

1 **Disc Large 1 expression is altered by Human Papillomavirus E6/E7 proteins in organotypic**  
2 **cultures of human keratinocytes.**

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14 **Running Title:** HPV E6/E7 proteins alter DLG1 expression.

15

16 **Abbreviations:** APC, Adenomatous polyposis coli; DLG1, human Disc large; HPV, Human  
17 Papillomavirus; Lgl, lethal giant larvae; Par, Partitioning defective; PATJ, PALS1 associated tight  
18 junction protein; PBM, PDZ-binding motif; PDZ, PSD-95/DLG/ZO-1 domains; PHK, primary  
19 human keratinocytes; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PTEN, phosphatase  
20 and tensin homolog; SDH, Human succinate dehydrogenase gene; SIL, squamous intraepithelial  
21 lesions; TJ, tight junction; URR, untranslated regulatory region; WB, western Blot.

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32

33 **ABSTRACT**

34 Loss of cell polarity is a fundamental process in cell transformation. Among polarity proteins, we  
35 focused on human Disc Large (DLG1), which is localized mainly at adherens junctions and  
36 contributes to the control of cell proliferation. We previously demonstrated that its expression is  
37 altered in HPV-associated cervical neoplastic lesions, but the mechanisms beyond this remain  
38 unknown. In this study, we analyzed the contribution of HPV proteins to the changes in DLG1  
39 expression in the squamous epithelium. We observed tissue and intracellular misdistribution of DLG1  
40 when high-risk HPV-18 E7 or E6/E7 proteins were expressed in organotypic raft cultures. The viral  
41 oncoproteins induce the loss of DLG1 from the cell borders and an increase in the level of DLG1  
42 protein, reflecting the pattern observed in cervical lesions. These findings were corroborated in  
43 cultures bearing the entire HPV-18 genome. Interestingly, changes in tissue distribution and  
44 abundance of DLG1 were also detected in organotypic cultures expressing the low-risk HPV-11 E7  
45 or E6/E7 proteins; suggesting a conserved function among different HPV types. However, for low-  
46 risk HPVs, the subcellular localization of DLG1 at cell-to-cell contacts was predominantly  
47 maintained. This report offers new evidence of the involvement of HPV proteins in DLG1 expression  
48 pattern and our data support previous observations regarding DLG1 expression in cervical lesions.

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## 53 INTRODUCTION

54 Human Disc large 1 (DLG1) is a modular scaffolding protein bearing different protein interaction  
55 modules, including the PSD-95/DLG/ZO-1 (PDZ) domains that allow multiprotein complexes  
56 formation (Lue et al., 1994, Roberts *et al.*, 2012). DLG1 is able to assemble different proteins into  
57 signal transduction networks where DLG1 has structural and signaling functions. DLG1 functions in  
58 controlling cell polarity were first shown in *Drosophila*, where it was also demonstrated to be a  
59 regulator of cell proliferation. Besides, functional loss of DLG1 has been associated with neoplastic  
60 transformation (Bilder, 2004). In mammalian epithelial cells, DLG1 localizes in the cytoplasm and at  
61 sites of cell contacts in association with adherens junctions (Laprise et al., 2004), being part of the  
62 Scribble polarity complex, which is crucial for the establishment and maintenance of apicobasal  
63 polarity (Assemat et al., 2008).

64 Most of DLG1 biological functions rely on its ability to interact with several regulatory proteins such  
65 as protein 4.1/ERM family members (Lue et al., 1994), several kinases (Gaudet et al., 2000, Sabio et  
66 al., 2005, Gaudet et al. 2011) and two important tumour suppressors: phosphatase and tensin homolog  
67 (PTEN) and Adenomatous polyposis coli (APC) (Sotelo et al., 2012). Remarkably, the DLG1:APC  
68 binding is important for the negative regulation of cell growth (Ishidate et al., 2000) and the  
69 interaction with PTEN is required for PTEN stability, cooperating with the inactivation of the  
70 proliferative pathways (Sotelo *et al.*, 2012; Valiente *et al.*, 2005). DLG1 was shown to have a dual  
71 role in the regulation of both cell polarity and proliferation, highlighting that tissue polarity and cell  
72 cycle should be fine-tuned linked processes during tumour suppression.

73 Several reports using human biopsies have described changes in DLG1 abundance and distribution  
74 during malignant progression (Facciuto et al., 2012). Interestingly, while a marked reduction of DLG1  
75 levels in poorly differentiated tumours was described, over-expression and changes in DLG1  
76 distribution at earlier stages of cervical, colon and breast cancers have been observed by different  
77 groups (Watson et al., 2002; Cavatorta et al., 2004; Fuja et al., 2004; Gardiol et al., 2006).  
78 Importantly, the loss of DLG1 expression at cell contacts during neoplastic progression has been  
79 consistently reported in studies using histological samples. It has been suggested that the intracellular  
80 localization of DLG1 is critical for its biological functions, most likely because the precise  
81 distribution of DLG1 may define the probable interacting partners, implying the orchestration of  
82 different specific signalling pathways.

