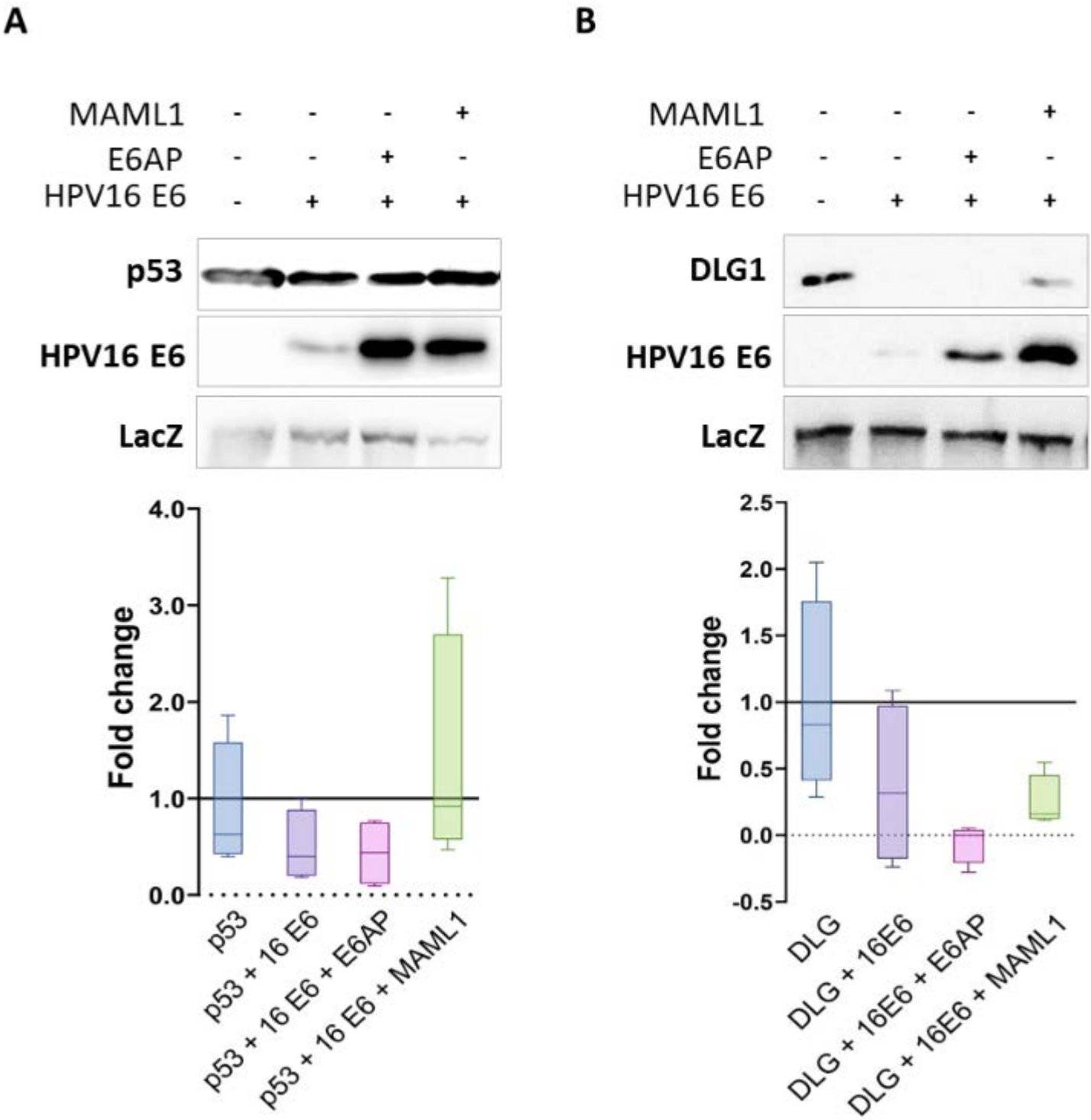

A
HPV8
HPV16
HPV18
MAML1
MAML1 LHHLL
HEK-293
MAML1
E6
LacZ
HEK-293 E6AP KO
MAML1
E6
LacZ
B
αHA-IP
Input
MAML1
MAML1 LHHLL
MAML1
HPV8 E6

