

PDZRN3/LNX3 Is a Novel Target of Human Papillomavirus Type 16 (HPV-16) and HPV-18 E6

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High-risk human papillomavirus (HPV) E6 proteins have a C-terminal PDZ binding motif through which they bind, and target for proteasome-mediated degradation, a number of PDZ-containing cellular targets. Recent studies have suggested that the RING-containing ubiquitin-protein ligase PDZRN3 might also be an HPV E6 target. In this analysis, we show that HPV-16 and HPV-18 E6 can target PDZRN3 in a PDZ- and proteasome-dependent manner and provide a connection between the HPV life cycle and differentiation-related STAT signaling.

High-risk human papillomaviruses (HPVs) are the causative agents of cervical cancer, other anogenital cancers, and some head and neck cancers (1, 2). The E6 oncoproteins of high-risk HPV types have a C-terminal PDZ-binding motif (PBM) that is important for a normal viral life cycle and progeny production (for a review, see reference 3). PBM deletion causes viral genome integration, loss of replicative competence, and an increased potential for oncogenic transformation (4–8).

E6 targets several PDZ-containing proteins (9–12), and recently, a proteomic screen of DNA tumor virus interactions (13) and an HPV-16 E6 C-terminal peptide screen of the human PDZome (14) both identified the E3 ubiquitin-protein ligase PDZRN3 (LNX3/SEMCAP3) (15) as a potential HPV-16 E6 binding partner.

This was particularly interesting because HPV replication re-

quires keratinocyte differentiation and PDZRN3 functions in the selection of stem cell differentiation pathways (16–19), inhibiting

