Protein tyrosine phosphatase H1 is a target of the E6 oncoprotein of high-risk genital human papillomaviruses

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The E6 proteins of high-risk genital human papillomaviruses (HPV), such as HPV types 16 and 18, possess a conserved C-terminal PDZ-binding motif, which mediates interaction with some cellular PDZ domain proteins. The binding of E6 usually results in their ubiquitin-mediated degradation. The ability of E6 to bind to PDZ domain proteins correlates with the oncogenic potential. Using a yeast two-hybrid system, GST pull-down experiments and coimmunoprecipitations, we identified the protein tyrosine phosphatase H1 (PTPH1/PTPN3) as a novel target of the PDZ-binding motif of E6 of HPV16 and 18. PTPH1 has been suggested to function as tumour suppressor protein, since mutational analysis revealed somatic mutations in PTPH1 in a minor fraction of various human tumours. We show here that HPV16 E6 accelerated the proteasome-mediated degradation of PTPH1, which required the binding of E6 to the cellular ubiquitin ligase E6-AP and to PTPH1. The endogenous levels of PTPH1 were particularly low in HPV-positive cervical carcinoma cell lines. The reintroduction of the E2 protein into the HPV16-positive cervical carcinoma cell line SiHa, known to lead to a sharp repression of E6 expression and to induce growth suppression, resulted in an increase of the amount of PTPH1. Our data suggest that reducing the level of PTPH1 may contribute to the oncogenic activity of high-risk genital E6 proteins.

INTRODUCTION

Human papillomaviruses (HPV) are small DNA tumour viruses infecting the basal cells of the skin or mucosa. The resulting tumours are initially benign, however, infections with some HPV types imply a high risk to undergo malignant conversion. A subset of mucosotropic HPVs, including HPV16 and 18, are associated with cervical cancer (Munoz, 2000). In these cancers, the expression of the two viral oncogenes E6 and E7 is usually upregulated. E6 and E7 transform cells by interfering with cell-cycle regulation and counteracting apoptosis (reviewed by Longworth & Laimins, 2004; Münger *et al.*, 2004). High-risk genital HPV E6 proteins target a number of cellular proteins for proteasome-mediated degradation, including p53. In complex with the cellular ubiquitin-protein isopeptide ligase E3, called E6-AP, E6 binds to the tumour suppressor p53, which leads to its ubiquitination and degradation (reviewed by Scheffner & Whitaker, 2003). The elimination of the function of p53 significantly contributes to E6-mediated oncogenesis by increasing the risk of accumulation of mutations. However, high-risk

genital E6 have oncogenic activities independent of targeting p53 (Kiyono *et al.*, 1997, 1998; Liu *et al.*, 1999). High-risk genital E6 proteins bind to PDZ (PSD95/Dlg/ ZO-1) domain proteins via four conserved C-terminal amino acids encoding a PDZ-binding motif (Songyang *et al.*, 1997). E6 proteins reveal specificity for particular PDZ domains; however, the molecular basis for this specificity is unknown (Thomas *et al.*, 2001). To date, several cellular PDZ domain proteins such as hDlg, hScribble, MUPP1 and MAGI-1-2-3 have been identified as targets of high-risk HPV E6. Most of these factors are prone to ubiquitin-mediated proteolysis (reviewed by Scheffner & Whitaker, 2003). The PDZ-binding motif of E6 proteins is essential for transformation of established rodent cell lines (Kiyono *et al.*, 1997) and for the induction of epithelial hyperplasia in transgenic mice expressing HPV16 E6 (16E6) (Nguyen *et al.*, 2003; Simonson *et al.*, 2005), indicating that the interaction with and presumably the degradation of the PDZ domain proteins contributes to the oncogenic potential of E6. However, the biologically relevant targets involved in this process still have to be determined.

Phosphorylation of tyrosine residues controlled by protein tyrosine kinases (PTKs) and phosphatases (PTPs) is a

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central feature of many cellular signalling pathways, including those affecting growth, differentiation, cell-cycle regulation, apoptosis and invasion (reviewed by Ostman et al., 2006). Although a variety of PTK genes have been directly linked to tumorigenesis through somatic activating mutations, only a few PTP genes have been implicated in cancer. A comprehensive mutational analysis of the PTP gene superfamily identified somatic mutations in six PTP genes in 26 % of colorectal cancers and occasionally in other cancers. Expression of wild-type, but not mutated, tyrosine phosphatases inhibited growth of cancer cells, indicating that the tyrosine phosphatases are tumour suppressors. Among them was PTPH1, also named PTPN3 (Wang et al., 2004). PTPH1 belongs to the subfamily of the non-receptor PTPs and encodes within the N terminus a region with homology to a band 4.1 ezrin radixin moesin (FERM) domain, responsible for targeting proteins to the cytoskeleton–membrane interface. PTPH1 has a C-terminal catalytic phosphatase and one central PDZ domain (Arpin et al., 1994; Yang & Tonks, 1991; Zhang et al., 1995). Overexpression of PTPH1 resulted in growth inhibition of NIH3T3 cells, which may rely on the dephosphorylation of the cell-cycle regulator VCP (valosin-containing protein) (p97/CDC48), one of the few substrates of PTPH1 identified (Zhang et al., 1999).

Here, we report the identification of PTPH1 as a target protein of the PDZ-binding motif of 16E6 by a yeast two-hybrid system. We demonstrate that the interaction induced the degradation of PTPH1 in vivo and in vitro, which required the binding of E6 to the ubiquitin ligase E6-AP as well. We thus confirm observations made by Jing et al. (2007), who identified PTPH1/PTPN3 as a target of the 16E6–E6-AP complex by a substrate trapping assay. Beyond that, we show that the levels of endogenous PTPH1 were particularly low in HPV-positive cervical cancer-derived cell lines that express E6. Overexpression of the HPV16-encoded E2 protein, a repressor of the expression of E6, led to increased PTPH1 concentrations in HPV16-positive SiHa cells, supporting the notion that 16E6 degrades endogenous PTPH1 in these cells. Moreover, the E6 protein of high-risk cutaneous HPV8 was also able to bind to PTPH1, implying that targeting of PTPH1 may be conserved among high-risk E6 proteins.

METHODS

Cells and tissue culture. HPV-negative C33A cells and HPV-positive cervical carcinoma-derived cell lines SiHa, Caski and HeLa were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FCS and antibiotics. RTS3b cells (Purdie et al., 1993) were grown in E-medium. C33A and SiHa cells were transfected by the $CaPO_4$ procedure as described previously.

Expression vectors. PCR products were cloned into the vector pGEX2T (Promega) to obtain GST–16E6-C and GST–16E6-N and into pcDNAFLAG3.1 (Müller-Schiffmann et al., 2006) to express 8E6, 16E6 and truncated versions thereof with an N-terminally fused FLAG epitope. pcDNA-FLAG16E6Δ123-126 and pcDNA-FLAG-16E6F47R were obtained by site-directed in vitro mutagenesis. The

PTPH1-encoding insert isolated from the yeast plasmid pACT-PTPH1 was cloned into the vectors pCMV-Myc (Clontech), pcDNA3.1-FLAG and pGEX-5X (Promega). Myc–PTPH1ΔPDZ, lacking the amino acids 504–598 was obtained by in vitro mutagenesis and HA–PTPH1+Ex12 (haemagglutinin-conjugated) was described by Zhang et al. (1997). The ORF for FLAG–p53 was isolated from pBS-FLAG-p53 and cloned into pcDNA 3.1. Myc–NAP-1 expression vector has been described by Rehtanz et al. (2004) and expression vectors for HA–16E6, HA–E6-AP and HA–E6-APΔBS have been described by Kuballa et al. (2007).