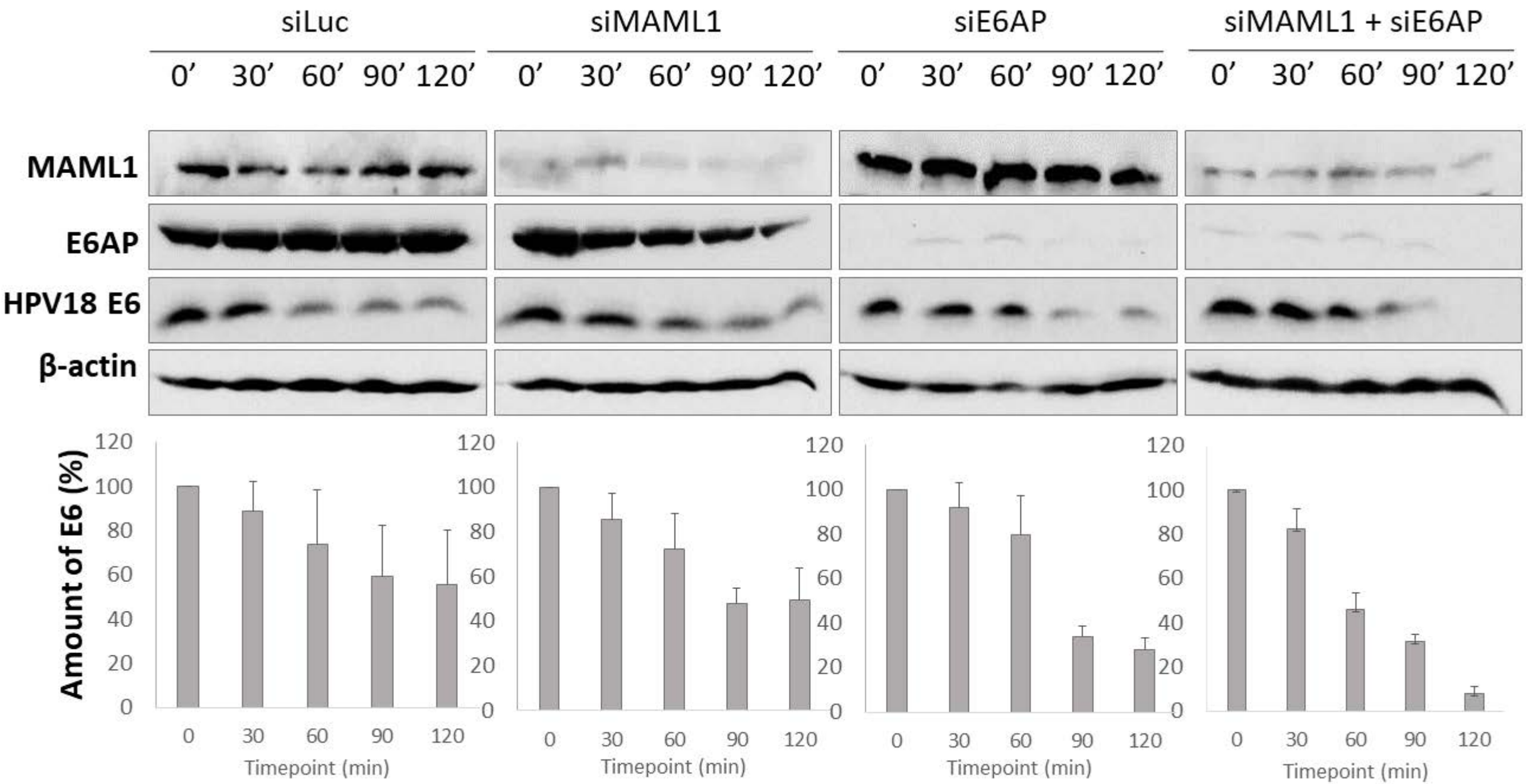

A
siLuc + - - + - -
siMAML1 - + - - + -
siE6AP - - + - - +
BTZ - - - + + +
MAML1
E6AP
p53
HPV18 E6
β-actin
B
siLuc + - - + - -
siMAML1 - + - - + -
siE6AP - - + - - +
BTZ - - - + + +
MAML1
E6AP
p53
β-actin
C
siLuc + - - + - -
siMAML1 - + - - + -
siE6AP - - + - - +
BTZ - - - + + +
MAML1
E6AP
HPV8 E6
β-actin