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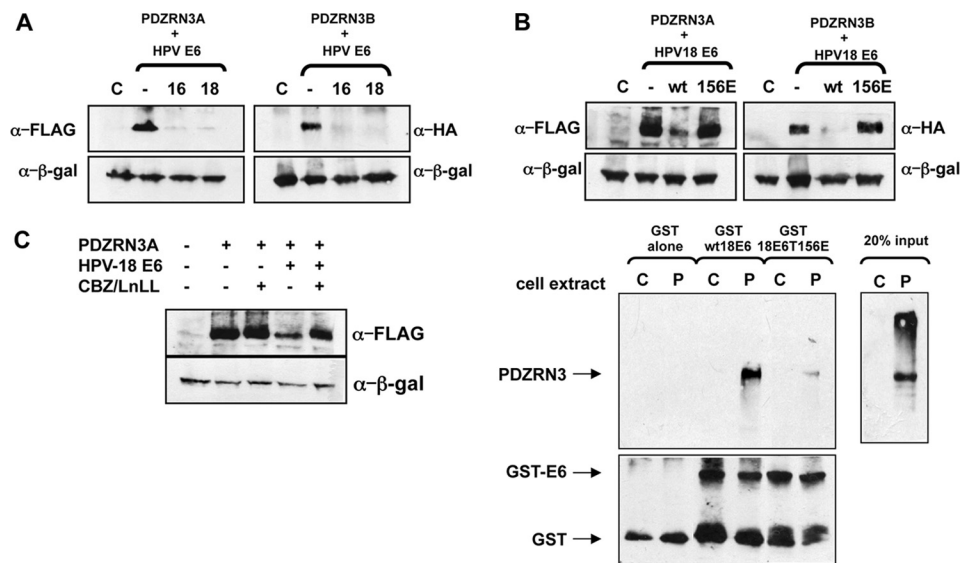
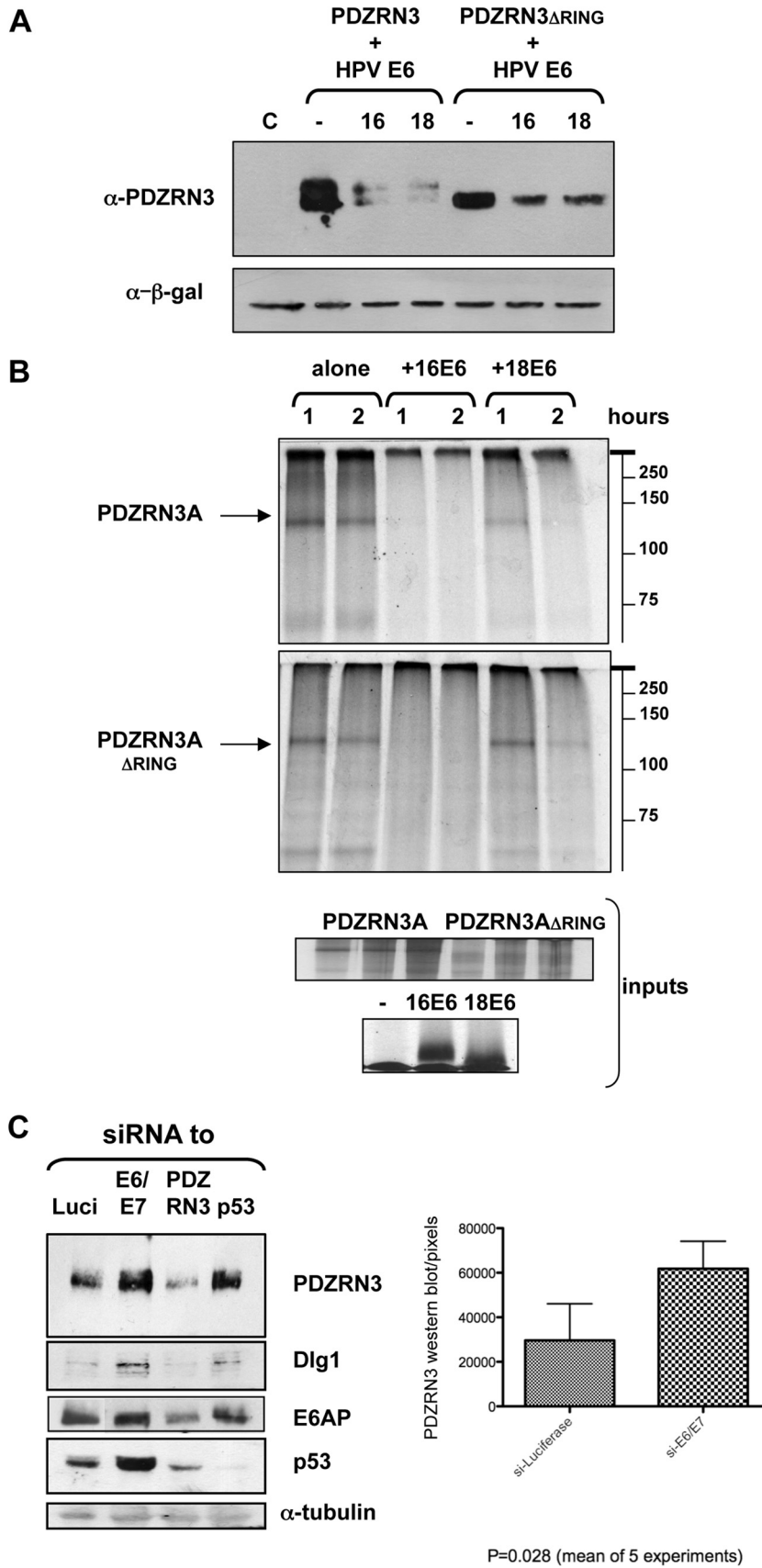