83 However, the molecular mechanisms responsible for such alterations, the contribution of high DLG1  
84 expression to tumour prognosis and the precise temporal de-regulation of this protein at different  
85 stages of cancer progression are not fully understood. Besides, despite the fact that DLG1 has been

86 postulated as a tumor suppressor some paradoxical data indicate that, under certain conditions, it may  
87 have oncogenic activities (Frese et al., 2006; Krishna Subbaiah et al., 2012).

88 Cervical cancer is a common malignancy, etiologically associated with infection by a group of Human  
89 papillomavirus (HPV) types. Previous studies have shown a striking DLG1 over-expression in the  
90 cervical precursor squamous intraepithelial lesions (SIL). In these samples DLG1 was lost from cell  
91 contacts and re-distributed to the cytoplasm, especially in the high grade SIL (HSIL) (Watson et al.,  
92 2002; Cavatorta et al., 2004).

93 The continuous expression of the two main HPV oncoproteins, E6 and E7, is important for tumor  
94 progression and maintenance of the transformed phenotype. One of the reported oncogenic activities  
95 of E6 from high-risk HPV types is its capacity to interact with a select group of PDZ domain-  
96 containing proteins, including DLG1, through the C-terminal class 1 PDZ binding motif (PBM) (X-  
97 S/T-X-V/L) (Gardioli et al., 1999; Pim et al., 2012). This interaction results in DLG1 protein  
98 degradation and/or alteration of subcellular distribution, depending on the experimental conditions  
99 and HPV type. However, the real contribution of HPV proteins to changes in DLG1 expression,  
100 mainly in the context of HPV infection of the squamous epithelia, remains to be established. In this  
101 context, it will be interesting to verify E7 influence on cell polarity proteins expression. E7 has been  
102 proposed as the main HPV oncogene and several reports show its ability to interact and interfere with  
103 a large number of factors that regulate cell proliferation, gene expression and cytoskeleton  
104 organization. For instance, E7 can induce changes in the expression of E-cadherin, a key protein  
105 involved in the formation of adherens junctions and epithelial polarity establishment (Caberg et al.,  
106 2008; Hellner et al., 2009).

107 As mentioned, an integrated picture of the mechanisms controlling DLG1 expression is lacking.  
108 Therefore, a better understanding of the phenomena involved could be relevant considering the  
109 changes in DLG1 levels observed in cervical lesions (Cavatorta et al., 2004). In the present study we  
110 analyzed the effect of HPV oncoproteins to changes in DLG1 expression levels and cell distribution  
111 in epithelial organotypic raft cultures of keratinocytes expressing HPV E7 and HPV E6/E7 proteins  
112 from high- and low- risk HPV types.

113 Using this system, we observed that cultures expressing HPV-18 E7 or E6/E7 exhibited a  
114 misdistribution of DLG1 throughout the tissue thickness. The presence of the viral proteins also  
115 induced DLG1 loss from cell borders to the cytoplasm, reflecting the expression pattern observed in  
116 SIL biopsies. In addition, E7 and E6/E7 from HPV-18 increased DLG1 levels. Our observations were  
117 corroborated in organotypic cultures of keratinocytes transfected with HPV-18 full-length genome.  
118 Interestingly, changes in DLG1 distribution and abundance were also detected in raft cultures  
119 expressing the low-risk HPV-11 E7 or E6/E7 proteins, although to a lesser extent. This suggests a

120 conserved mechanism among HPV types associated with different pathologies. In the case of cultures  
121 expressing the low-risk viral proteins, however, DLG1 subcellular localization at cell contacts was  
122 predominantly maintained. This report offers new evidence concerning HPV proteins involvement in  
123 DLG1 expression pattern, and our data support previous observations regarding DLG1 expression in  
124 cervical lesions.

125

## 126 **RESULTS**

### 127 **HPV-18 oncoproteins interfere with the pattern of DLG1 expression in organotypic raft** 128 **cultures.**

129 We first analyzed DLG1 expression pattern in the context of the squamous epithelium in the presence  
130 or absence of the HPV-18 E7 or E6/E7 oncoproteins. We focused on HPV-18 since DLG1 was shown  
131 to be a preferential target for this virus (Thomas et al., 2008). Therefore, we generated organotypic  
132 cultures from primary human keratinocytes (PHK) previously infected with retroviral vectors  
133 expressing E7 or E6/E7. Raft cultures obtained from PHK were used as a control (Fig. 1a). A section  
134 of each paraffinized organotypic culture was stained with haematoxylin-eosine, in order to analyze  
135 the morphology of the tissue (Fig. 1a). As expected, the presence of E7 or E6/E7 was associated with  
136 the thickening of the stratified cell layers. Nuclei retention throughout the epithelium including the  
137 uppermost layers was also observed, as previously reported (Delury et al., 2013). The expression of  
138 the HPV-18 genes was determined by RT-PCR (Fig. 1b). In addition, the functionality of the viral  
139 proteins was assessed by evaluating the expression of E7 and E6 cellular targets: retinoblastoma (Rb)  
140 and p53. E7 expression induces a reduction of Rb and an increase in p53 protein levels as described  
141 previously (Fig. 1c) (Thomas & Laimins, 1998; Seavey et al., 1999; Flores et al., 2000, Munger et  
142 al., 2001). p53 expression slightly decreased in E6/E7 expressing rafts when compared to the E7 rafts  
143 and in relation to the loading control (Fig. 1c) (Flores et al., 2000). The effect of the viral proteins  
144 was also corroborated by immunohistochemistry (IHC) on sections of the rafts cultures. The results  
145 presented in Fig. S1 clearly indicate the expression and functionality of both viral proteins.