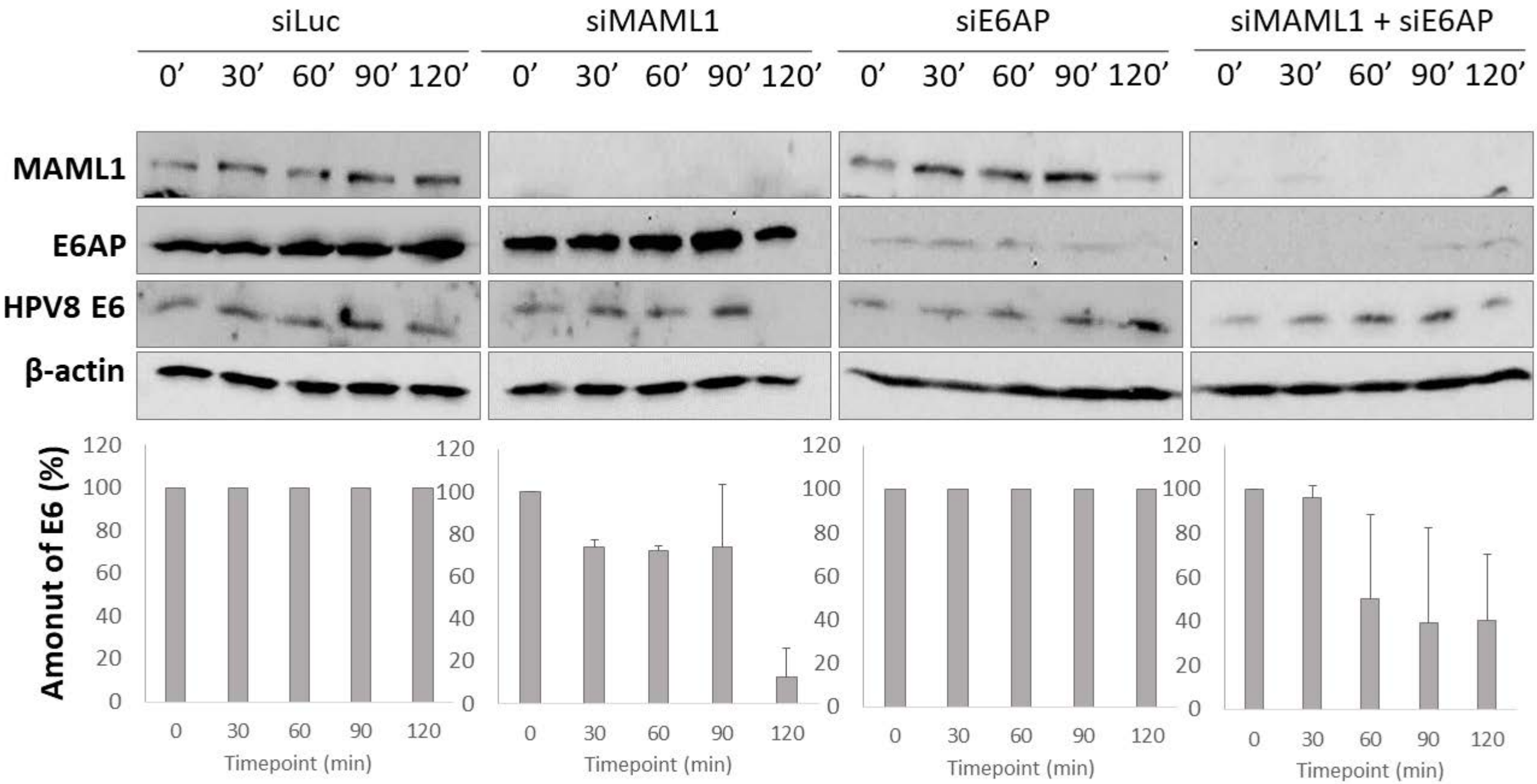

A
DAPI
E6AP
p53
merge
siLuc
siE6AP

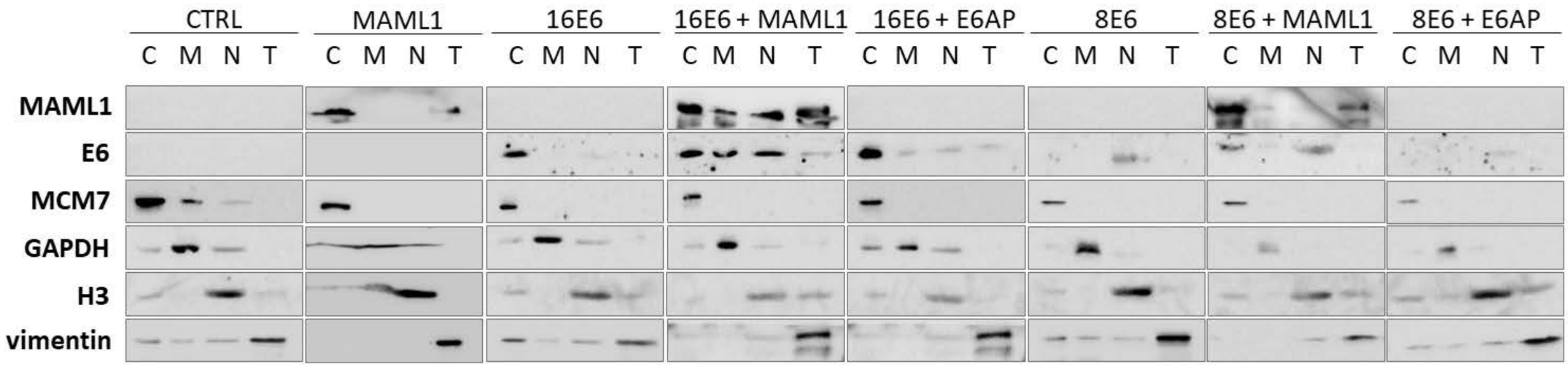

B
DAPI
MAML1
p53
merge
siLuc
siMAML1