FIG 1 (A) HPV-16 and HPV-18 E6 proteins induce degradation of PDZRN3. Plasmids expressing FLAG-tagged PDZRN3A and HA-tagged PDZRN3B were transfected into 293 cells alone or with plasmids expressing HPV-16 or HPV-18 E6. Cell extracts were analyzed by Western blotting with anti-HA and anti-FLAG antibodies; β -galactosidase was included as a control for transfection efficiency. (B) HPV-18 E6-induced degradation of PDZRN3 is PDZ binding dependent. (Top) Plasmids expressing FLAG-tagged PDZRN3A and HA-tagged PDZRN3B were transfected into 293 cells alone or with plasmids expressing wild-type HPV-18 E6 or the PDZ binding-defective HPV-18 E6T156E mutant. Cell extracts were analyzed by Western blotting with anti-HA and anti-FLAG antibodies; β -galactosidase was included as a control for transfection efficiency. (Bottom) Extracts of 293 cells transfected with pCDNA3.1 (lanes C) or pCDNA-FLAG-PDZRN3A (lanes P) were incubated with GST, GST-HPV18E6, or GST-HPV18E6T156E, as indicated. After washing, the bound proteins were analyzed by a Western blot assay probed with anti-PDZRN3 and anti-GST antibodies to confirm equal loading of GST proteins. (C) HPV-18 E6-induced degradation of PDZRN3 is proteasome dependent. A plasmid expressing FLAG-tagged PDZRN3A was transfected into 293 cells alone or with an HPV-18 E6-expressing plasmid. After overnight incubation, the cells were treated with the proteasome inhibitors carbobenzoxy-Leu-Leu-leucinal (CBZ) and *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal for 3 h before harvesting. Cell extracts were analyzed by Western blotting with anti-FLAG antibody; β -galactosidase was included as a control for transfection efficiency.



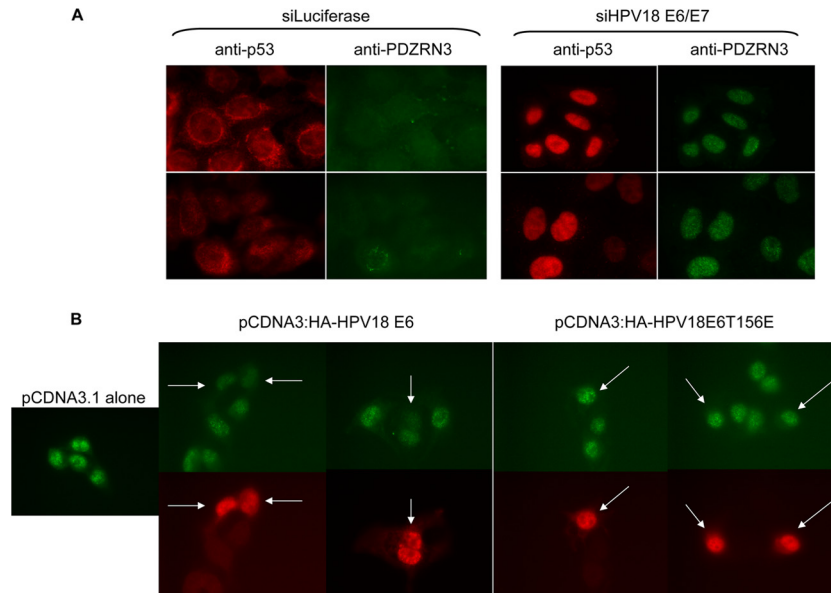


FIG 3 (A) Knockdown of HPV-18 E6 in HeLa cells increases nuclear PDZRN3 levels. HeLa cells were seeded onto coverslips and transfected with siRNA for luciferase or HPV-18 E6/E7 (Dharmacon). After 48 h, the cells were fixed, permeabilized, and then probed for p53 (red) and PDZRN3 (green). Each panel shows two different fields of view. (B) In C33A cells, transfection of HPV-18 E6 reduces nuclear PDZRN3 in a PBM-dependent manner. C33A cells were seeded onto coverslips and transfected with pCDNA3.1, pCDNA3:HA-HPV-18E6, or pCDNA3:HA-HPV18E6T156E. After 24 h, the cells were fixed, permeabilized, and then probed for HA (red) and PDZRN3 (green). Arrows indicate cells expressing E6 with a corresponding reduction in nuclear PDZRN3 with wild-type E6 but no change with the T156E mutant.

some but enhancing others and thus playing various roles in diverse differentiation pathways.