Protein–protein interaction studies. GST pull-down assays were performed according to Müller et al. (2002) and coimmunoprecipitations were described by Rehtanz et al. (2004). Endogenous PTPH1 was detected by Western blot with the monoclonal antibody 2-117, kindly provided by N. Tonks (Zhang et al., 1997, 1999). For immunoprecipitations to detect endogenous PTPH1, 1.2 mg of total cell extracts, prepared as described by Rehtanz et al. (2004), was incubated with 5 μl of the antibody 2-117 for 1 h at 4 °C; antigen–antibody complexes were collected with protein-A-Sepharose followed by Western blotting.

In vitro and in vivo degradation assays. Proteins were translated using the Promega TNT-coupled transcription/translation system in the presence of ^{35}S-Cys and mixed in a ratio of 2 : 3 PTPH1 or p53 and the respective E6 protein. Reactions were incubated at 30 °C for the indicated time before analysis. In order to investigate the in vivo degradation, 15 μg C33A cells extracts, prepared 24 h after transfection, were subjected to Western blotting with the anti-Myc, anti-HA (both Roche Diagnostics) or the anti-p53 (DO-1; Santa Cruz Biotechnology) antibodies to detect the respective proteins.

RESULTS

PTPH1 binds to 16E6

To search for cellular factors targeted by the PDZ-binding motif of 16E6, we performed a classical yeast two-hybrid screen with the 12 most C-terminal amino acids of 16E6, including the PDZ-binding motif as bait and a cDNA library derived from the keratinocyte cell line HaCat. In addition to the cellular factors hScrib, hDlg and Tip-1, all known as targets of the PDZ-binding motif of 16E6 (Hampson et al., 2004; Kiyono et al., 1997; Nakagawa & Huibregtse, 2000), we isolated 24 cDNA clones containing the open reading frame for PTPH1 (data not shown). The interaction between PTPH1 and 16E6 was confirmed in vitro by GST pull-down experiments. As shown in Fig. 1(a), GST–16E6-C (from amino acids 85 to 151) efficiently retained ^{35}S-labelled PTPH1 in contrast to GST–16E6-N (from amino acids 1 to 84) or GST, indicating that PTPH1 interacts with the C-terminal half of 16E6 in vitro. GST–PTPH1 was able to precipitate ^{35}S-labelled 16E6, in contrast to 16E6ΔETQL, a 16E6 protein lacking the C-terminal four amino acids ETQL, the PDZ-binding motif. An internal deletion mutant, 16E6Δ123-126, which is defective in degradation of p53 (Foster et al., 1994), was still able to bind to PTPH1 (Fig. 1a). Thus, PTPH1 binds to the C-terminal four amino acids of 16E6.

The interaction also occurs within the cell, as demonstrated by coimmunoprecipitations. The HPV-negative cervical cancer cell line C33A was transiently transfected with
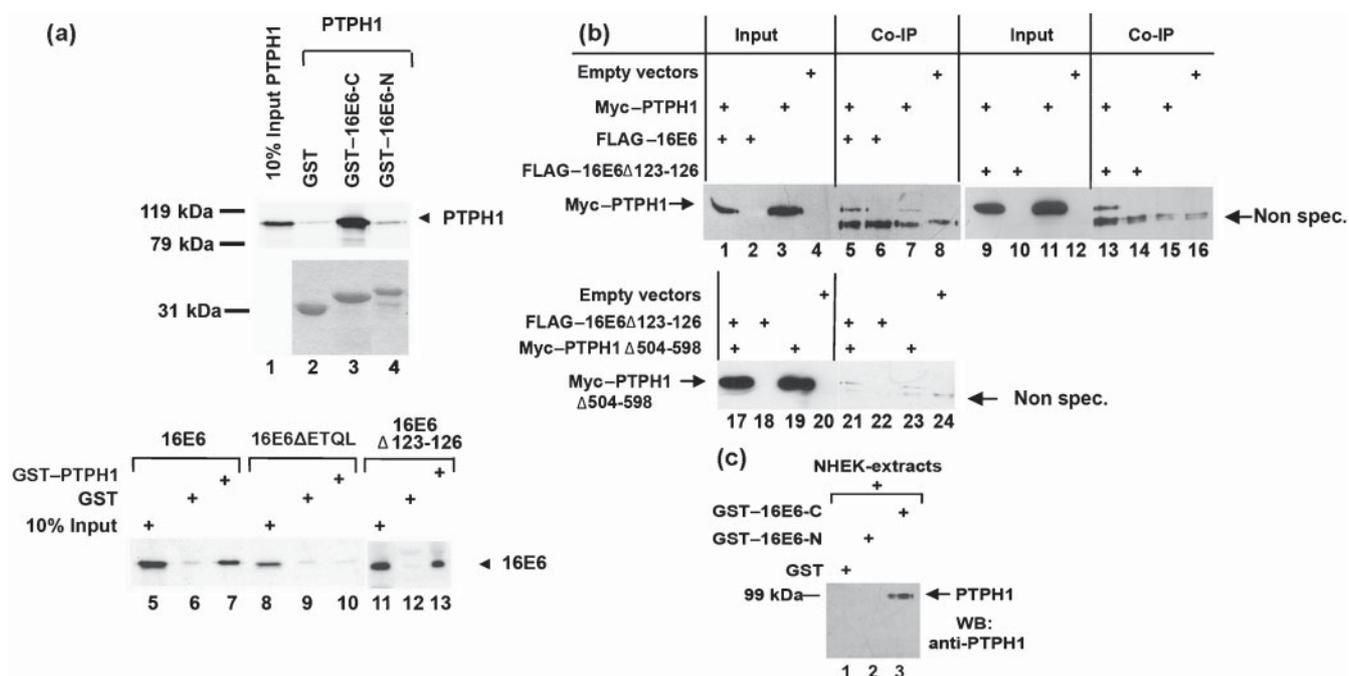
Fig. 1. 16E6 binds to PTPH1 *in vitro* and *in vivo*. (a) GST pull-down assays. GST (lane 2), GST–16E6-C (amino acids 85–151) (lane 3) and GST–16E6-N (amino acids 1– 84) (lane 4) were incubated with ^{35}S-labelled *in vitro*-translated PTPH1. Lane 1 was loaded with 10 % of its input . An SDS-polyacrylamide gel stained with Coomassie blue showing the corresponding GST proteins used for the interaction assay is shown beneath the interaction assay. In lanes 5–13, GST or GST–PTPH1 were incubated with ^{35}S-labelled *in vitro*-translated 16E6, 16E6ΔETQL and 16E6Δ123-126. In lanes 5, 8, and 11, 10 % of the input of each *in vitro*-translated 16E6 version was loaded. The positions of bound *in vitro*-translated PTPH1 (upper part) or 16E6 (lower part) and some marker proteins are indicated. (b) Coimmunoprecipitation. Extracts from C33A cells, transiently transfected with empty vectors or expression vectors for Myc-tagged PTPH1 (upper part) or Myc–PTPH1Δ504-598, lacking the PDZ domain (lower part), and FLAG–16E6 or FLAG–16E6Δ123-126 in different combinations, as indicated in the figure, were incubated with anti-FLAG-M2 affinity beads. Bound Myc–PTPH1 as well as PTPH1Δ504-598 was detected in a subsequent Western blot developed with the anti-Myc antibody. To verify the presence of Myc–PTPH1, an aliquot of the extract was used in a Western blot as an input control (Input). (c) 16E6 binds to endogenous PTPH1. Extract prepared from primary keratinocytes (NHEK) were incubated with GST, GST–16E6-C or GST–16E6-N. Bound endogenous PTPH1 was detected by a Western blot developed with an antibody directed against PTPH1.

