Cutaneous papillomavirus E6 proteins must interact with p300 and block p53-mediated apoptosis for cellular immortalization and tumorigenesis

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Running title: Binding and inhibition of p300 by E6-proteins of CRPV and HPV38

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Abstract

Binding of the papillomavirus E6 protein to E6AP and induction of p53 degradation is a common feature of genital high-risk human papillomaviruses (HPV), but cutaneous HPV lack this capacity. Nevertheless several cutaneous HPV types like HPV38 are associated with tumor formation when combined with a genetic predisposition, immunosuppression or UV-exposure. In an animal model system, the cottontail rabbit papillomavirus (CRPV) rapidly induces skin cancer without additional cofactors and CRPVE6 and E7 immortalize rabbit keratinocytes *in vitro*. However, CRPVE6 neither interacts with E6AP, with p53 nor induces p53 degradation. In this study, we show that interaction of CRPV or HPV38E6 with the histone acetyltransferase p300 is crucial to inhibit the ability of p53 to induce apoptosis. Strikingly, E6 mutants deficient for p300 binding are incapable of preventing p53 acetylation, p53 dependent transcription and apoptosis induction. Moreover E6 mutants deficient for p300 binding can not contribute to HPV38-induced immortalization of human keratinocytes or CRPV-induced tumor formation.

Our findings highlight changes in the p53 acetylation status mediated by the viral E6 protein as a crucial requirement in the ability of high-risk cutaneous papillomaviruses to immortalize primary keratinocytes and induce tumors.

Introduction

Although more than 100 different human papillomaviruses (HPVs) were characterized on the basis of sequence homologies, only a limited number was shown to be associated with cancer development so far. Thirteen so called high-risk types of the alpha genus HPVs (α-HPVs) play a critical role in the development of cervical cancer and have been implicated in other anogenital cancers and a subset of head and neck carcinoma (1, 2). In addition β-HPVs have been linked to the development of non-melanoma skin cancers (NMSC), which represents the most common malignancy in Caucasians worldwide (3). Beta-papillomaviruses such as HPV5 and 8 were described to be associated with skin carcinogenesis in patients with the rare genetic disorder epidermodysplasia verruciformis (EV). EV patients develop warts, which progress in up to 60% of the individuals mainly into primary squamous cell carcinoma (4). Furthermore, up to 70% of long-term immunosuppressed patients develop skin cancer of which more than 80% contain HPV-DNA (5-7). A causative relationship between β-HPV infection and NMSC development in immunocompetent individuals is suggested by epidemiological data and by the fact that the associated risk factors age, UV-mediated local and transplantation-related immunosuppression point to an infectious agent most probably belonging to the ß2-group that includes HPV38. (8, 9).

An excellent animal model to study papillomavirus induced skin cancer formation is the New Zealand White rabbit, where infections with cottontail rabbit papillomavirus (CRPV) causes the development of tumors within 3 to 6 weeks post-infection that progress within 6 to 12 month into invasive cancer without additional cofactors (10, 11). Recently, we reported that the CRPV oncoproteins E6 and E7 are able to immortalize primary rabbit keratinocytes (12),

a common feature of all carcinogenic HPV types (13), which is believed to be a key requirement for cancer formation.

High-risk HPV disrupt cell cycle control by the interaction of E7 with pRB family members and activate telomerase and inhibit p53 via E6 (14). Comparable to high-risk HPV16, CRPVE7 alone immortalizes primary rabbit keratinocytes and coexpression of E6 increases the efficiency of immortalization (12, 15, 16). Like HPV16 E7, CRPV E7 binds to and induces degradation of pRB and thus induces cell cycle progression (12, 17, 18). However, in contrast to HPV16 immortalized cells, no reduction of p53 levels in CRPVE6/E7 immortalized cells was observed (12, 13). Comparable findings were described for β-HPV, where E6 interacts with the ubiquitin ligase E6AP, but is unable to degrade p53. Consistent with UV as a major risk factor, β-HPV types like HPV38, were shown to prevent UVB-induced apoptosis by degradation of Bak (19, 20). This activity is surprisingly shared with high-risk genital HPV18, causing cervical cancer where UV is no known co-factor. In contrast, CRPVE6 neither interacts with p53 nor with E6AP and therefore is not able to degrade p53 and probably also not Bak (12, 20, 21).Currently, it is not understood why the increased p53 levels in the CRPVE6/E7 immortalized cells do not interfere with immortalization or tumor development (12).