To determine whether PDZRN3 is an E6 target, we transfected 293 cells with pCDNA3:FLAG-PDZRN3A and pCDNA3:HA-PDZRN3B (isoforms of PDZRN3) plus pCDNA3:HPV-16E6 and pCDNA3:HPV-18E6 by Ca_2PO_4 precipitation (20). After 24 h, total protein was analyzed by SDS-PAGE and Western blotting with anti-hemagglutinin (HA; Roche), anti-FLAG (Stratagene), and anti- β -galactosidase (Promega) antibodies and a horseradish peroxidase-conjugated anti-mouse secondary antibody (Dako). The results in Fig. 1A show that PDZRN3 protein levels are reduced in the presence of HPV-16 and HPV-18 E6.

To determine whether the interaction is PDZ dependent, we transfected 293 cells with pCDNA3:FLAG-PDZRN3A and pCDNA3:HA-PDZRN3B alone or with pCDNA3:HPV-18E6 or pCDNA3:HPV-18E6T156E (a PBM-defective mutant [9]). After 24 h, the total protein was analyzed as before, and the results in Fig. 1B (upper panels) show that the PBM-defective mutant of HPV-18 E6 cannot induce PDZRN3 degradation. To confirm interaction, extracts from 293 cells transfected with pCDNA3:FLAG-PDZRN3A or pCDNA3 alone were used in a pull-down assay with glutathione *S*-transferase (GST)-HPV18E6 or GST-HPV18E6T156E. The results in the lower panels show that the PBM mutation abolishes interaction between E6 and PDZRN3.

To determine whether E6-induced degradation of PDZRN3 is

proteasome mediated, we transfected 293 cells with pCDNA3:FLAG-PDZRN3A with or without pCDNA3:HPV-18E6. After 24 h, the cells were treated with the proteasome inhibitors carboben-zoxy-Leu-Leu-leucinal (CBZ) and *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal for 3 h before the proteins were assayed as before. The results in Fig. 1C show that proteasome inhibitors reduce the destabilization of PDZRN3. Thus, HPV-18 E6 targets PDZRN3A for degradation via the proteasome.

The PDZRN3 RING domain may have a ubiquitin-ligase function; to investigate whether E6 uses this to induce PDZRN3 autodegradation, we transfected 293 cells with plasmids expressing wild-type and Δ RING mutant PDZRN3 with or without pCDNA3:HPV-16E6 or pCDNA3:HPV-18E6. After 24 h, the total protein was analyzed as before with an anti-PDZRN3 antibody (Santa Cruz). Figure 2A shows that mutant PDZRN3 Δ RING is degraded efficiently in the presence of HPV-16 and HPV-18 E6, suggesting that the RING domain is not required. Wild-type PDZRN3A appears to run as a doublet (also shown in Fig. 1C), but both bands are degraded equally in the presence of HPV-16 or HPV-18 E6. A degradation assay (Fig. 2B) with the radiolabeled, *in vitro*-translated PDZRN3 and HPV E6 proteins (TNT kit; Promega), showing that HPV-16 and HPV-18 E6 induces degradation of PDZRN3 Δ RING *in vitro*, confirms that the interaction is robust.

To determine whether HPV-18 E6 also targets endogenous PDZRN3, HPV-18-transformed HeLa cells, which continuously

FIG 2 The RING domain of PDZRN3 is not required for E6-induced degradation. (A) Plasmids expressing PDZRN3A and PDZRN3 Δ RING were transfected into 293 cells alone or with plasmids expressing HPV-16 or HPV-18 E6. Cell extracts were analyzed by Western blotting with an anti-PDZRN3 antibody (upper panel); β -galactosidase was included as a control for transfection efficiency (lower panel). (B) *In vitro*-translated radiolabeled PDZRN3A and PDZRN3 Δ RING (arrow) and HPV-16 or HPV-18 E6 were incubated together at 30°C for 1 and 2 h, as indicated, and then analyzed by SDS-PAGE and autoradiography; positions of molecular weight markers are shown on the right. The inputs are shown in the lower panels. (C) Knockdown of HPV-18 E6 in HeLa cells results in increased levels of endogenous PDZRN3. HeLa cells were transfected with siRNA to luciferase (Luci), HPV-18 E6/E7, PDZRN3, or p53 (Dharmacon), and total cell extracts were harvested at 48 h posttransfection. Cell extracts were analyzed by Western blotting for the proteins indicated; α -tubulin was included as a loading control. The histogram shows the collated results of five experiments quantitated with ImageJ software and subjected to statistical analysis by the Prism program.