expression vectors encoding FLAG-tagged 16E6 and Myc-tagged PTPH1. When coexpressed with FLAG–16E6, Myc–PTPH1 was recovered in FLAG–16E6 immunoprecipitates. In contrast, immunoprecipitates from cells which did not express FLAG–16E6 did not contain PTPH1. In addition, FLAG–16E6Δ123-126 was able to specifically precipitate Myc–PTPH1 (Fig. 1b). The PDZ domain is a specific type of protein–protein interaction module with a structurally well defined interaction 'pocket' that can be filled by a PDZ-motif 'ligand' (Dev, 2004). This mode of association seems to occur in the case of the interaction between 16E6 and PTPH1, since FLAG–16E6Δ123-126 failed to precipitate Myc–PTPH1Δ504-598, with a deletion of its PDZ domain (Fig. 1b). Moreover, GST–16E6-C specifically retained endogenous PTPH1 from extracts of primary keratinocytes (Fig. 1c). Taken together, 16E6 binds via its C-terminal PDZ-binding motif to the PDZ domain of PTPH1 *in vitro* and *in vivo*.

Binding of E6 to PTPH1 enhances its degradation

The interaction of 16E6 with its PDZ partners usually leads to their accelerated degradation (Kiyono *et al.*, 1997; Massimi *et al.*, 2004), although exceptions have been described (Hampson *et al.*, 2004). To test whether 16E6 induces the degradation of PTPH1, *in vitro* degradation assays were performed by coincubating ^{35}S-labelled 16E6 and PTPH1, both obtained by *in vitro* translation via a rabbit reticulocyte lysate, for various time points. As indicated in Fig. 2(a), the amount of PTPH1 gradually decreased with increasing incubation time only in the presence of 16E6. After 2 h, the PTPH1 specific band disappeared, demonstrating that 16E6 reduced the stability of PTPH1. The kinetic of the 16E6-mediated degradation of PTPH1 was comparable to that of p53 (Fig. 2a, lanes 7–12). The targeting of PTPH1 was essential for degradation, since the stability of 16E6-binding deficient
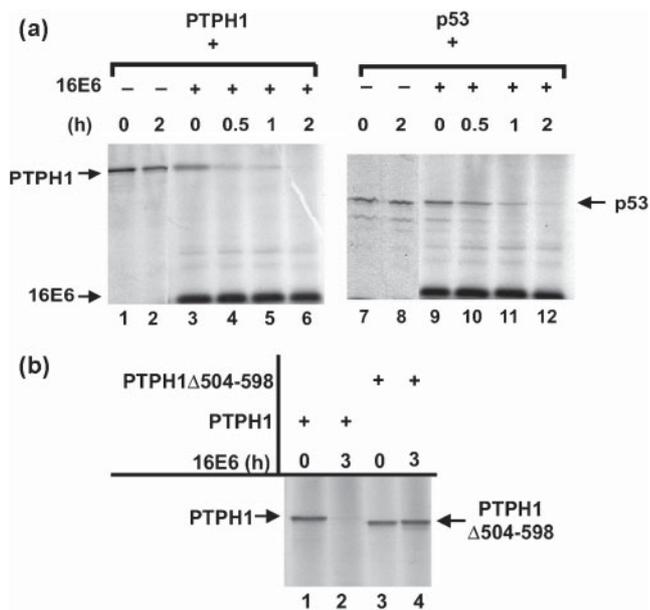
Fig. 2. 16E6 induces the accelerated degradation of PTPH1 *in vitro*. (a) *In vitro* degradation assay. *In vitro*-translated, ^{35}S-labelled PTPH1 (lanes 1–6) or p53 (lanes 7–12) were incubated for various time points, as indicated in the figure, either in the absence or in the presence of ^{35}S-labelled *in vitro*-translated 16E6 before analysis by SDS-PAGE, followed by autoradiography. The positions of PTPH1, p53 and 16E6 are indicated. (b) The PDZ domain of PTPH1 is required for degradation. A similar experiment as in (a) was performed with the exception that either PTPH1 or PTPH1Δ504-598 were incubated for 0 and 3 h with ^{35}S-labelled 16E6.

PTPH1Δ504-598, with the deletion of the PDZ domain, was not affected after 3 h incubation with 16E6 (Fig. 2b).

To gain insights into the sequence requirements of 16E6, the capacity of different E6 mutants to degrade PTPH1 was analysed *in vitro* and *in vivo*. For *in vivo* degradation assays, C33A cells were cotransfected with expression vectors for Myc–PTPH1 and for the various 16E6 mutants, respectively. To exclude variations of the Myc–PTPH1 levels due to different transfection efficiencies, we included an expression vector for Myc-tagged nucleosome assembly protein-1 (NAP-1), which was not affected by 16E6, as internal control. As shown in Fig. 3(a, b), the binding of 16E6 to PTPH1 is a prerequisite to induce the degradation of PTPH1, since 16E6ΔETQL failed to reduce the amount of PTPH1 *in vitro* and *in vivo*. The PDZ-binding motif of 16E6 (ETQL) differs slightly from the consensus PDZ-motif, which is S/TXV (Nourry *et al.*, 2003). Replacing the C-terminal L by V, giving rise to 16E6+ETQV, resulted in a more potent inducer of PTPH1 degradation *in vitro* and *in vivo*. This finding supports the importance of targeting 16E6 to PTPH1 via the PDZ-binding motif. Although interacting with PTPH1 (Fig. 1b), the C-terminal moiety of 16E6 (amino acids 85–151) was not sufficient to reduce the

level of PTPH1, independently of whether the ETQL was present or not (Fig. 3a, b). The proteasomal degradation of many cellular partner proteins bound by the PDZ-binding motif requires the complex formation of E6 with E6-AP, although other ubiquitin ligases may be involved as well, as suggested for hDlg (Pim *et al.*, 2000). To address the question whether E6-AP is the ubiquitin ligase mediating the proteasomal degradation of PTPH1, we tested two known 16E6 mutants, differing in binding to E6-AP. The deletion of amino acids 123–126 abolished interaction with E6-AP, the degradation of p53 and conferred a defect in immortalization (Foster *et al.*, 1994; Gewin *et al.*, 2004; Kiyono *et al.*, 1998; Klingelhutz *et al.*, 1996; Liu *et al.*, 1999). This deletion eliminated the capacity of 16E6 to accelerate the degradation of PTPH1 (Fig. 3a, b). The second mutant we used was 16E6F47R, described by Nomine *et al.* (2006). The exchange of F to R at position 47 did not affect the binding to E6-AP, but eliminated the capacity to induce the degradation of p53 by a defect in recruiting p53 to the complex 16E6–E6-AP (Nomine *et al.*, 2005, 2006; Zanier *et al.*, 2005). This was analysed in the context of the additional mutation of the C in position 6 to S (Nomine *et al.*, 2006). We observed that in the context of the wild-type 16E6, the mutation F47R abrogated the ability to degrade p53 as well (data not shown), but did not affect the degradation of PTPH1 *in vitro* and *in vivo* (Fig. 3a, b). Thus, there is a correlation between binding of 16E6 to E6-AP and the ability to induce the degradation of PTPH1. We also determined the effect of the proteasome inhibitor MG132 upon PTPH1 protein levels in the presence of 16E6 in transiently transfected C33A cells. PTPH1 was stabilized upon treatment with MG132, indicating that PTPH1 is normally degraded by the proteasome. In the presence of 16E6, a dramatic decrease of the level of PTPH1 could be observed, which was restored when the cells were cultured in the presence of the proteasome inhibitor (Fig. 3b, lanes 8–11), supporting the notion that the proteasome is involved in the 16E6-mediated degradation of PTPH1.