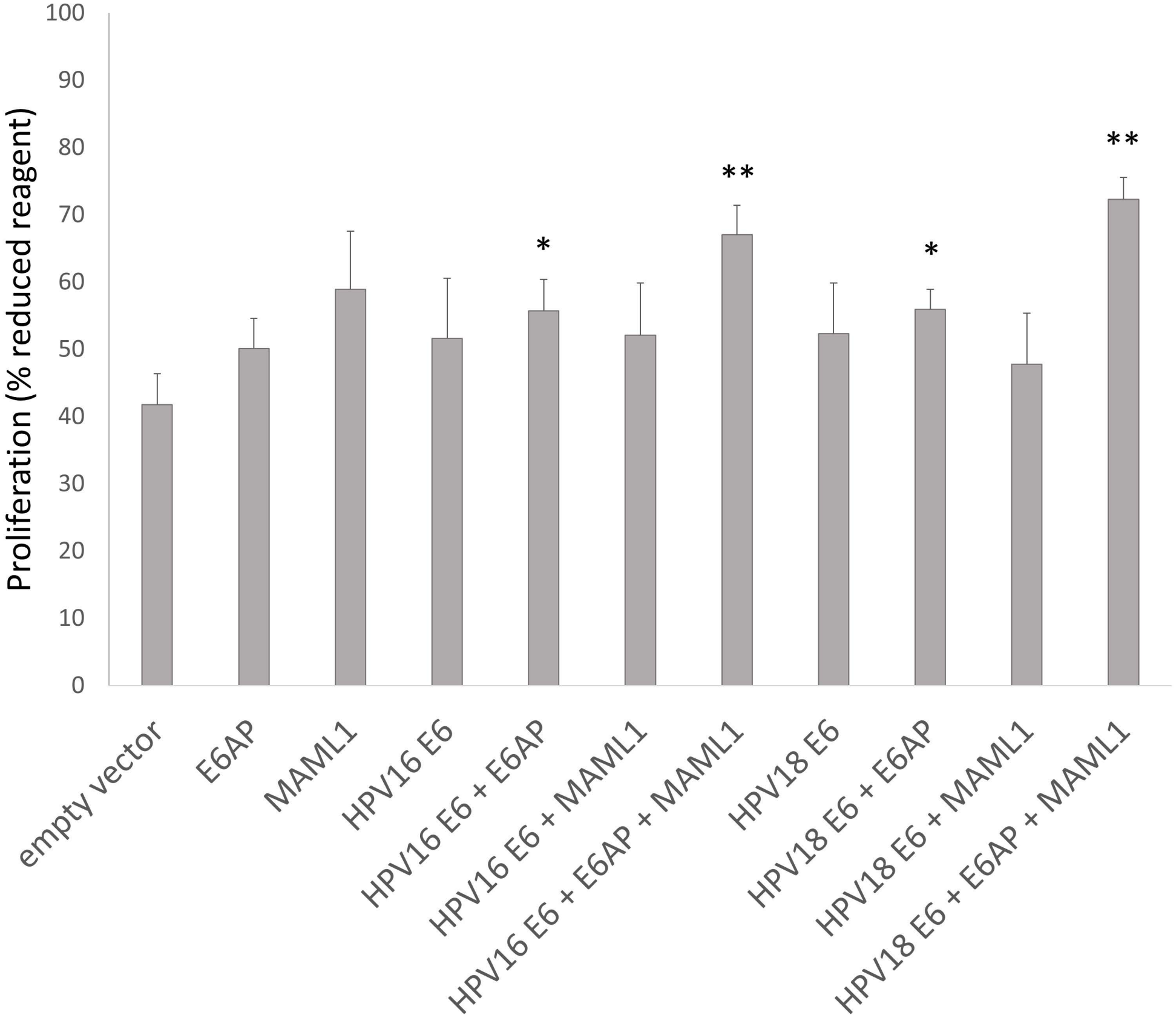

C
DAPI
E6AP
8E6
merge
siLuc
siE6AP
D
DAPI
MAML1
8E6
merge
siLuc
siMAML1

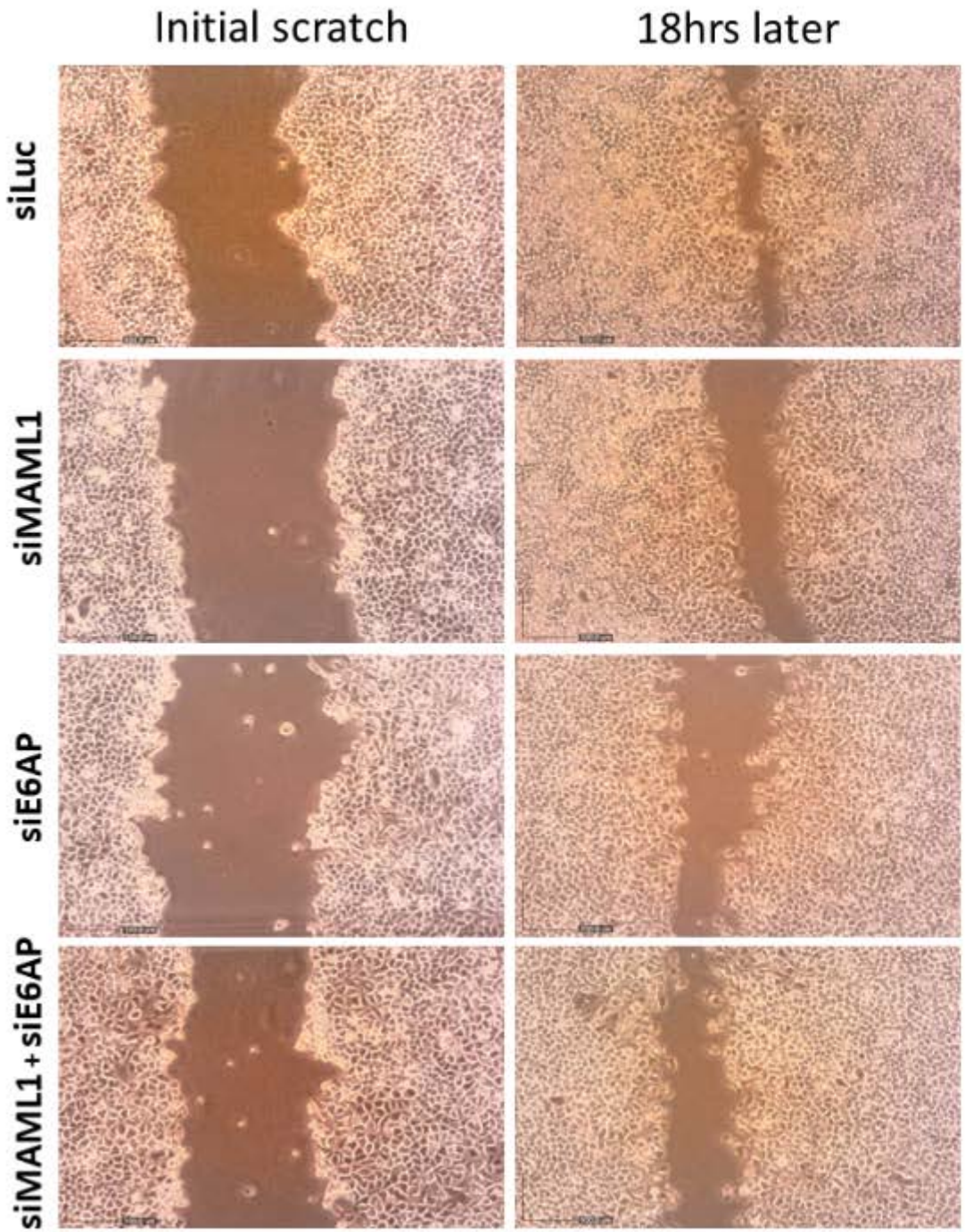

A
MAML1 - - - +
E6AP - - + -
HPV16 E6 - + + +
p53
HPV16 E6
LacZ
Fold change
4.0
3.0
2.0
1.0
0.0
p53
p53 + 16 E6
p53 + 16 E6 + E6AP
p53 + 16 E6 + MAML1
B
MAML1 - - - +
E6AP - - + -
HPV16 E6 - + + +
DLG1
HPV16 E6
LacZ
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
DLG
DLG + 16E6
DLG + 16E6 + E6AP
DLG + 16E6 + MAML1