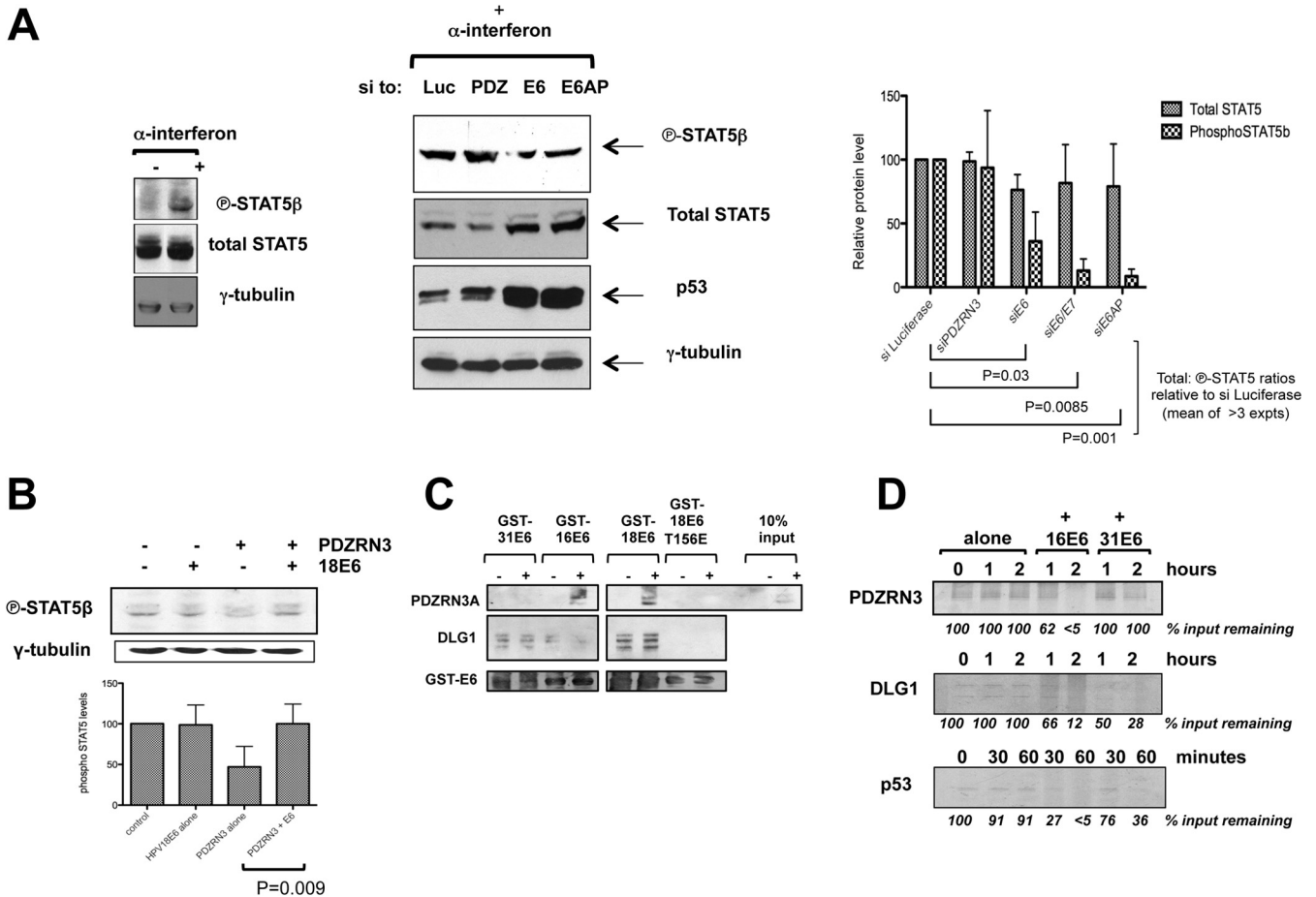


FIG 4 (A) Knockdown of HPV-18 E6 in HeLa cells decreases levels of phosphorylated STAT5β. HeLa cells were transfected with siRNA to luciferase, PDZR3, HPV18 E6, or E6AP, and total cell extracts were harvested at 72 h posttransfection. (Left panel) IFN-α treatment prior to harvesting specifically induces phosphorylation of STAT5β. (Right panel) Cell extracts were analyzed by Western blotting for the proteins indicated; γ-tubulin was included as a control. The histogram shows the collated results of at least three experiments quantitated with ImageJ software and subjected to statistical analysis by the Prism program. (B) Transfection of PDZR3 in 293 cells reduces levels of phosphorylated endogenous STAT5β, which is rescued by cotransfection of HPV-18 E6. HEK293 cells were transfected with plasmids expressing PDZR3 with or without plasmids expressing HPV-18 E6. Empty plasmid was transfected as a control. The cells were extracted in the presence of phosphatase inhibitors, and the extracts were analyzed by SDS-PAGE and Western blotting for the proteins indicated. The histogram shows the collated results of at least three experiments quantitated with ImageJ software and subjected to statistical analysis by the Prism program. (C) HPV-31 E6 binds to PDZR3 more weakly than HPV-16 or HPV-18 E6. Extracts from 293 cells transfected with pCDNA3.1 (–) or pCDNA-FLAG-PDZR3A (+) were incubated with GST-HPV31E6, GST-HPV16E6, GST-HPV18E6, or GST-HPV18E6T156E as indicated. After washing, the bound proteins were analyzed by a Western blot assay probed with anti-PDZR3 and anti-GST antibodies to confirm equal loading of GST proteins. The blot was reprobed for DLG1 to confirm GST-E6 integrity. (D) HPV-31 E6 is less efficient than HPV-16 E6 at inducing PDZR3 degradation *in vitro*. *In vitro*-translated PDZR3 was incubated at 30°C either alone or with *in vitro*-translated HPV-16 or HPV-31 E6 as indicated. The remaining PDZR3 was analyzed by SDS-PAGE and autoradiography. Dlg1 and p53 were included as controls. The values below the lanes are the percentages of the input protein remaining and are the mean results of two assays.