This was further confirmed by the use of the stable H1299-K3 cell line, in which E6-AP expression was stably suppressed by RNA-interference, as described by Kuballa *et al.* (2007). While parental H1299 cells efficiently supported the degradation of PTPH1 by 16E6, this was markedly reduced in H1299-K3 cells. The ability of 16E6 to degrade PTPH1 in H1299-K3 cells was restored after overexpression of an E6-AP protein that was resistant to the siRNA (small interfering RNA) used to abrogate expression of endogenous E6-AP (see Kuballa *et al.*, 2007). The deletion of amino acids 378–395 of E6-AP (E6-APΔBS), encoding the E6-binding site, failed to rescue the degradation of PTPH1 (Fig. 3c). The differences in the amounts of PTPH1 protein were not due to different expression levels of 16E6, as shown by the Western blot in Fig. 3(c). Taken together, the proteasomal degradation of PTPH1 by 16E6 requires the binding of E6 to the ubiquitin ligase E6-AP as well as to PTPH1.

During our studies we realized that all 24 cDNAs for PTPH1, isolated by the initial yeast two-hybrid screen, lacked amino acids 334–379. This segment is located between the band 4.1-FERM homology region and the
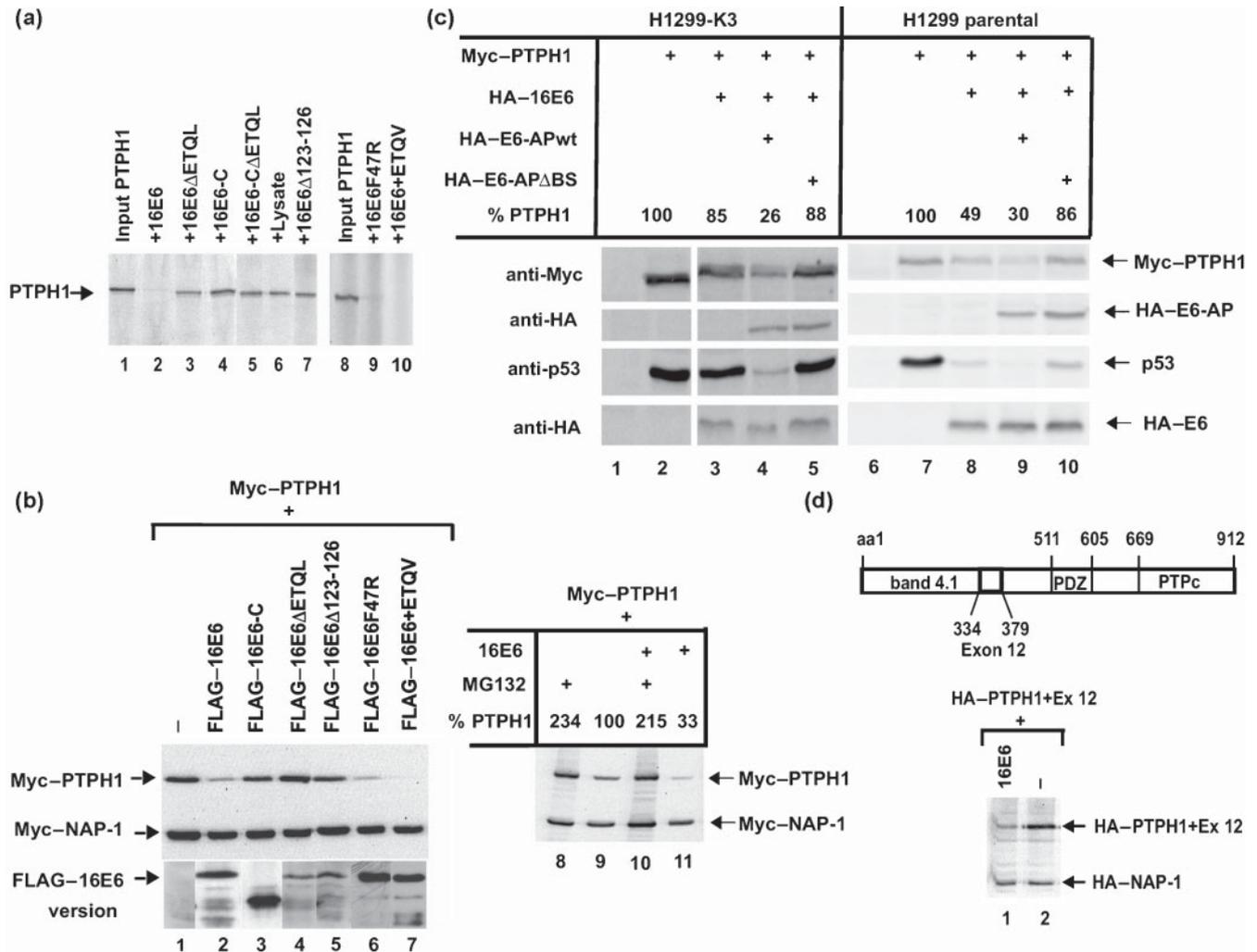

Fig. 3. The degradation of PTPH1 requires the interaction between 16E6 and PTPH1 and involves the proteasome. (a) *In vitro* degradation assay with ^{35}S-labelled, *in vitro*-translated PTPH1 and 16E6 or various mutants thereof. The presence of PTPH1 was analysed by autoradiography after overnight incubation of the proteins. (b) *In vivo* degradation assays. An expression vector for Myc–PTPH1 was cotransfected with expression vectors for FLAG–16E6 or various mutants of 16E6, as indicated in the figure (lanes 1–7). An expression vector for Myc–hNAP-1 was included as internal transfection control. Myc–PTPH1 and Myc–NAP-1 were detected by Western blot developed with the anti-Myc antibody. To analyse the expression of the various E6 proteins, extracts of the transfected C33A cells were incubated with FLAG affinity matrix and bound E6 proteins were detected by Western blot developed with the anti-FLAG antibody. In lanes 8–11, transfected cells, as indicated in the figure, were incubated in the presence of either 25 μM MG132 (lanes 8, 10) or solvent (lanes 9, 10) before harvesting and analysis as above. The percentage of PTPH1 was quantified. (c) Parental H1299 cells (lanes 6–10) or H1299-K3 cells (lanes 1–5), in which the expression of E6-AP had been stably suppressed by RNA interference (Kuballa *et al.*, 2007), were transiently transfected with expression vectors for Myc–PTPH1, HA–16E6, HA–E6-AP wild-type (wt) or E6-APΔBS, with a deletion of the E6-binding site from amino acids 378–395 (Kuballa *et al.*, 2007), in different combinations, as indicated in the figure. The presence of Myc–PTPH1, p53, HA–16E6 and HA–E6-AP in the extracts was analysed by Western blot developed with the anti-Myc, -HA or -p53 antibodies. The signals corresponding to Myc–PTPH1 were quantified and the ratios are given. (d) 16E6 also degrades PTPH1+Ex12. *In vivo* degradation assay with extracts from C33A cells transiently transfected with expression vectors for HA-tagged PTPH1+Ex12, HA-NAP-1 and FLAG–16E6. HA–PTPH1+Ex12 and HA–NAP-1 were detected by Western blot. The structure of PTPH1, with the positions of the region homologous to band 4.1, the PDZ domain and the phosphatase (PTPc) domain, as well as exon 12, is shown.