146 Our data indicate that in control tissues DLG1 was expressed mainly in the basal and parabasal layers  
147 of the epithelium but was absent in the uppermost differentiated cellular strata. DLG1 was localized  
148 preferentially in the cytoplasm of the basal cells, but in the suprabasal areas it was predominantly  
149 present at cell contacts (Fig. 1d). Isolated nuclei of some epithelial cells showed positive staining,  
150 especially in the basal layer. This observation is in agreement with the DLG1 pattern previously  
151 reported in normal cervical samples (Cavatorta *et al.*, 2004).

152 For organotypic cultures expressing HPV oncoproteins the overall intracellular staining for DLG1  
153 was more intense compared to control samples. In this case, DLG1 expression was observed

154 throughout the epithelial strata. Besides, DLG1 cell localization was cytoplasmic in the basal as well  
155 as in the uppermost layers, where DLG1 at intercellular contacts was reduced (Fig. 1d). This effect  
156 was more striking in samples expressing both E6 and E7, where DLG1 expression at the cell borders  
157 was greatly diminished; interestingly, an increase in nuclear staining was also observed. Remarkably,  
158 these findings are similar to DLG1 expression in SIL (Cavatorta et al., 2004).

159 Our results demonstrate that HPV-18 E7 can induce changes in DLG1 distribution along the  
160 squamous epithelium and in the subcellular localization, these effects being more marked when both  
161 HPV-18 E6 and E7 proteins are expressed together.

162 In order to confirm our results, DLG1 expression pattern was analyzed within the context of the whole  
163 HPV genome, using raft cultures established from PHK bearing full-length HPV-18 genome (FK18B)  
164 at passage 27, which morphologically resemble mild dysplasia *in vivo* (Steenbergen et al., 1998).  
165 DLG1 staining was much more intense comparing to the control, and, in agreement with the data  
166 presented for the E6/E7 cultures, it was expressed throughout the epithelium thickness with a  
167 predominant cytoplasmic distribution and loss from cell contacts (Fig. 2).

168

### 169 **HPV-18 E7 protein induces an increase in the levels of DLG1**

170 In addition to changes in DLG1 distribution by HPV-18 E7 or E6/E7, a slight change in DLG1  
171 abundance could be also appreciated (Fig. 1d). In order to apply a more quantitative assay, we  
172 analyzed DLG1 levels by Western Blotting (WB) using protein extracts from the organotypic  
173 cultures. HPV-18 E7 and E6/E7 proteins induce an increase in DLG1 levels compared to control  
174 samples (Fig. 3a). This raise could be due to the fact that in tissues expressing the HPV proteins,  
175 DLG1 expression extends throughout all the cell strata, while it was only present in the less  
176 differentiated layers in the control (Fig. 1d). Considering this, we evaluated if the presence of E7  
177 could be involved in the up-regulation of DLG1 intracellular levels, independently of the tissue  
178 context. For this, DLG1 abundance was analyzed by WB using protein extracts from HEK293 cells  
179 transiently transfected with plasmids expressing HPV-18 proteins. RT-PCR assays were used to  
180 confirmed viral gene expression (Fig. S2). In these experimental conditions HPV-18 E6 was shown  
181 to promote the degradation of DLG1 in a PBM-dependent manner (Gardiol et al., 1999; Pim et al.,  
182 2012). However, it is important to understand the regulation of PDZ proteins in the context of HPV  
183 infection where both oncoproteins are expressed together. As observed in Fig. 3b, E7 alone or together  
184 with E6 results in a moderate increase in DLG1 protein level, in agreement with the observations  
185 obtained using the organotypic cultures and, apparently, overriding the reported effects of E6-  
186 mediated DLG1 degradation.