express E6 and E7, were transfected with small interfering RNAs (siRNAs; Dharmacon) to HPV-18 E6/E7 and PDZR3, using RNAiMax (Invitrogen), with siRNAs for luciferase and p53 as controls. After 48 h, cell extracts were analyzed by Western blotting as before. The results in Fig. 2C show that HPV-18 E6/E7 knockdown increases the levels of p53, Dlg1, and E6AP (mouse monoclonal antibodies from Santa Cruz and BD Biosystems, respectively). The PDZR3 levels also increase, indicating that endogenous PDZR3 is targeted for degradation in HPV-18 E6-positive cells. The results from five such assays were quantitated by ImageJ software and subjected to statistical analysis with the Prism program. The histogram in Fig. 2C shows a statistically significant ($P = 0.028$) 2-fold increase in PDZR3 levels in HeLa cells upon treatment with siRNA for HPV-18 E6/E7, relative to those treated with siRNA for luciferase.

To investigate whether specific pools of the protein are targeted by E6, HeLa cells were transfected with siRNA for luciferase or siRNA for HPV18E6/E7 and, after 48 h, subjected to immunofluorescence analysis, probing for PDZR3 and p53 as a positive control for HPV18E6/E7 silencing. The secondary antibodies were fluorescein isothiocyanate (FITC) and rhodamine conjugated (Invitrogen). The images in Fig. 3A, obtained with a Leica DMLB fluorescence microscope, show little PDZR3 staining in HeLa cells transfected with siRNA to luciferase. However, in HeLa cells transfected with siRNA to HPV18E6/E7 that show nuclear p53 staining (indicating HPV-18 E6 knockdown [21]), strong nuclear PDZR3 staining is also evident. Indeed, the strength of p53 staining, demonstrating the degree of E6 knockdown, also correlates with the strength of nuclear PDZR3 staining (siRNA for E6/E7, lower panels). To confirm that this effect on the nuclear