PDZ domain (for overview, see Fig. 3d), but does not exhibit structural similarity to any known protein motifs. The 45 amino acids lacking in all our clones are exactly encoded by exon 12 (data not shown), as revealed by a BLAST search. The mRNA for PTPH1 contains 24 exons. Thus, our cDNAs for PTPH1 presumably represent an alternative splice product. The full-length HA-tagged PTPH1, HA–PTPH1+Ex12 (Zhang *et al.*, 1997), was degraded by 16E6 as was wild-type (Fig. 3d), demonstrating that the amino acids encoded by exon 12 do not affect the interaction between 16E6 and PTPH1.

The targeting of PTPH1 is conserved among E6 proteins

As already mentioned, the PDZ-binding motif is conserved among E6 proteins of high-risk genital HPVs and, as expected, HPV18 E6 was able to induce the degradation of PTPH1 *in vitro* and *in vivo* (Fig. 4a). In contrast, high-risk cutaneous E6 proteins do not encode a C-terminal

PDZ-binding motif, although possessing transforming activity as well, as shown in the case of the high-risk cutaneous HPV8 (Iftner *et al.*, 1988; Kiyono *et al.*, 1992). Moreover, HPV8 E6 (8E6) does not bind to and induce the degradation of p53 (Kiyono *et al.*, 1992; Steger & Pfister, 1992). Surprisingly, 8E6 specifically precipitated coexpressed Myc–PTPH1 (Fig. 4b). While 8E6-N (from amino acid 1 to 93) interacted with PTPH1, 8E6-C (amino acids 94–155) failed to do so, indicating that the N-terminal moiety was responsible for the binding. In correlation, 8E6Δ132-136, deficient in interaction with the cellular coactivator p300 (Müller-Schiffmann *et al.*, 2006), precipitated PTPH1 as well. Although the binding of C-terminal PDZ motifs to PDZ domains appears to be the typical mode of interaction, it has been described that PDZ domains can contact internal motifs as well (Hung & Sheng, 2002; Nourry *et al.*, 2003). However, PTPH1Δ504-598, lacking the PDZ domain, still interacted with 8E6, as demonstrated in the coimmunoprecipitation shown in Fig. 4(b). Obviously, the interaction between PTPH1 and 8E6
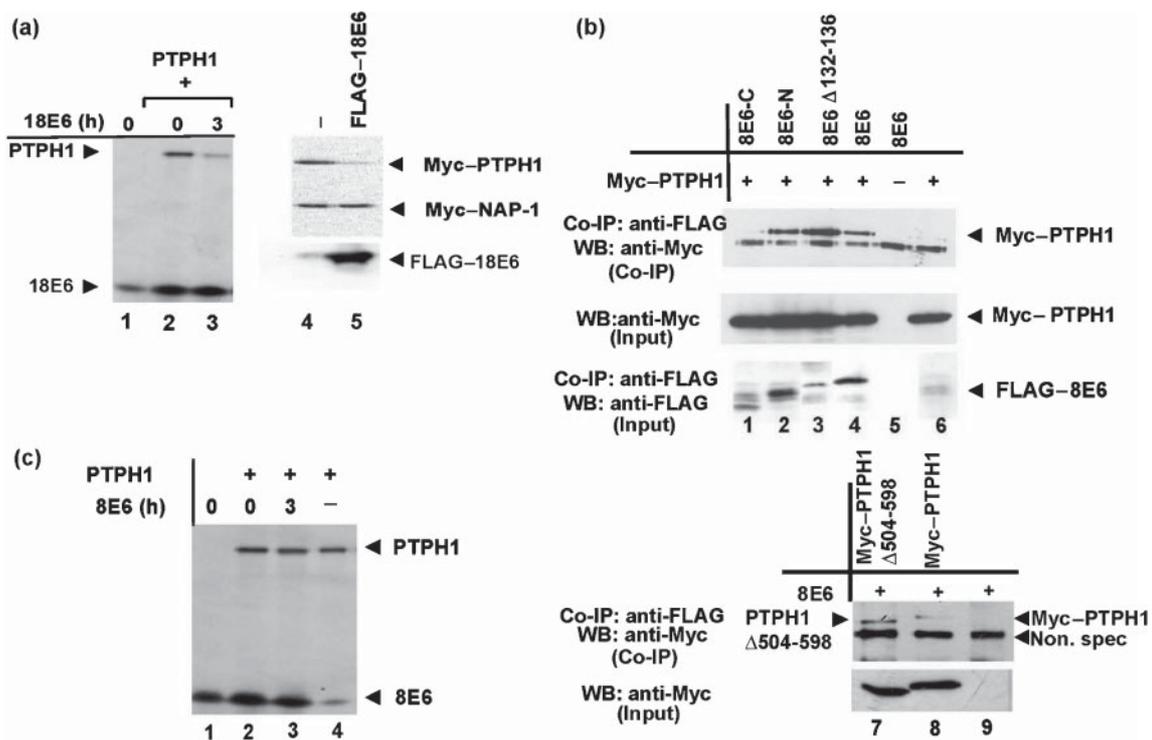

Fig. 4. (a) 18E8 induces the accelerated degradation of PTPH1 as well. *In vitro* (lanes 1–3) and *in vivo* (lanes 4, 5) degradation assays were performed as described in Fig. 3(a, b), except that 18E6 was used. (b) 8E6 binds to PTPH1, but does not affect its stability. Coimmunoprecipitation: C33A cells were transiently transfected with expression vectors for Myc–PTPH1 and for FLAG-tagged 8E6, 8E6-N (amino acids 1–93) or 8E6-C (amino acids 94–155), as indicated in the figure. An aliquot of extracts was used in a Western blot to determine the level of Myc–PTPH1 as input control. One milligram of the extracts was used for an immunoprecipitation with the FLAG affinity beads and coprecipitated Myc–PTPH1 was detected by Western blot developed with the anti-Myc antibody. To confirm the expression of the 8E6 versions, FLAG-immunoprecipitates were subjected to a Western blot developed with the anti-FLAG antibody. (c) *In vitro* degradation assay. 8E6 and PTPH1, both ^{35}S-labelled and obtained by *in vitro* translation, were coincubated for 0 or 3 h before analysis by SDS-PAGE, followed by autoradiography.

does not involve the PDZ module, but requires other regions. It became obvious in Fig. 4(b), that the amount of Myc–PTPH1 was not reduced when 8E6 was coexpressed, suggesting that 8E6 could not induce the accelerated degradation of PTPH1. This was confirmed by the *in vitro* degradation assay shown in Fig. 4(c).