The HPV38E6/E7 genes immortalize normal human keratinocytes (22) and induce hyperproliferation and neoplasia in a transgenic mouse model (23). This was proposed to occur by a p53-dependent transcriptional mechanism through ΔNp73 accumulation (24). Comparable to CRPV-positive cells, p53 accumulates, but does not induce growth arrest or apoptosis in HPV38E6/E7 immortalized keratinocytes (20, 22). ΔNp73 represses the expression of p53-dependent target genes by binding site competition with p53 (24, 25).

However, the mechanism of ΔNp73 induction bypassing the p53-ΔNp73-feedback loop and the individual contributions of HPV38 E6 and E7 have not been addressed.

Thus it seems possible that ß-HPVs and CRPV block p53-dependent apoptosis independent of proteasomal degradation. Besides ΔNp73 activation, E6 can affect p53 functions by inhibition of p53-binding to DNA, mislocalization or post-translational modifications of p53 (26). An interesting mechanism is the inhibition of p53 regulated genes via interaction of E6 with the histone acetylase p300/CBP (27-29). p300/CBP modulate p53 activity via regulation of degradation by mdm2 (30, 31), co-activation of p53 regulated genes (32), and the acetylation of p53 (33). Prevention of p53 acetylation via interaction of E6 with p300 has been demonstrated for high-risk, low-risk genital types and for bovine papillomavirus type 1, but not for β-papillomaviruses or CRPV. Inhibition of p53 acetylation prevents p21 induction, which has been reported to alter growth of keratinocytes (34) or to be anti-apoptotic in human mammary epithelial cells (35). What remains to be determined are the functional consequences of the prevention of p53 acetylation mediated by p300 with regard to carcinogenesis and whether skin cancer-associated papillomaviruses manipulate this pathway.

Here we show for the first time that CRPVE6 binds efficiently to p300 and prevents p300-mediated p53-induced apoptosis *in vitro* and *in vivo* which is required for tumor formation by CRPV *in vivo*. HPV38E6 also binds p300 via a motif present in CRPVE6, but not in HPV16E6, and this interaction is necessary for keratinocyte immortalization by HPV38 E6/E7. Taken together, our results imply a novel mechanism for p53 inactivation by ß-HPV types and CRPV that is important for skin carcinogenesis.

Material and Methods

Vectors and plasmids

Constructs were generated by PCR using the primers in supplementary figure 1. pLA2-CRPV (61) was used as template to generate plasmids expressing CRPVE6 and the respective mutants. Alanine mutants were generated by overlap extension PCR or by insertion of synthetic AvrII-BamHI-oligonucleotides. Mutant CRPV genomes were constructed by replacing the BspEI fragment in pLA2-CRPV with fragments from the respective pMalc2x-CRPVSE6 plasmids. pMal, pcDNA or pMSCVpuro Expression vectors for HPV38E6 and 38E6Ala80-84 were generated by overlap extension PCR. The expression vector Rc/CMV-p53 was kindly provided by M. Scheffner.

Cell culture and transient siRNA cotransfection

Primary keratinocytes were isolated from normal skin tissue (12). C33A, HEK293T and Saos-2 cell lines were authenticated by standard methods and provided by the American Type Culture Collection. Cells were aliquoted, immediately frozen, replated for the purposes of the experiments and used within 6 month. Cells were cultivated as described previously (12). 4.5 x 10^5 Saos-2 cells were transfected 24h after seeding with 150ng siRNA (sip300-2: GGACUACCCUAUCAAGUAA) and 12µl HiPerFect reagent (Qiagen). The next day, cells were cotransfected with 3.4µg of p53 expression vector or empty vector by calcium phosphate precipitation. RNA was isolated 30h post transfection.