187

188 **Expression of low-risk HPV E7 and E6/E7 proteins also alter DLG1 expression in squamous**  
189 **raft cultures.**

190 In several neoplastic lesions, tissue disorganization has been associated with alterations in DLG1  
191 expression pattern (Facciuto et al., 2012). However, there are no data about possible changes in  
192 lesions associated to low-risk HPV that, even linked to benign lesions that rarely progress to  
193 malignancy, cause cell hyper proliferation and aberrant differentiation (Jian et al., 1999; Cheng et al.,  
194 1995; McCord et al., 2014). We analyzed the effects of E7 and E6/E7 proteins derived from low-risk  
195 HPV-11 on DLG1 expression in organotypic cultures. PHK were infected with retroviral vectors  
196 expressing HPV-11 E7 or E6/E7. In tissues expressing the HPV-11 proteins, the epithelium was  
197 thicker than that from control cultures, due to an increased number of cell layers in the spinous stratum  
198 (Fig. 4a). However, the morphological alterations were less evident than in cultures expressing high-  
199 risk HPV proteins (Fig. 1a). The expression of the HPV-11 genes was assessed by RT-PCR (Fig. 4b).  
200 Then, DLG1 immunostaining was evaluated and, interestingly, some changes were observed when  
201 the low-risk HPV proteins were expressed (Fig. 4c). There was a general increase in DLG1 abundance  
202 and its expression was also detected in the uppermost strata. However, DLG1 was present at high  
203 levels at cell borders, especially in the samples were only E7 was expressed. Besides, only a moderate  
204 reduction of DLG1 at that precise cell localization was observed in the E6/E7 expressing samples. In  
205 both conditions an increased in the cytoplasmic DLG1 expression could also be appreciated.

206 Our data suggest that low-risk HPV also induce a redistribution of DLG1 along the squamous  
207 epithelium but did not markedly alter the subcellular localization at the cell contacts, as when high-  
208 risk HPV proteins are expressed.

209 We next evaluated whether the presence of the HPV-11 E7 and E6/E7 produce a change in DLG1  
210 abundance by WB. As can be seen, the protein extracts derived from organotypic cultures expressing  
211 HPV-11 E7 exhibited a slight increase in DLG1, and this effect was more striking for those samples  
212 where HPV-11 E6 and E7 were present (Fig. 5a).

213 In order to study the potential effect of HPV-11 proteins on DLG1 levels within the cell, we performed  
214 experiments using transiently transfected HEK293 cells with expression plasmids for the viral  
215 proteins. The expression of the viral sequences was tested by RT-PCR (Fig. S2). As known, the  
216 presence of HPV-11 E6 alone do not change DLG1 levels, since low-risk E6 proteins cannot bind  
217 and degrade the PDZ DLG1 protein (Gardiol et al., 1999). As can be seen in Fig. 5b, the presence of  
218 HPV-11 E7 and E6/E7 also induces an increase in DLG1 levels.

219 The overall data suggest that low- and high-risk HPV share a conserved mechanism leading to  
220 changes in DLG1 expression levels that should be important for virus replication. This idea points

221 out the hypothesis that common functions of E7 or E6/E7 proteins among low- and high-risk HPVs  
222 might participate in molecular pathways that induce these changes.

223

## 224 **Discussion**

225 In this study, we describe the changes in DLG1 expression in the presence of HPV proteins. Several  
226 studies demonstrated alterations in DLG1 abundance and localization during malignant progression,  
227 although it is not clear whether these alterations are a cause or a consequence of the neoplastic  
228 hyperproliferation. In order to better understand the complex pattern of DLG1 expression, we  
229 analyzed this in a model that is relevant for HPV infection and the associated lesions. As HPV life  
230 cycle is entirely dependent on the epithelium differentiation, we set up organotypic cultures that *in*  
231 *vitro* mimic the tissue structure.

232 The presence of HPV-18 E7 or E6/E7 proteins induces a change in tissue and cell distribution of  
233 DLG1 when compared to control cultures. Our results clearly resemble DLG1 expression patterns  
234 observed in HPV-associated SIL (Cavatorta et al., 2004). In the presence of viral proteins, DLG1 was  
235 expressed throughout the thickness of the epithelium, in contrast to control cultures where it was  
236 absent in the uppermost differentiated cells. HPV E7 or E6/E7 expression is expected to alter the  
237 epithelium differentiation program. Therefore, regarding the differentiation status, cells that compose  
238 the tissue strata might not be equivalent to those present in the control tissue. HPV E6 and E7  
239 oncogenes have been shown to disturb the differentiation schedule of the host cell (Pei *et al.*, 1998;  
240 Zehbe et al., 2009). Moreover, it was recently reported that E6 and/or E7 from high-risk HPVs are  
241 able to down-regulate the expression of differentiation genes (Gyongyosi et al., 2012). This would be  
242 important for viral cycle, inducing the cellular replication machinery in differentiated keratinocytes.  
243 In this sense, HPV-18 E6/E7 induced the expression of the cell cycle and proliferation markers: cyclin  
244 A and proliferating cell nuclear antigen (PCNA) in the epithelium suprabasal layers (Fig. S3), as  
245 described previously (Flores et al., 2000; Wang et al., 2009). Although differences in cell cycle  
246 activity may contribute to DLG1 altered expression observed in organotypic cultures, our results do  
247 not rule out the involvement of other mechanisms, as discussed below.