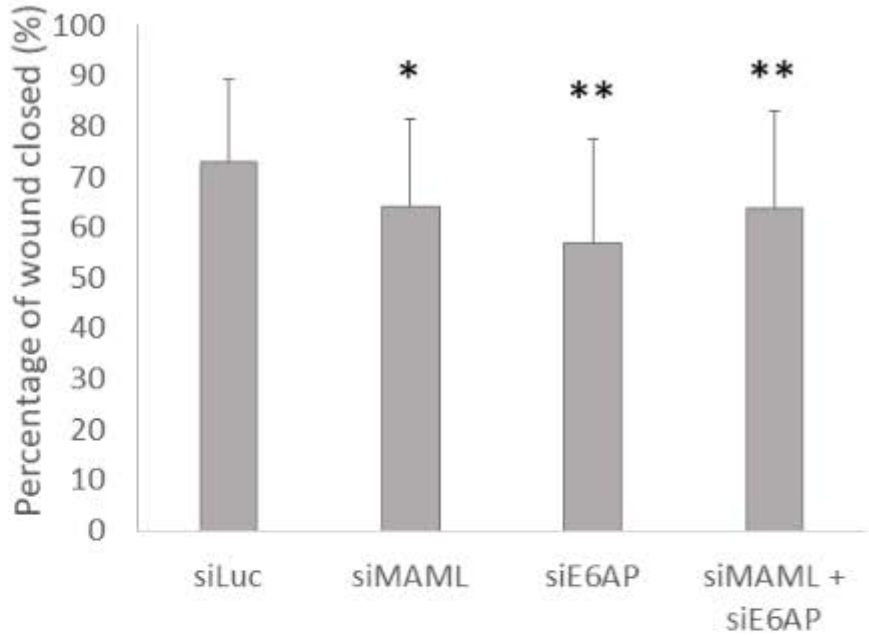

siLuc
siMAML1
siE6AP
siMAML1 + siE6AP
0' 30' 60' 90' 120'
MAML1
E6AP
HPV18 E6
β-actin
Amount of E6 (%)
0
20
40
60
80
100
120
0 30 60 90 120
Timepoint (min)

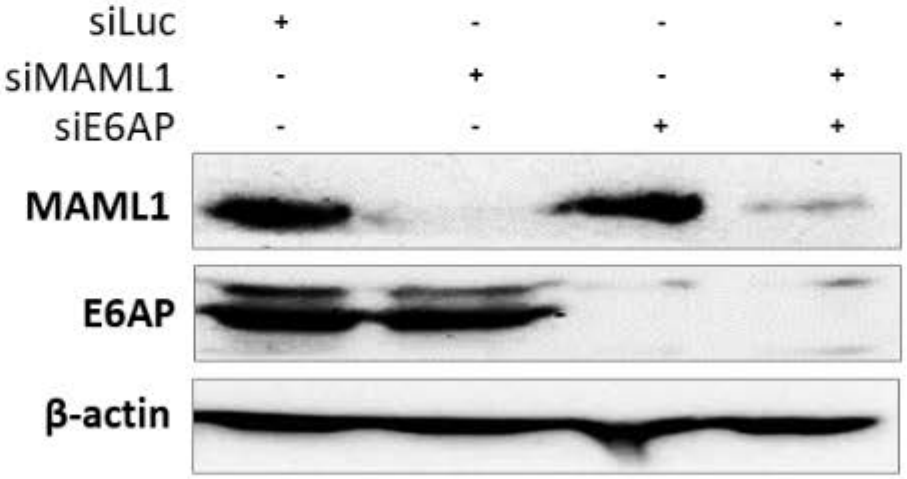

siLuc
siMAML1
siE6AP
siMAML1 + siE6AP
0’ 30’ 60’ 90’ 120’
MAML1
E6AP
HPV8 E6
β-actin
Amonut of E6 (%)
120
100
80
60
40
20
0
0 30 60 90 120
Timepoint (min)