Reducing the intracellular amounts of E6 correlates with increased levels of endogenous PTPH1 in SiHa cells

The findings described until here imply that, in established HPV-positive cervical cell lines, PTPH1 protein levels should be low, due to its degradation by E6. To test this, we compared the amount of PTPH1 in HPV-positive cell lines and in HPV-negative cells by immunoprecipitation. Fig. 5(a) shows that less PTPH1 was precipitated from extracts
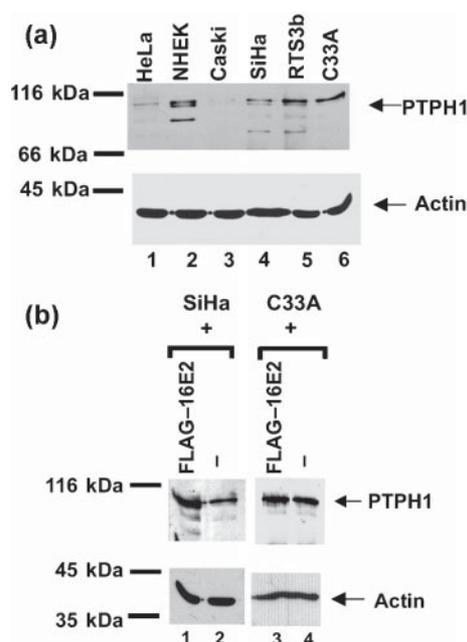
Fig. 5. (a) HPV-positive cervical carcinoma-derived cell lines have low amounts of endogenous PTPH1. Immunoprecipitation: total cell extracts from the HPV16-positive cell lines SiHa and Caski and the HPV18-positive cell line HeLa, the HPV-negative cell lines C33A and RTS3b as well as from normal human epidermal keratinocytes (NHEK) were subjected to an immunoprecipitation with an antibody directed against PTPH1. The presence of PTPH1 was confirmed in a Western blot developed with the PTPH1 antibody. As control, the presence of actin in the extracts was determined by Western blot. (b) Introducing an expression vector for E2 into SiHa cells leads to increased level of PTPH1. SiHa or C33A cells were transfected with an expression vector for FLAG–16E2 or the empty pcDNA3.1-FLAG vector. Transfected cells were selected 24 h later with G418 for 48 h. Cell extracts were used to immunoprecipitate PTPH1 by the anti-PTPH1 antibody. As control, the presence of actin was determined with 15 μg of each extract.

of HPV16-positive cervical carcinoma-derived cell lines SiHa, Caski and HPV18-positive HeLa cells, compared with primary human epithelial keratinocytes (NHEK), C33A and RTS3b cells, which all are HPV negative. Moreover, HaCat cells revealed higher levels of PTPH1 as well, in accordance with previous observations (data not shown) (Zhang *et al.*, 1997). Thus, the reduced amounts of PTPH1 in HPV-positive cervical cancer cell lines may be a result of its degradation by E6, expressed in these cell lines.

Integration of HPV into the host genome is an important step in cervical neoplastic progression and usually causes deletion or disruption of the ORF for the viral transcription factor E2, while the expression of the E6 and E7 genes is retained (reviewed by Longworth & Laimins, 2004; Münger *et al.*, 2004). It is well established that over-expression of E2 from heterologous promoters in HPV-positive cervical carcinoma-derived cell lines represses the early HPV promoter, resulting in a sharp reduction in E6 and E7 expression (Desaintes *et al.*, 1997; Goodwin & DiMaio, 2000; Goodwin *et al.*, 1998; Thierry & Yaniv, 1987). In order to test whether this E2-mediated repression correlates with a change in the level of PTPH1 protein, we transfected SiHa cells with an expression vector for FLAG-tagged HPV16 E2 (16E2). The introduction of E2 into cervical carcinoma cell lines such as HeLa has been shown to result in growth arrest or apoptosis (Francis *et al.*, 2000; Nishimura *et al.*, 2000) (reviewed by Blachon & Demeret, 2003). In order to minimize these pathways, the transfected cells were enriched by selection with G418 for only 48 h. In accordance with previous observations, overexpression of E2 led to a significant reduction of the E6 mRNA level, as revealed by RT-PCR (data not shown). Compared to the empty vector, the amount of PTPH1 was significantly elevated in the extracts of SiHa cells transfected with the 16E2 vector. E2 per se had no effect on PTPH1, since the overexpression of 16E2 in C33A cells did not change the levels of PTPH1 (Fig. 5b). Thus, higher concentrations of PTPH1 in SiHa cells may be the result of reduced amounts of 16E6 due to E2-mediated repression of E6 transcription, which is in line with the model that 16E6 induces the accelerated degradation of endogenous PTPH1 in SiHa cells.

DISCUSSION

The binding to PDZ partner proteins by the E6 oncoproteins of high-risk genital HPVs contributes to the oncogenic potential. The deletion of the PDZ-binding motif impairs the ability of E6 to transform established rodent cell lines, to immortalize keratinocytes and to induce epithelial hyperplasia in transgenic mice (Kiyono *et al.*, 1997, 1998; Liu *et al.*, 1999; Mantovani & Banks, 2001; Nguyen *et al.*, 2003; Simonson *et al.*, 2005). This transforming activity inherent to the PDZ-binding motif presumably does not relate to one specific target but to a combined degradation of different PDZ domain proteins. Our results presented here suggest that PTPH1 is an

additional target for the PDZ motif of high-risk genital HPVs.

The specificity of the interaction was demonstrated by the use of recombinant PTPH1 and 16E6 proteins synthesized in different systems such as yeast, bacteria, *in vitro* translation via a rabbit reticulocyte lysate and in mammalian cells. Moreover, GST–16E6-C precipitated endogenous PTPH1, which underlines that the native protein can be targeted by 16E6 and that the binding is not restricted to recombinant PTPH1. In addition to the interaction of both proteins via their PDZ module, the proteasome-mediated degradation of PTPH1 by 16E6 presumably depends on the binding of E6 to E6-AP. This is supported by the use of an inhibitor of the proteasome, the analysis of the degradation capacity of various 16E6 mutants and by reconstitution experiments in H1299 cells clone K3, in which expression of endogenous E6-AP was stably ablated by RNA interference. Our results are in line with the findings of Jing *et al.* (2007), demonstrating that the complex 16E6 + E6-AP associates with and targets the degradation of PTPH1, although we cannot exclude that another ubiquitin ligase is involved, as suggested for the E6-mediated degradation of hDlg and the MAGI family of proteins (Grm & Banks, 2004; Pim *et al.*, 2000, 2002).

The exon 12 of PTPH1, which was not present in the splice variant of PTPH1 which we have isolated from HaCat cells, has no effect on targeting of PTPH1 by 16E6. The fact that all 24 cDNA clones we have recovered from HaCat cells during the initial yeast two-hybrid screen lacked exon 12 indicates that this splice variant may be the major isoform of PTPH1 expressed in HaCat cells. A serine at position 359, located within the segment from amino acid 334 to 379 encoded by exon 12, has been shown to represent one out of two binding sites to 14-3-3β protein. The previous observation that in HaCat cells less PTPH1 was found in complex with 14-3-3 (Zhang *et al.*, 1997) may rely on the presence of this splice variant in these cells.