248 One of the interesting features shown in Fig. 1d is the clear reduction of DLG1 at cell contacts while  
249 the staining increased in the cytoplasm in presence of HPV-18 proteins. Our results indicate a possible  
250 role of the HPV proteins in DLG1 changes observed for HPV-associated neoplasias (Watson, 2002;  
251 Cavatorta, 2004). In fact, the accumulation of DLG1 in the cytoplasm may have an oncogenic  
252 significance since it was shown that specific cellular pools of DLG1 in the presence of viral  
253 oncoproteins could have oncogenic functions (Frese et al., 2006; Krishna Subbaiah et al., 2012).



254 Several studies demonstrated that E6 can interact and induce the proteasome-mediated degradation  
255 of DLG1; however, this degradation involves specific subcellular pools. Furthermore, degradation  
256 seems to be incomplete since significant levels remain in HPV cancer cell lines and certain pools of  
257 DLG1 are actively maintained by the continuous expression of E6/E7 (Krishna Subbaiah et al., 2012).  
258 In a recent study it was shown that the residual cytoskeletal-bound forms of DLG1 influences RhoG  
259 activity by making a complex with SGEF and this association is maintained by the presence of E6  
260 and E7 oncoproteins. These cooperative functions between E6 and E7, promoting the activity of  
261 RhoG, a protein which has been involved in cell proliferation and differentiation, could be useful for  
262 virus replication in the tissue context (Krishna Subbaiah et al., 2012). These observations may  
263 contribute to explain the data shown in Fig. 1d, where a considerable amount of DLG1 is maintained  
264 in the cytoplasm perhaps complexed with cell proteins involved in cell growth.

265 E7 expression in epithelial cells was demonstrated to cause a drop in the levels of the adhesion protein  
266 E-cadherin, which is involved in DLG1 localization at cell contacts (Reuver and Garner, 1998;  
267 Hellner et al., 2009). This may account for the slight decrease in DLG1 immunostaining at cell  
268 borders in the presence of HPV-18 E7. Moreover, DLG1 reduction at cell contacts was more striking  
269 in E6/E7 cultures. Therefore, it is possible to speculate that E6 could contribute to DLG1 down-  
270 regulation, perhaps by preferentially targeting those DLG1 forms for degradation (Massimi et al.,  
271 2006).

272 At present, it is not possible to discard that other mechanisms induced by the presence of the viral  
273 oncoproteins could also promote changes in DLG1 protein binding capacity, stimulating its  
274 interaction with different partners that redirect its localization. This is consistent with the hypothesis  
275 that diverse DLG1 pools may have different functions and, possibly, opposite activities regarding the  
276 malignant progression.

277 It is important to highlight that the presence of E6/E7 also promotes an increase in DLG1 the nuclear  
278 localization. It was shown that in conditions where cell polarity can be altered, cell junction proteins  
279 are disassembled and can migrate to the nucleus and regulate transcriptional activity (Polette et al.,  
280 2005). Moreover, Narayan and colleagues (2009) have shown that DLG1 nuclear localization is  
281 highly dependent on phosphorylation by cycling kinases during cell cycle progression, and high-risk  
282 HPV oncoproteins are known to promote cell cycle entry even in differentiated cells (Fig. S3).

283 Moreover, changes in both DLG1 abundance and localization were also observed in tissues derived  
284 from the FK18B-passage 27 cells (Fig. 2), highlighting the significance of our findings. These results  
285 indicate that viral proteins expression in the context of HPV-18 entire genome is responsible of those  
286 alterations, and, in view of our previous data, E7 or E6/E7 expression is most likely involved. These  
287 results are also relevant in light of DLG1 expression in cervical biopsies. FK-18B-passage 27 cultures

288 exhibit morphological alterations suggestive of mild/moderate dysplasia (Steenbergen et al., 1998)  
289 and we previously demonstrated that DLG1 was over-expressed and exhibited altered cellular  
290 distribution in SIL HPV-positive lesions, with a progressive loss from cell contacts (Cavatorta et al.,  
291 2004).

292 Most of the previous studies about HPV interference with cell polarity were focused on high-risk  
293 HPVs. No data were available about polarity proteins expression in general, or specifically for DLG1,  
294 in low-risk HPV associated lesions. Unexpectedly, we also found some changes in DLG1 expression  
295 in the presence of HPV-11 proteins. We observed that raft cultures expressing HPV-11 sequences  
296 exhibit morphological changes suggesting a low-grade dysplasia, albeit to a lesser extent than for  
297 high-risk HPVs (Fig. 1a and 4a) (Thomas et al., 2001; Fang et al., 2006). In organotypic cultures  
298 expressing HPV-11 E7 or E6/E7, DLG1 immunostaining was altered when compared to control  
299 cultures (Fig. 4c). DLG1 was observed throughout the tissue strata, possibly due to changes in cellular  
300 differentiation status (Thomas et al., 2001; Fang et al., 2006). However, unlike the results described  
301 for HPV-18, DLG1 was present at cell borders in cultures expressing HPV-11 proteins, and this may  
302 be relevant considering the different capacity of each virus in transforming cells and in promoting  
303 malignant progression. DLG1 reduction at cell contacts may contribute to alterations in signal  
304 transduction pathways controlling cell proliferation, as part of DLG1 oncosuppressing functions.