Quantitative real time PCR

RNA was isolated with the RNAeasy kit (Qiagen) and cDNA was synthesized from 1µg total RNA using the QuantiTectReverse Transcription kit (Qiagen). PCR reactions (20µl) consisted of 10µl LightCycler 480 SYBR Green I Master (Roche), 50ng cDNA and 3µM forward and

reverse primers (for sequences see Supplementary figure 1). Relative transcript levels were calculated using phosphoglycerate kinase 1 (PGK1) transcripts as a reference (36).

Generation and analysis of stable keratinocyte cell lines

Generation of amphotropic recombinant retroviruses and infection of NHK was performed as previously described (37). Transduced cells were selected with puromycin (0.4µg/ml) or G418 (50µg/ml) for 14 days. To determine the generation time, cells were trypsinized at 80% confluence and counted. The total cell number was then extrapolated.

P53 stability assays

CRPV E6/E7 transformed rabbit keratinocytes, HPV16 E6/E7 or HPV38 E6/E7 immortalized human keratinocytes were treated with DMSO alone, 30 µg/ml cycloheximide (Roth) or 20µg/ml MG132 (Merck) , both dissolved in DMSO. Treated and untreated cells were harvested at the indicated time points by scraping into cold PBS, pelleted by centrifugation and lysed by boiling in 4xSDS sample buffer. Aliquots were then analysed by immunoblotting.

Maltose binding protein pulldown and coimmunoprecipitation assays

MBP-pulldown assays were performed as desribed previously (12).

C33A cells were cultivated in DMEM supplemented with 10% fetal calf serum and Gentamicin (0.5 mg/mL). $8x10^6$ cells were transfected with pcDNA-E6-HA or pcDNA-E6Ala200-204-HA using FuGENE HD transfection reagent (Roche). 48h later cells were lysed on ice in 700µl IP-buffer (100mM NaCl, 50mM Tris pH 8.0, 1mM EDTA, 0,1% Igepal CA-630, 0.1% β-mercaptoethanol) and insoluble proteins were removed by centrifugation. An

aliquot of the cell lysates was removed (input control) and the rest of the lysates were mixed with 50µl pre-washed µMACS Anti-HA Micro Beads (Miltenyi Biotec) and incubated for 3h at 4°C. Beads were collected in µColumns (Miltenyi Biotec) and washed 5 times with IP-buffer. Bound proteins were eluted with 4xSDS sample buffer (95°C) and analysed by immunoblotting.

Animal model

Infections of New Zealand White rabbits with CRPV DNA were performed as previously described (10). Tumor numbers and maximum diameter of each tumor were determined over a period of 5 months. Biopsies were removed 3 months post infection, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek), snap-frozen, and stored at -80°C or DNA was extracted using a BioRobot EZ1 workstation with the EZ1 DNA Tissue Kit (Qiagen). Viral DNA was amplified using primers CRPV444-463F and CRPV1124-1104R and sequenced.

Immunofluorescence microscopy and TUNEL staining

Cells were grown on MatTek glassbottom culture dishes (MatTek Corporation), fixed for 10minutes in 2% paraformaldehyde and incubated with HA antibody (sc-805, Santa Cruz) in PBS/3% BSA for 1hour at RT, washed and incubated with FITC-conjugated anti-rabbit IgG antibodies. Cells were counterstained with DAPI. For TUNEL staining cells were fixed 48 hours post transfection with 4% neutral-buffered formaldehyde for 5 minutes and permeabilized with 20 µg/ml proteinase K (Merck) for 5 minutes at room temperature. The terminal deoxynucleotidyl transferase mediated dUTP biotin nick end labeling assay (TUNEL) was performed using the QIA39 FragEL in situ apoptosis detection kit (Merck). Fluorescence signals were visualized with a Zeiss Axiovert 200M microscope (Carl Zeiss

MicroImaging GmbH). For each sample, at least 5 different fields were examined and counted to determine the rate of apoptosis.