expression pattern of PDZRNA3 is mediated by the E6 PBM, we transfected HPV-negative C33A cells with either wild-type HA-HPV-18E6 or HA-HPV18E6T156E. The immunofluorescence analysis in Fig. 3B shows that the nuclear localization of PDZRN3 is disrupted by transfection of wild-type HPV18E6 but not by transfection of the T156E mutant.

HPV-18 E6 targeting of PDZRN3 suggests that PDZRN3 might inhibit viral replication. The HPV life cycle depends upon keratinocyte differentiation, and PDZRN3 can affect differentiation through the Wnt or STAT5 signaling pathway, respectively (17, 18). Since inhibition of STAT5 signaling has been shown to reduce the genome amplification of HPV-31 (22), we transfected HeLa cells with siRNAs to PDZRN3, HPV-18 E6, and HPV-18 E6/E7 or E6AP to examine the effects on STAT5 β activation. In parallel, cells were treated for 5 h with alpha interferon (IFN- α), which has been shown to induce the phosphorylation specifically of STAT5 β , but not STAT5 α , in HeLa cells (23). The proteins were then analyzed by Western blotting with rabbit monoclonal antibodies specific to phosphorylated (i.e., active) STAT5 β and to total STAT5 (Cell Signaling). The left panel of Fig. 4A shows that IFN- α increases the level of phosphorylated STAT5, indicating that it is indeed STAT5 β ; the right panel shows that knockdown of E6, E6/E7, or E6AP reduces phospho-STAT5 β levels, whereas PDZRN3 knockdown modestly increases phospho-STAT5 β levels. The results of at least three assays were analyzed as before, and the histogram shows a slight reduction in the total STAT5 levels and a significantly greater reduction in phospho-STAT5 β upon E6, E6/E7, or E6AP knockdown ($P = 0.03$, $P = 0.0085$, $P = 0.001$). Figure 4B shows that transfection of PDZRN3 into 293 cells decreases the phospho-STAT5 β level by half ($P = 0.011$), relative to that of vector alone, and this is rescued upon cotransfection of HPV-18 E6 ($P = 0.009$). Together, these results suggest that one downstream effect of HPV-18 E6-induced degradation of PDZRN3 is an increase in STAT5 β activation. In Fig. 2C, the p53 and Dlg1 levels appear to be reduced upon PDZRN3 knockdown; STAT5 activation has been shown to affect p53 function, and it is tempting to speculate that this might be the case here.

We have shown that high-risk HPV E6 proteins target PDZRN3 for PDZ-dependent, proteasome-mediated degradation, validating previous proteomic analyses (13, 14), and that HPV-18 E6-induced PDZRN3 degradation can lead to an increase in STAT5 β activation. In HPV-31, E7 was shown to activate STAT5 directly and E6 was not required (22). However, different HPV types are known to differ in the strategies employed to achieve the same state (24–26). To address this point, extracts from 293 cells transfected with pCDNA3: FLAG-PDZRN3A or pCDNA3 alone were used in a pull-down assay with GST-HPV31E6, GST-HPV16E6, GST-HPV18E6, or GST-HPV18E6T156E. The Western blot analysis in Fig. 4C shows that PDZRN3 binds much more weakly to GST-HPV31E6 than to GST-HPV16 or GST-HPV18E6, whereas the binding to endogenous DLG1 is equivalent, showing that the GST-HPV31E6 PBM is functional. Figure 4D also shows that HPV-31 E6 is much less efficient than HPV-16 E6 at inducing PDZRN3 degradation *in vitro*.

Thus, it seems possible that different HPV types may activate the PPAR γ pathway by activating STAT5 β through E7 in some cases and by degrading its inhibitor, PDZRN3, through E6 in others.

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