305 Nevertheless, HPV-11 proteins were also capable to induce an increase in DLG1 levels in both  
306 organotypic and monolayer epithelial cell cultures (Fig. 5). It is possible to speculate that this augment  
307 in DLG1 abundance may be a critical conserved viral activity in order to favor virus replication. Some  
308 function conservations between the E6/E7 proteins from high- and low-risk HPV types were shown  
309 (Pim and Banks, 2010) and probably E6/E7 from both HPV could induce molecular mechanisms  
310 involved in DLG1 levels regulation. In addition, infections by low-risk HPV types also induce  
311 unscheduled host DNA synthesis in a fraction of post mitotic, differentiated cells (Cheng et al., 1995,  
312 Jian et al., 1999, McCord, 2014). We observed that HPV-11 E6/E7 induced cyclin A and PCNA  
313 proteins expression in the epithelium suprabasal layer (Fig.3S), although to a lesser extent than that  
314 observed in HPV-18 cultures. It is possible that DLG1 expression and cell cycle progression may be  
315 functionally linked explaining the differences observed in the different conditions analyzed in this  
316 study.

317 How the abundance of low and high- risk HPV proteins could influence DLG1 expression pattern  
318 during viral cycle is still unknown. However, deregulated viral protein expression might have an  
319 impact on DLG1 expression. This is supported by the observation that in cervical cancer samples,  
320 where HPV oncoproteins expression is deregulated and no viral replication occurs, there is a dramatic  
321 reduction of DLG1 levels, when comparing with intraepithelial precursors lesions (Cavatorta et al.,

322 2004). Further studies are needed to clarify this important issue and for a complete comprehension of  
323 HPV associated pathogenesis.

324 In summary, the data from this study demonstrated that E7 and E6/E7 proteins derived from both  
325 HPV-11 and HPV-18 induce changes in the distribution and abundance of DLG1 polarity protein in  
326 organotypic cultures. The most significant difference was the DLG1 expression at cell contacts, which  
327 may reflect the difference in the pathologies associated with low- or high-risk HPV. Moreover,  
328 alterations in DLG1 expression was also observed in raft cultures bearing the entire HPV-18 genome.  
329 DLG1 protein expression is frequently altered in a variety of human cancers and the results presented  
330 here suggest a role for E7 and E6/E7 proteins in these changes for the HPV-associated neoplastic  
331 lesions.

332

### 333 **Methods**

#### 334 **Organotypic raft cultures**

335 Organotypic raft cultures were generated as described elsewhere (Boccardo et al., 2010). Low  
336 passage-pooled neonatal foreskin keratinocytes (LonzaWalkersville) were grown in serum-free  
337 medium (Invitrogen). Cells were infected with the recombinant retroviruses and after 24 h were  
338 selected with the correspondent antibiotic. After 2 days, when 100% of mocked infected controls  
339 were dead, infected cells were used to seed the epithelial raft. Recombinant retroviral vectors  
340 containing HPV-11 E7 and E7/E6 genes were kindly provided by Dennis J. McCance and are  
341 described elsewhere (Guess and McCance, 2005). Recombinant retrovirus vectors containing  
342 untraslated regulatory region (URR)-E7 or -E6/E7 sequences from HPV-18 are described elsewhere  
343 (Boccardo et al., 2004). After 10 days, organotypic raft cultures were harvested for protein or RNA  
344 analysis or fixed for histological investigation as described below.

345 Organotypic raft cultures from the keratinocyte cell line FKB18B, which harbors the HPV-18 full-  
346 length genome were established as previously described (Pinheiro et al., 2014). Briefly, PHK were  
347 transfected with HPV-18 full-length genome (FK18B) and grown during different passages before  
348 raft culture seeding (Steenbergen et al., 1998).

#### 349 **Cell culture, plasmids and transfection**

350 HEK293 epithelial cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented  
351 with 10% (v/v) fetal bovine serum (PAA). The respective HPV-18 E6 or E7 genes were cloned under  
352 the control of CMV promoter (Facciuto *et al.*, 2014). Plasmids expressing HPV-11 E6 or E7 proteins  
353 were kindly provided (Guess and McCance, 2005). Cells were transfected with the indicated  
354 constructions using calcium phosphate precipitation as described (Matlashewski et al., 1987).

