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

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

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ABSTRACT

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

INTRODUCTION

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

It is important to highlight that the presence of E6/E7 also promotes an increase in DLG1the nuclear localization. It was shown that in conditions where cell polarity can be altered, cell junction proteins are disassembled and can migrate to the nucleus and regulate transcriptional activity (Polette et al., 2005). Moreover, Narayan and colleagues (2009) have shown that DLG1 nuclear localization is highly dependent on phosphorylation by cycling kinases during cell cycle progression, and high-risk HPV oncoproteins are known to promote cell cycle entry even in differentiated cells (Fig. S3).

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

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

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

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

How the abundance of low and high- risk HPV proteins could influence DLG1 expression pattern during viral cycle is still unknown. However, deregulated viral protein expression might have an impact on DLG1 expression. This is supported by the observation that in cervical cancer samples, where HPV oncoproteins expression is deregulated and no viral replication occurs, there is a dramatic reduction of DLG1 1evels, when comparing with intraepithelial precursors lesions (Cavatorta et al.,

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

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

Methods

Organotypic raft cultures

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

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

Cell culture, plasmids and transfection

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

Western Blotting

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

RNA isolation, cDNA synthesis and RT-PCR

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

Immunohistochemistry

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

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

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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 μm.
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. γ-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 μm.
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 μm.
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

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

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

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

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

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

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

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

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

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