Western Blotting

Cells were lysed in RIPA buffer (Sigma). Normalized amounts of protein (Micro BCA protein assay reagent kit; Thermo Scientific) were separated by 10% SDS-PAGE and transferred to nitrocellulose. Primary antibodies were: α-p53 (DO-1, Santa Cruz); α-p53-Lys382 (2525S, New England Biolabs), α-Tubulin (DM1A, Merck) or α-GFP (FL 32, Santa Cruz). Secondary antibodies conjugated to horseradish peroxidise (Dako) were detected using SuperSignal West substrates (Thermo Scientific) and a Fluor-S Max MultiImager (Bio-Rad). MBP pulldown and coimmunoprecipitation eluates were run on 5-12% SDS-polyacrylamide gels and transferred to nitrocellulose. The membranes were probed with antibodies detecting p300 (C-20, sc-585 (Santa Cruz); ab37142; ab10485 (both Abcam)) and MBP (E8038 (New England Biolabs)).

Blots were developed with SuperSignal West (Thermo Scientific) substrate and visualized by the Fluor-S Max MultiImager (Bio-Rad). Band intensities were quantified using the Quantity One quantification software package (version 4) (Bio-Rad).

Results

P53 is not degraded by E6 proteins of cutaneous papillomaviruses

The addition of the proteasome inhibitor MG132 to CRPV and HPV38 E6/E7 immortalized keratinocytes has indicated that the respective E6 proteins do not induce p53 degradation (12, 24). To further confirm these findings, HPV16, HPV38 or CRPV E6/E7 immortalized keratinocytes were treated with cycloheximide, an inhibitor of protein biosynthesis (Fig 1A).

In HPV16E6/E7 positive cells, cycloheximide treatment resulted in a rapid decrease of detectable p53 levels, whereas p53 was unaffected in CRPV or HPV38 E6/E7 immortalized cells. Consistent with this MG132 treatment only increased p53 in HPV16 E6/E7 cells, but not in HPV38 E6/E7 cells. These data clearly show that p53 is not degraded by CRPV or HPV38 E6 proteins.

CRPVE6 represses transcriptional activity of p53

To evaluate if rabbit p53 (rp53) is similar to human p53 (hp53) with regard to transcriptional activation, we expressed rp53 and hp53 in p53-deficient Saos-2 cells and tested for p21 induction. After 48 hours a 4-fold induction of p21 was observed by rp53 and hp53. P21-induction of rp53 was strongly impaired by cotransfection of CRPVE6 (Fig. 1B) although it does neither interact with nor degrade p53 (12, 20, 21).

CRPVE6 interacts with p300

Immobilized E6 proteins fused to maltose-binding protein (MBP) were incubated with HEK293T cell extracts containing high amounts of p300 protein. Similar to high-risk HPV, CRPV encodes two forms of E6. Short E6 (SE6) is generated by the second methionine (M98) within the full length E6 (long E6, LE6). Both CRPVE6 proteins revealed a much stronger interaction with p300 than the E6 proteins of HPV11, 16 or 18 (Fig 1C). As the smaller SE6 protein also bound p300 this indicated that the binding motif is located within a region common to both proteins.

The interaction of HPV16E6 with p300 was described to rely on glycine 134 (G134), which is conserved among genital HPVE6 proteins (28, 38). The corresponding glycine at position 135 (CRPVLE6) and 38 (CRPVSE6) was replaced by valine (MBP-SE6 G38V) (Table 1; HPV16-

G134 and corresponding glycines underlined) and tested for interaction with p300 by MBP pulldown assays (Fig. 1D). Surprisingly, both wildtype CRPVSE6 and the mutant SE6 G38V bound comparable amounts of p300 suggesting that the CRPVE6 interaction domain with p300 is different from HPV16E6.

Identification of the p300-binding region within CRPVE6

As both LE6 and SE6 interact with p300, we generated subfragments of SE6 to map the interaction domain by MBP pulldown assays (Fig 2A). For better understanding, the amino acid positions are based on the respective amino acids in the full length CRPVE6 protein (LE6). The minimal interaction domain consisted of aa 166-214 (Fig. 2B). Further C- or N-terminal truncations (aa 178-214 or aa166-197) resulted in a complete loss of p300 binding. To further narrow down the binding motif, we performed an alanine scanning mutagenesis of aa 166-178 and aa197-214 by replacing four to five consecutive residues with alanines (Fig. 2B). Quantification of the binding affinities derived from two to five individual MBP pulldown experiments revealed no effect by mutation of aa 210-214, and aa 166-169. Mutation of aa 170-173, 174-177, 178-181 or 205-209 revealed an intermediate loss of p300-binding (25-75% compared to wildtype protein). In contrast, mutation of aa 195-199 or aa 200-204 resulted in a reduction in p300-binding affinity to 15% to 20% of the wildtype SE6 protein. This is similar to the background p300-binding activity of MBP (9% of wt SE6).