#### 355 **Western Blotting**

356 Protein extraction from organotypic raft cultures was performed as previously reported (Boccardo et  
357 al., 2004) using extraction buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1mM EDTA, 0.5%  
358 NP40) containing protease inhibitors. HEK293 cells were lysed in ice-cold lysis buffer (50mM Tris-  
359 HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP40, 0.5% deoxycolate) containing Halt Protease  
360 Inhibitor single use cocktail (Thermo Scientific Pierce). Subsequently, WB experiments were carried  
361 out as described (Gardiol et al., 1999). Equal amounts of proteins were separated by SDS-PAGE and  
362 transferred to appropriate membranes. Specific protein levels were determined by immunoblot  
363 analysis using mouse monoclonal anti-DLG1 (2D11, Santa Cruz Biotechnology); mouse monoclonal  
364 anti p53 (DO-1, Santa Cruz Biotechnology); rabbit polyclonal anti pRb (M-153, Santa Cruz  
365 Biotechnology) and mouse monoclonal anti- $\gamma$  tubulin (T6557, Sigma Aldrich). The secondary  
366 antibody used was anti-mouse (NXA931; Amersham GE) coupled to horseradish peroxidase and  
367 detected by chemiluminescence, using the SuperSignal West Pico Chemiluminescent Substrate  
368 reagent (Thermo Scientific Pierce).  $\gamma$  -tubulin was measured as a loading control. Protein band  
369 intensities were quantitated using Image J quantification program.

### 370 **RNA isolation, cDNA synthesis and RT-PCR**

371 For testing E7 or E6 gene expression, total RNA was extracted using TRIzol Reagent (Life  
372 Technologies) following the manufacturer's instructions. cDNA was synthesized from 2  $\mu$ g of RNA  
373 using 200 U MMuLV RevertAid reverse transcriptase (Fermentas) and oligo(dT) primers. cDNA  
374 from each sample was subjected to PCR amplification with specific forward (F) and reverse (R)  
375 primers. HPV-11E6 F: 5'-TTATAGATCTATGGAAAGTAAAGATGCCTCC-3', R: 5'-  
376 TTATAACTTTTAGGGTAACAAGTCTTCCATG-3'; HPV-18E6 F: 5'-  
377 TTATAGATCTATGGCGCGCTTTGAGGATCCAAC-3', R: 5'-  
378 TTATAAGCTTTTATACTTGTGTTTCTCTGCGTC-3'; HPV-11E7 F: 5'-  
379 GTGGACAAACAAGACGCACAA-3', R: 5'-TGCCAGCAAAGGTCTTG TA-3'; HPV-18E7  
380 F: 5'-TGCATGGACCTAAGGCAACA-3', R: 5'-CTCGTCGGGCTGGTAAATGT-3'. Human  
381 succinate dehydrogenase gene (SDH), used as housekeeping marker, was amplified with SDH-F, 5'-  
382 GCACACCCTGTCCTTTGT-3' and SDH-R, 5'-CACAGTCAGCCTCGTTCA-3' primers.

### 383 **Immunohistochemistry**

384 Organotypic raft cultures were harvested, fixed in buffered formalin, embedded in paraffin, cut into  
385 3  $\mu$ m sections and mounted on pretreated glasses. Sections were stained with hematoxylin and eosin  
386 to observe histology. The immunohistochemistry assays were performed as described previously  
387 (Cavatorta et al., 2004, Gardiol et al., 2006). Briefly, the samples were deparaffinized in xylene and  
388 rehydrated using a graded alcohol series. Endogenous peroxidase activity was blocked by immersing

389 sections in 3% hydrogen peroxide in methanol for 20 min. Sections were placed in 10mM Tris-1mM  
390 EDTA buffer (pH 8.3) and heated for 12 min on high power using a conventional microwave oven,  
391 to facilitate antigen retrieval. Samples were allowed to cool down. After blocking nonspecific binding  
392 by addition of normal horse serum (Vectastain ABC Kit; Vector) for 40 min, sections were incubated  
393 with the respective primary antibody overnight at 4°C in a humid chamber. The primary antibodies  
394 anti-DLG1 (2D11, 1:40), anti-pRb (M-153, 1:200) and anti-p53 (DO-1, 1:50) were purchased from  
395 Santa Cruz Biotechnology. The primary anti-cyclin A (NCL-cyclin A) and anti-PCNA (#18-0110)  
396 were purchased from Novocastra and Zymed, respectively. For detection, samples were treated  
397 successively with biotinylated secondary antibody for 30 min and with avidin–biotin peroxidase  
398 complex for a further 30 min at room temperature (DAKO). The reaction was developed using a  
399 diaminobenzidine chromogenic substrate kit for peroxidase (Vector), and sections were  
400 counterstained with hematoxylin. Negative controls were processed as described, except that primary  
401 antibody was omitted.

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561



562 **Legend to Figures**

563 **Figure 1. HPV-18 oncoproteins redistribute and alter subcellular localization of DLG1 in**  
564 **organotypic raft cultures.**

565 a- Paraffin-embedded sections of the different organotypic rafts cultures were stained with  
566 hematoxylin and eosin in order to show the morphological details of the tissues. Scale Bar: 20  $\mu$ m.

567 b- HPV-18 E7 and E6 gene expression transcription was ascertained by RT-PCR with specific  
568 primers. – RT lanes demonstrate the absence of residual contaminating viral DNA in DNase-treated  
569 mRNA samples.