The lower level of endogenous PTPH1 protein in HPV-positive cervical carcinoma-derived cell lines SiHa, Caski and HeLa, compared with HPV-negative cell lines and primary keratinocytes, correlates with the notion that the E6 oncoproteins expressed by the high-risk HPV types 16 and 18, present in these cell lines, induce the accelerated degradation of endogenous PTPH1. This assumption is consistent with the observation made by Jing *et al.* (2007), who found that the introduction of 16E6 into keratinocytes reduced the amount of endogenous PTPH1, and with our result, demonstrating that the PTPH1 level rose when E6 expression was repressed by the E2 transcription factor (Fig. 5b). PTPH1 upregulation due to overexpression of E2 did not result from E2-mediated apoptosis, since E2 was reported to induce apoptosis in the HPV-negative cervical cancer cell line C33A as well (Demeret *et al.*, 2003), but has no effect on PTPH1 in these cells (see Fig. 5b). The integration of the HPV genome into a host chromosome resulting in loss of the E2 transcriptional repressor is regarded as one of the key events during HPV-induced carcinogenesis (reviewed by Longworth & Laimins, 2004; Münger *et al.*, 2004). Upregulated E6/E7 expression confers the cells growth advantage (Pett *et al.*, 2004, 2006). Reduced levels of PTPH1, as a consequence of high amounts of E6, may contribute to promote cell growth. This is supported by the observation that ectopically expressed PTPH1 disrupts cell-cycle progression in NIH3T3 cells, thus slowing down or arresting cell growth (Zhang *et al.*, 1999), and inhibited growth of RTS3b cells as well (S.T. and G.S; unpublished results). The specific targets mediating growth inhibition by PTPH1 are unknown. Although the cell-cycle regulator VCP (valosin-containing protein), tyrosine-phosphorylated in response to T-cell stimulation, was identified as a substrate of PTPH1 (Zhang *et al.*, 1999), we did not detect any effect of 16E6 on the phosphorylation level of VCP. It seems feasible that other, not yet identified, substrates of PTPH1 may exist in human keratinocytes that mediate the growth regulatory effects of PTPH1. For instance, PTPH1 may be involved in phosphotyrosine-based signal transduction initiated by growth factor receptors. Jing *et al.* (2007) observed that siRNA-mediated targeting of PTPH1 reduced growth factor requirement of immortalized keratinocytes. This activity may be affected by E6, since E6-transduced immortalized keratinocytes revealed the same phenotype (Jing *et al.*, 2007). Moreover, the N-terminal band 4.1-FERM domain is thought to target PTPH1 to cytoskeleton–membrane interfaces (reviewed by Ostman *et al.*, 2006). This subcellular localization may determine, at least in part, its substrates. Thus, it is possible that the interaction of E6 with PTPH1 may play a role in the E6-mediated disruption of the epithelial tight junction and enhanced morphological transformation of immortalized human keratinocytes, which was reported to be dependent on the integrity of the PDZ-binding motif of E6 (reviewed by Mantovani & Banks, 2001; Watson *et al.*, 2003).

An interaction with PTPH1 may be conserved among cutaneous high-risk E6 proteins as well, since 5E6 was able to bind to PTPH1 in GST pull-down experiments (data not shown), in addition to 8E6. As expected, this interaction does not involve the PDZ domain of PTPH1, as high-risk cutaneous E6 proteins do not encode a PDZ-binding motif. Our preliminary studies revealed that 8E6 binds to the C-terminal part of PTPH1 encoding the phosphate activity (D. Jordanovski, Institute of Virology, University of Cologne, and G.S., data not shown). We are currently testing whether 8E6 will modulate the phosphate activity or substrate binding. Taken together, results suggest that targeting the putative tumour suppressor PTPH1 may be conserved among high-risk E6 proteins.

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REFERENCES

Arpin, M., Algrain, M. & Louvard, D. (1994). Membrane-actin microfilament connections: an increasing diversity of players related to band 4.1. *Curr Opin Cell Biol* **6**, 136–141.

Blachon, S. & Demeret, C. (2003). The regulatory E2 proteins of human genital papillomaviruses are pro-apoptotic. *Biochimie* **85**, 813–839.

Demeret, C., Garcia-Carranca, A. & Thierry, F. (2003). Transcription-independent triggering of the extrinsic pathway of apoptosis by human papillomavirus 18 E2 protein. *Oncogene* **22**, 168–175.

Desaintes, C., Demeret, C., Goyat, S., Yaniv, M. & Thierry, F. (1997). Expression of papillomavirus E2 protein in HeLa cells leads to apoptosis. *EMBO J* **16**, 504–514.

Dev, K. K. (2004). Making protein interactions druggable: targeting PDZ domains. *Nat Rev Drug Discov* **3**, 1047–1056.

Foster, S. A., Demers, G. W., Etscheid, B. G. & Galloway, D. A. (1994). The ability of human papillomavirus E6 proteins to target p53 for degradation in vivo correlates with their ability to abrogate actinomycin D-induced growth arrest. *J Virol* **68**, 5698–5705.

Francis, D. A., Schmid, S. I. & Howley, P. M. (2000). Repression of the integrated papillomavirus E6/E7 promoter is required for growth suppression of cervical cancer cells. *J Virol* **74**, 2679–2686.

Gewin, L., Myers, H., Kiyono, T. & Galloway, D. A. (2004). Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex. *Genes Dev* **18**, 2269–2282.

Goodwin, E. C. & DiMaio, D. (2000). Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes an orderly reactivation of dormant tumor suppressor pathways. *Proc Natl Acad Sci U S A* **97**, 12513–12518.

Goodwin, E. C., Naeger, L. K., Breiding, D. E., Androphy, E. J. & DiMaio, D. (1998). Transactivation-competent bovine papillomavirus E2 protein is specifically required for efficient repression of human papillomavirus oncogene expression and for acute growth inhibition of cervical carcinoma cell lines. *J Virol* **72**, 3925–3934.

Grm, H. S. & Banks, L. (2004). Degradation of hDlg and MAGIs by human papillomavirus E6 is E6-AP-independent. *J Gen Virol* **85**, 2815–2819.

Hampson, L., Li, C., Oliver, A. W., Kitchener, H. C. & Hampson, I. N. (2004). The PDZ protein TIP-1 is a gain of function target of the HPV16 E6 oncoprotein. *Int J Oncol* **25**, 1249–1256.

Hung, A. Y. & Sheng, M. (2002). PDZ domains: structural modules for protein complex assembly. *J Biol Chem* **277**, 5699–5702.

Iftner, T., Bierfelder, S., Csapo, Z. & Pfister, H. (1988). Involvement of human papillomavirus type 8 genes E6 and E7 in transformation and replication. *J Virol* **62**, 3655–3661.

Jing, M., Bohl, J., Brimer, N., Kinter, M. & Vande Pol, S. B. (2007). Degradation of tyrosine phosphatase PTPN3 (PTPH1) by association with oncogenic human papillomavirus E6 proteins. *J Virol* **81**, 2231–2239.

Kiyono, T., Hiraiwa, A. & Ishibashi, M. (1992). Differences in transforming activity and coded amino acid sequences among E6 genes of several papillomaviruses associated with epidermodysplasia verruciformis. *Virology* **186**, 628–639.

Kiyono, T., Hiraiwa, A., Fujita, M., Hayashi, Y., Akiyama, T. & Ishibashi, M. (1997). Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila disc large tumor suppressor protein. *Proc Natl Acad Sci U S A* **94**, 11612–11616.

Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J.K., Galloway, D. A. & Klingelhutz, A. J. (1998). Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* **396**, 84–88.

Klingelhutz, A. J., Foster, S. A. & McDougall, J. K. (1996). Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* **380**, 79–81.

Kuballa, P., Matentzoglu, K. & Scheffner, M. (2007). The role of the ubiquitin ligase E6-AP in human papillomavirus E6-mediated degradation of PDZ domain containing proteins. *J Biol Chem* **282**, 65–71.

Liu, Y., Chen, J. J., Gao, Q., Dalal, S., Hong, Y., Mansur, C. P., Band, V. & Androphy, E. J. (1999). Multiple functions of human papillomavirus type 16 E6 contribute to the immortalization of mammary epithelial cells. *J Virol* **73**, 7297–7307.

Longworth, M. S. & Laimins, L. A. (2004). Pathogenesis of human papillomaviruses in differentiating epithelia. *Microbiol Mol Biol Rev* **68**, 362–372.

Mantovani, F. & Banks, L. (2001). The human papillomavirus E6 protein and its contribution to malignant progression. *Oncogene* **20**, 7874–7887.

Massimi, P., Gammoh, N., Thomas, M. & Banks, L. (2004). HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation. *Oncogene* **23**, 8033–8039.