To verify this, we performed a co-immunoprecipitation with an HA-tagged CRPV full length E6 protein (Fig. 2C) in C33A cells, an HPV-negative keratinocyte cell line. To avoid expression of SE6 from the LE6 expression vectors the methionine at residue 98 was exchanged to serine in the wildtype (LE6M98S) and both mutant proteins (LE6M98SAla195-199, LE6M98SAla200-204). CRPVE6M98S, HA-tagged CRPVE6M98SHA,

E6M98SHAAla195-199 or E6M98SHAAla200-204 mutants were transfected into C33A cells. Cell lysates were precipitated with HA-antibody and the immunoprecipitates were analyzed for p300 by immunoblotting. Only the HA-tagged wildtype E6 protein was able to precipitate p300. The mutant E6 proteins were present even at higher levels than the wildtype , but did not immunoprecipitate p300 (Fig. 2C). These results demonstrate that CRPVE6 interacts with p300 and that residues 195-204 are crucial for this interaction and do not impair protein stability. Immunofluorescence analyses revealed that wtE6 and both mutants (E6Ala195-199, E6Ala200-204) were present in the nucleus and to a minor extent in the cytosol (Fig. 2D) which is consistent with previous reports (21). Localization of E6M98SHAAla195-199 and E6M98SHAAla200-204 were indistinguishable from E6M98SHA. From these data, we conclude that these mutations in LE6 specifically prevent interaction with p300 but do not change subcellular localization.

Interaction of CRPVE6 with p300 inhibits p53 acetylation

Four different siRNAs against p300 were constructed and tested for their protein knockdown efficiency (Fig. 3A upper panel). The most effective siRNA (sip300-2) was further tested for p300 mRNA reduction (Fig 3A middle panel) and further used for the functional assays. The knock-down of p300 reduced p21 induction by rabbit and human p53 2-3 fold (Fig. 3A lower panel). It is known that p300 acetylates p53 at lysine-382 which stabilizes binding to the p21 promoter (39). Therefore we exchanged the corresponding lysine-380 in rp53 to arginine (rp53K380R). In contrast to wildtype rp53, rp53R380K no longer induced p21 transcription in Saos-2 cells (Fig. 3B). This indicated that rp53 like hp53 depends on p300 as a coactivator. We then transfected Saos-2 cells with hp53 and CRPVE6 and analyzed the amount of p53 and p53 acetylated at lysine-382 (Fig. 3C). Cells transfected with p53 alone or p53 together with

CRPVE6 revealed similar amounts of total p53. However, coexpression of CRPVE6 led to a loss of p53 acetylation at K382. When we cotransfected the p300 binding deficient CRPVE6 (E6M98SAla200-204), acetylated hp53 was clearly visible, indicating that the interaction of wildtype CRPVE6 with p300 is responsible for inhibition of p53 acetylation.

CRPVE6 inhibits p53 induced apoptosis

As E6 prevents acetylation of p53 by p300 and therefore p21 induction, we next investigated the effect of CRPVE6 on p53-mediated apoptosis. Saos-2 cells were cotransfected with expression vectors for hp53, rp53, rp53K380R and wt CRPVE6 or E6M98SAla200-204. Rp53 and hp53 induced similar numbers of apoptotic cells (19%) as determined by TUNEL staining whereas rp53R380K did not (9.7 ± 1.3%) (Fig. 3D). Interestingly, coexpression of CRPVE6 reduced apoptosis induction by rp53 to 10.8 ± 2.6%, whereas E6M98SAla200-204 did not (20.4 ± 1.3%). This suggests that the ability of CRPVE6 to abrogate p300-mediated acetylation of p53 is crucial for cell survival.