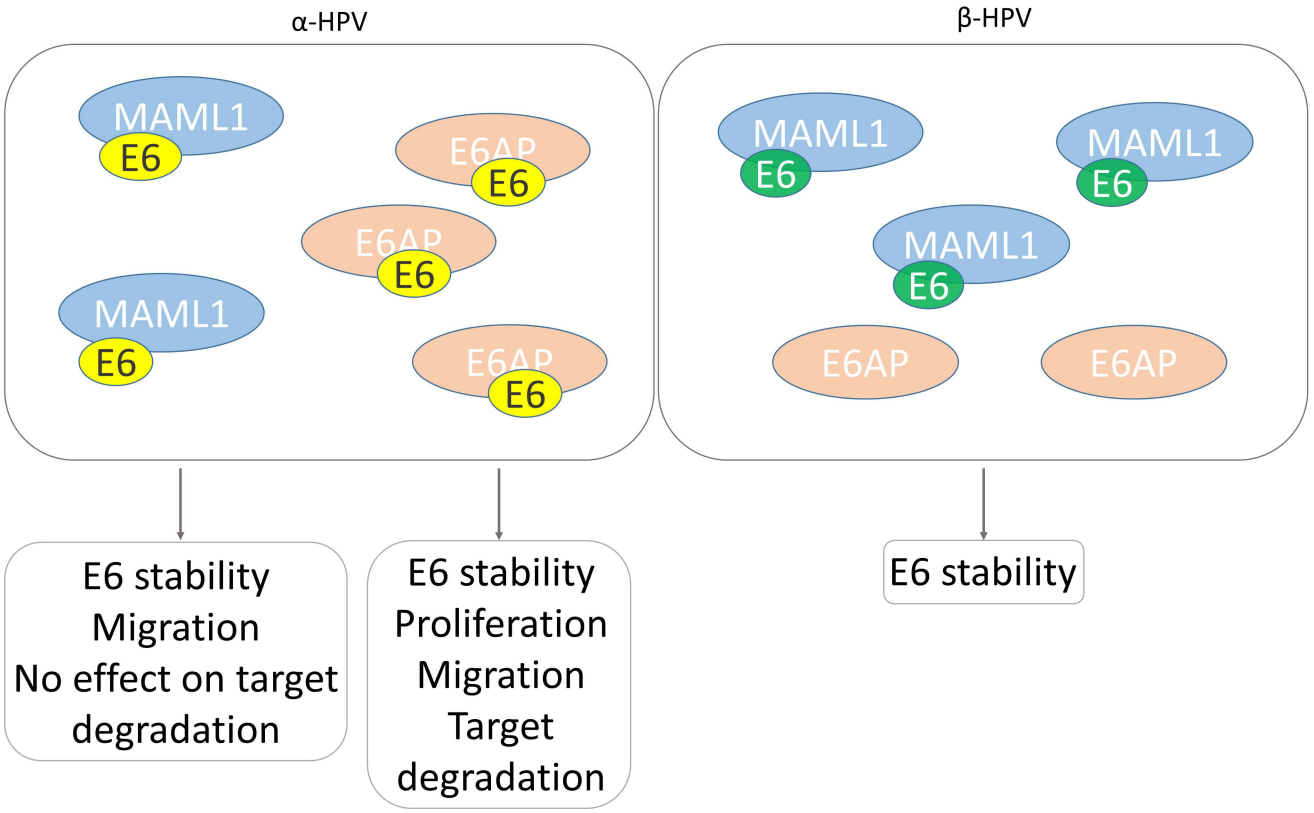

CTRL
MAML1
16E6
16E6 + MAML1
16E6 + E6AP
8E6
8E6 + MAML1
8E6 + E6AP
C M N T
MAML1
E6
MCM7
GAPDH
H3
vimentin


Proliferation (% reduced reagent)
100
90
80
70
60
50
40
30
20
10
0
*
**
*
**
empty vector
E6AP
MAML1
HPV16 E6
HPV16 E6 + E6AP
HPV16 E6 + MAML1
HPV16 E6 + E6AP + MAML1
HPV18 E6
HPV18 E6 + E6AP
HPV18 E6 + MAML1
HPV18 E6 + E6AP + MAML1


Initial scratch
18hrs later
siLuc
siMAML1
siE6AP
siMAML1 + siE6AP


Percentage of wound closed (%)
100
90
80
70
60
50
40
30
20
10
0
*
**
**
siLuc
siMAML
siE6AP
siMAML + siE6AP


siLuc + - - -
siMAML1 - + - +
siE6AP - - + +
MAML1
E6AP
β-actin


α-HPV
β-HPV
MAML1
E6
E6AP
E6
E6AP
E6
MAML1
E6
E6AP
E6
MAML1
E6
MAML1
E6
MAML1
E6
E6AP
E6AP
E6 stability
Migration
No effect on target degradation
E6 stability
Proliferation
Migration
Target degradation
E6 stability