570 c- HPV-18 E7 and E6 functional protein expression was confirmed by ascertaining the level of the  
571 cellular targets, RB and p53, by Western Blot.  $\gamma$ -tubulin was used as loading control.

572 d- Analysis of the expression and localization of DLG1 in control, HPV-18 E7 and HPV-18 E6/E7  
573 raft cultures by immunohistochemistry. Representative paraffin-embedded sections of rafts  
574 immunostained with anti-DLG1 (brown staining) and counterstained with haematoxylin are shown.  
575 Blue arrows indicate DLG1 localization at the cell borders in control culture and some areas of the  
576 HPV-18 E7 raft. Red arrows show DLG1 misdistribution from the cell contacts to the cytoplasm in  
577 HPV-18 E7 and E6/E7 cultures. Scale Bar: 20  $\mu$ m.

578

579 **Figure 2. DLG1 pattern of expression is altered in organotypic cultures bearing the full-length**  
580 **HPV-18 genome.**

581 Analysis of the expression and localization of DLG1 in control and FKB18 (passage 27,p27) raft  
582 cultures by immunohistochemistry. Representative paraffin-embedded sections of rafts  
583 immunostained with anti-DLG1 (brown staining) and counterstained with haematoxylin are shown.

584 Blue arrows indicate DLG1 localization at the cell borders in control culture. Red arrows show DLG1  
585 misdistribution from the cell contacts to the cytoplasm in HPV-18 entire genome culture. Scale Bar:  
586 20  $\mu$ m.

587

588 **Figure 3. HPV-18 E7 and E6/E7 proteins induce an increase in DLG1 expression levels.**

589 a- Protein extracts from control and HPV-18 E7 or E6/E7 expressing organotypic cultures were  
590 analyzed by WB for DLG1 expression.

591 b- HEK293 cells were transfected with the corresponding empty or HPV-18 E7 or HPV-18 E6/E7  
592 expressing vectors. After 24 hs, cells were harvested and protein extracts were assessed by WB for  
593 DLG1 levels.

594 Numbers are folds of band intensity for DLG1 in rafts (a) or cells (b) expressing viral proteins, with  
595 respect to the corresponding control sample (considered as 1). Right panels, densitometry analysis of

596 Western blots for DLG1 (mean  $\pm$  SD,  $n \geq 3$ ) showing DLG1 level (DLG1/ $\gamma$ -Tubulin ratio) in rafts (a)  
597 or cells (b) expressing viral proteins, relative to each control sample, set as 1. The intensity of each  
598 band was normalized to  $\gamma$ -tubulin expression, used as loading control.

599

600 **Figure 4. HPV-11 E7 and E6/E7 proteins induce a redistribution of DLG1 along the squamous**  
601 **epithelium in organotypic raft cultures.**

602 a- HPV-11 E7 and E6/E7 induce a thickening of the stratified epithelium and mildly modify tissue  
603 morphology. Paraffin-embedded sections of the different organotypic raft cultures were stained with  
604 hematoxylin and eosin in order to show the morphological details of the tissues. Scale Bar: 20  $\mu$ m.

605 b- HPV-11 E7 and E6 gene expression was ascertained by RT-PCR with specific primers. –RT lanes  
606 demonstrate the absence of residual contaminating viral DNA in DNase-treated mRNA samples.

607 c- Analysis of the expression and localization of DLG1 in control, HPV-11 E7 and HPV-11 E6/E7  
608 raft cultures by immunohistochemistry. Representative paraffin-embedded sections of rafts  
609 immunostained with anti-DLG1 (brown staining) and counterstained with haematoxylin are shown.  
610 Blue arrows indicate DLG1 localization at the cell borders in control, HPV-11 E7 and HPV-11 E6/E7  
611 cultures. Red arrows show cytoplasmic DLG1 expression in HPV-11 E7 and HPV-11 E6/E7  
612 culture. Scale Bar: 20  $\mu$ m.

613

614 **Figure 5: HPV-11 E7 and E6/E7 proteins increase DLG1 expression levels.**

615 a- Protein extracts from epithelial control cultures and from rafts expressing HPV-11 E7 or E6/E7  
616 were analyzed by WB for DLG1 expression.

617 b- HEK293 cells were transfected with the corresponding empty vector or expressing HPV-11 E7 or  
618 HPV-11 E6/E7 proteins. After 24 hs, cells were harvested and protein extracts were assessed by WB  
619 for DLG1 levels.

620 Numbers are folds of band intensity for DLG1 in rafts (a) or cells (b) expressing viral proteins, with  
621 respect to the corresponding control sample (considered as 1). Right panels, densitometry analysis of  
622 Western blots for DLG1 (mean  $\pm$  SD,  $n \geq 3$ ) showing DLG1 level (DLG1/ $\gamma$ -Tubulin ratio) in rafts (a)  
623 or cells (b) expressing viral proteins, relative to each control sample, set as 1. The intensity of each  
624 band was normalized to  $\gamma$ -tubulin expression, used as loading control.

625

626