E6-p300 binding is important for carcinogenesis

To analyze the importance of the ability of E6 to bind p300 and inhibit the acetylation of p53 *in vivo*, we created p300-binding deficient (pLA2-CRPVE6Ala195-199 and pLA2-CRPVE6Ala200-204) and competent (pLA2-CRPVE6Ala210-214) E6 mutant proteins in the context of the CRPV genome. Wildtype and mutant CRPV genomes were each injected at six sites in the skin of the back of two New Zealand white rabbits. Tumor development and growth were monitored for five months. Wildtype CRPV and pLA2-CRPVE6Ala210-214 produced tumors comparable in size and growth at all injection sites in both animals (Fig. 4A; Table 2). Multiple small tumors became visible around 25 days post infection and grew up to

1-2 cm in diameter at all injection sites in both animals within 55 days. In contrast, pLA2-CRPVE6Ala195-199 and pLA2-CRPVE6Ala200-204 produced single, isolated, very small tumors, that never exceeded a diameter of 3 mm (Fig. 4A; Table 2). The tumors induced by the mutant viruses retained their slower growth over the course of the experiment (Fig 4A, Table 2). To examine if the reduced growth rate of the tumors was due to increased apoptosis, biopsies were taken 85 days post infection. Cryo-sections of the biopsies were TUNEL-stained for apoptotic cells and analyzed by fluorescence microscopy. Whereas the tumors from both wildtype and pLA2-CRPVE6Ala210-214 genomes showed no signs of apoptotic cells, the tumors induced by p300-binding-deficient genomes showed increased numbers of apoptotic cells (Fig 4B). CRPVE6/E7 genes were subjected to sequence analysis which revealed that only the introduced mutations but no other mutations in the E6/E7 region were present in the tumor cells. This strongly suggests that the retarded tumor growth is a consequence of increased apoptosis due to a lack of interaction between CRPVE6 and p300.

The novel p300 binding motif of CRPVE6 and the ability to repress p53 functions is conserved in HPV38E6

A search for homologies of the CRPVE6 p300 binding motif (aa 195-204) in cutaneous HPVE6 proteins revealed the highest similarity with the E6 protein of NMSC-associated HPV38, where aa 81 to 83 are identical to CRPVE6 aa 201-203. To investigate if HPV38 E6 is able to interact with p300 and whether this interaction is mediated by aa 81-83, wildtype and a mutant HPV38 E6 (HPV38 E6Ala80-84) were expressed as MBP fusion proteins. Pulldown experiments revealed that p300 bound to HPV38E6, but not to the mutant (Fig 5A). In contrast, both the wildtype and the mutant HPV38 E6 protein bound to E6AP (40), suggesting that the mutation is specific for p300 (Fig. 5A). Consistent with the observations

for CRPVE6, only wildtype HPV38E6 but not 38E6Ala80-84 was able to repress p21 induction by p53 and acetylation of p53K382 in a p300-binding dependent manner (Fig.5B,C)

p300 binding to HPV38E6 is important for immortalization

Comparable to high-risk genital HPV types, the E6/E7 proteins of HPV38 are able to immortalize primary human foreskin keratinocytes which normally undergo senescence within approximately 20 days after isolation. (22). To address if the E6-p300-interaction influences the immortalization capacity of HPV38 E6 (wt or 38E6Ala80-84) and E7 were expressed from separate retroviral vectors (pLXSN and pMSCVpuro). Normal human foreskin keratinocytes from two different donors were infected in two independent experiments with E7 alone, wtE6 and E7, or 38E6Ala80-84 and E7 (Fig 5D). For the first 20 days all transfected cells displayed a slow proliferation rate, comparable to normal keratinocytes followed by a period of 30-50 days without proliferation. Then a phase of exponential cell proliferation only in cells infected with wildtype HPV38 E6 and E7 started to continue for more than 15 passages. In contrast, cells transduced with HPV38 E7 alone or E7 together with 38E6Ala80-84 did not proliferate, but continuously detached from the culture dish until all cells were lost. From this data we conclude, that p300 binding is essential for immortalization of primary human keratinocytes by HPV38 E6/E7.

Discussion

While epidemiological studies hint to a possible role for HPV types of the β2-genus in the development of skin cancer (8) the underlying molecular mechanisms are not understood in great detail. Recent work has suggested that the E7 proteins of CRPV and the skin-cancer associated ß2-subgenus type HPV38 interfere with pRB function similar to HPV16 and 18 (12, 22). In contrast, very little is known about how these viruses prevent p53 mediated apoptosis provoked by E7-activated E2F proteins (41). The E6 proteins of both viruses lack the ability to degrade p53 (21, 22), indicating that they rely on alternative mechanisms to inactivate p53.