Müller, A., Ritzkowsky, A. & Steger, G. (2002). Cooperative activation of human papillomavirus type 8 gene expression by the E2 protein and the cellular coactivator p300. *J Virol* **76**, 11042–11053.

Müller-Schiffmann, A., Beckmann, J. & Steger, G. (2006). The E6 protein of the cutaneous human papillomavirus type 8 can stimulate the viral early and late promoters by distinct mechanisms. *J Virol* **80**, 8718–8728.

Münger, K., Baldwin, A., Edwards, K. M., Hayakawa, H., Nguyen, C. L., Owens, M., Grace, M. & Huh, K. (2004). Mechanisms of human papillomavirus-induced oncogenesis. *J Virol* **78**, 11451–11460.

Munoz, N. (2000). Human papillomavirus and cancer: the epidemiological evidence. *J Clin Virol* **19**, 1–5.

Nakagawa, S. & Huibregtse, J. M. (2000). Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. *Mol Cell Biol* **20**, 8244–8253.

Nguyen, M. L., Nguyen, M. M., Lee, D., Griep, A. E. & Lambert, P. F. (2003). The PDZ ligand domain of the human papillomavirus type 16 E6 protein is required for E6's induction of epithelial hyperplasia in vivo. *J Virol* **77**, 6957–6964.

Nishimura, A., Ono, T., Ishimoto, A., Dowhanick, J. J., Frizell, M. A., Howley, P. M. & Sakai, H. (2000). Mechanism of human papillomavirus E2-mediated repression of viral oncogene expression and cervical cancer cell growth inhibition. *J Virol* **74**, 3752–3760.

Nomine, Y., Charbonnier, S., Miguet, L., Potier, N., Van Dorsselaer, A., Atkinson, R. A., Trave, G. & Kieffer, B. (2005). ^{1}H and ^{15}N resonance assignment, secondary structure and dynamic behaviour of the C-terminal domain of human papillomavirus oncoprotein E6. *J Biomol NMR* **31**, 129–141.

Nomine, Y., Masson, M., Charbonnier, S., Zanier, K., Ristriani, T., Deryckere, F., Sibler, A. P., Desplancq, D., Atkinson, R. A. & other authors (2006). Structural and functional analysis of E6 oncoprotein:

insights in the molecular pathways of human papillomavirus-mediated pathogenesis. *Mol Cell* **21**, 665–678.

Nourry, C., Grant, S. G. & Borg, J. P. (2003). PDZ domain proteins: plug and play! *Sci STKE* **2003**, RE7.

Ostman, A., Hellberg, C. & Bohmer, F. D. (2006). Protein-tyrosine phosphatases and cancer. *Nat Rev Cancer* **6**, 307–320.

Pett, M. R., Alazawi, W. O., Roberts, I., Dowen, S., Smith, D. I., Stanley, M. A. & Coleman, N. (2004). Acquisition of high-level chromosomal instability is associated with integration of human papillomavirus type 16 in cervical keratinocytes. *Cancer Res* **64**, 1359–1368.

Pett, M. R., Herdman, M. T., Palmer, R. D., Yeo, G. S., Shivji, M. K., Stanley, M. A. & Coleman, N. (2006). Selection of cervical keratinocytes containing integrated HPV16 associates with episome loss and an endogenous antiviral response. *Proc Natl Acad Sci U S A* **103**, 3822–3827.

Pim, D., Thomas, M., Javier, R., Gardiol, D. & Banks, L. (2000). HPV E6 targeted degradation of the discs large protein: evidence for the involvement of a novel ubiquitin ligase. *Oncogene* **19**, 719–725.

Pim, D., Thomas, M. & Banks, L. (2002). Chimaeric HPV E6 proteins allow dissection of the proteolytic pathways regulating different E6 cellular target proteins. *Oncogene* **21**, 8140–8148.

Purdie, K. J., Sexton, C. J., Proby, C. M., Glover, M. T., Williams, A. T., Stables, J. N. & Leigh, I. M. (1993). Malignant transformation of cutaneous lesions in renal allograft patients: a role for human papillomavirus. *Cancer Res* **53**, 5328–5333.

Rehtanz, M., Schmidt, H.-M., Warthorst, U. & Steger, G. (2004). Direct interaction between nucleosome assembly protein-1 and the papillomavirus E2 proteins involved in activation of transcription. *Mol Cell Biol* **24**, 2153–2168.

Scheffner, M. & Whitaker, N. J. (2003). Human papillomavirus-induced carcinogenesis and the ubiquitin-proteasome system. *Semin Cancer Biol* **13**, 59–67.

Simonson, S. J. S., Difilippantinio, M. J. & Lambert, P. F. (2005). Two distinct activities contribute to human papillomavirus 16 E6's oncogenic potential. *Cancer Res* **65**, 8266–8273.

Songyang, Z., Fanning, A. S., Fu, C., Xu, J., Marfatia, S. M., Chishti, A. H., Crompton, A., Chan, A. C., Anderson, J. M. & Cantley, L. C.

(1997). Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* **275**, 73–77.

Steger, G. & Pfister, H. (1992). In vitro expressed HPV 8 E6 protein does not bind p53. *Arch Virol* **125**, 355–360.

Thierry, F. & Yaniv, M. (1987). The BPV1 E2 trans-acting protein can be either a repressor or activator of the HPV18 regulatory region. *EMBO J* **6**, 3391–3397.

Thomas, M., Glaunsinger, B., Pim, D., Javier, R. & Banks, L. (2001). HPV16 E6 and MAGUK protein interactions: determination of the molecular basis for specific protein recognition and degradation. *Oncogene* **20**, 5431–5439.

Wang, Z., Shen, D., Parsons, D. W., Bardelli, A., Sager, J., Szabo, S., Ptak, J., Silliman, N., Peters, B. A. & other authors (2004). Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science* **304**, 1164–1166.

Watson, R. A., Thomas, M., Banks, L. & Roberts, S. (2003). Activity of the human papillomavirus E6 PDZ-binding motif correlates with enhanced morphological transformation of immortalized human keratinocytes. *J Cell Sci* **116**, 4925–4935.

Yang, Q. & Tonks, N. K. (1991). Isolation of a cDNA clone encoding a human protein-tyrosine phosphatase with homology to the cyto-skeletal-associated proteins band 4.1, ezrin, and talin. *Proc Natl Acad Sci U S A* **88**, 5949–5953.

Zanier, K., Charbonnier, S., Baltzinger, M., Nomine, Y., Altschuh, D. & Trave, G. (2005). Kinetic analysis of the interactions of human papillomavirus E6 oncoproteins with the ubiquitin ligase E6AP using surface plasmon resonance. *J Mol Biol* **349**, 401–412.

Zhang, S.-H., Eckberg, W. R., Yang, Q., Samatar, A. A. & Tonks, N. K. (1995). Biochemical characterization of human band 4.1-related protein-tyrosine phosphatase, PTPH1. *J Biol Chem* **270**, 20067–20072.

Zhang, S.-H., Kobayashi, R., Graves, P. R., Piwnica-Worms, H. & Tonks, N. K. (1997). Serine phosphorylation-dependent association of the band 4.1-related protein-tyrosine phosphatase PTPH1 with 14-3-3β protein. *J Biol Chem* **272**, 27281–27287.

Zhang, S.-H., Liu, J., Kobayashi, R. & Tonks, N. K. (1999). Identification of cell cycle regulator VCP (p97/CDC48) as a substrate of band 4.1-related protein-tyrosine phosphatase PTPH1. *J Biol Chem* **274**, 17806–17812.