To address differences or mutations in the rabbit p53 that shares 85% aa identity with human p53, we analyzed rp53 DNA sequences from the immortalized rabbit keratinocytes and tumors of different stages and found only silent mutations in comparison to the reference (NM_001082404; data not shown). Furthermore, in this report we demonstrate that rp53 is very similar to hp53 with regard to p21 and apoptosis induction and the dependance upon acetylation by p300.

Both E6 of CRPV and HPV38 were able to prevent p300-mediated acetylation of p53 at lysine 382, probably through a direct interaction with p300. Interestingly we were able to detect weak p53 acetylation in Saos-2 cells transfected with p53 without using HDAC inhibitors that are commonly used to detect p53 acetylation. (39)

Recently it has been shown that loss of acetylation completely abolishes p53-dependent growth arrest and apoptosis (33). Acetylation of p53 abrogates Mdm-2 mediated repression by blocking the recruitment of Mdm2 and Mdmx to p53 responsive promoters (42-44). The six carboxy-terminal lysines of human p53 including K382 are acetylated by p300/CBP (45). In addition, lysine-120 is acetylated by Tip60/hMOF and lysine-164 by p300/CBP (33).

Whereas it seems that some acetylation defects in human p53 can be compensated by the modification of other sites, loss of acetylation at all major sites completely abolishes the ability of human p53 to activate p21 (33). In contrast just the rp53K380R mutation showed a major effect on p21 and apoptosis induction in Saos-2 cells, which could indicate a central role of this lysine for rabbit p53-induced apoptosis. As seven out of eight possible lysines of human p53 can be acetylated by p300/CBP (33, 45) and we demonstrated that E6 prevents p300-mediated acetylation it is conceivable that also the acetylation of other lysines by p300 is impeded. This needs to be tested in further experiments. In that case, however, lack of acetylation at the C-terminus may allow increased ubiquitination of p53 by Mdm2 and thereby affect p53 stability, which is clearly not the case in CRPV or HPV38 immortalized keratinocytes. The ubiquitin ligase activity of Mdm2 could, however, at least in CRPV immortalized cells be inhibited by the high rp19Arf levels (12). p53 hypoacetylation mediated by E6 could then allow binding of p53 together with Mdm2 and Mdmx to p53-responsive promoters and thereby prevent transcription of proapoptotic genes, which would explain the effects we observed in this work. In addition it has been recently shown that acetylation at K320, K373 and K382 is required for the transcription independent pro-apoptotic functions of p53 at the mitochondrion (46). Whether this also contributes to the anti-apoptotic effects of CRPVE6 remains to be determined.

Two known CRPV strains with low or high tumor progression potential show aa exchanges (Q225L or G200D) in the carboxy-terminal part of E6 (47), which is the newly identified p300 interaction domain. The reduced carcinogenicity of the E6G200D genome probably results from a decreased E6-p300-interaction as indicated by our data and as a consequence increased expression of p53-dependent proapoptotic target genes.

The highest similarity to the p300-binding domain of CRPV among cutaneous HPVE6 proteins was found with HPV38E6. This was notable, as HPV38 and CRPV are the only cutaneous PVs, that are able to immortalize normal keratinocytes. Consistent with the results obtained with CRPV, immortalization of primary keratinocytes by HPV38E6/E7 is dependent on the E6-p300-interaction. It was reported previously, that p53 accumulates in HPV38 E6/E7 immortalized keratinocytes and induces ΔNp73, but is unable to displace ΔNp73 from p53 responsive promoters (24). This is consistent with our findings as binding of p53 to its responsive promoters is dependent upon the acetylation state (45, 48). The observed lack of immortalization of keratinocytes by a p300-binding deficient HPV38E6 could be due to acetylation of p53 by p300 which then can overcome the ΔNp73 blockage and induce senescence or apoptosis. Interestingly the cutaneous PV types behave similar to the adenoviral E1A protein, which binds to the TAZ2 domain of p300/CBP and competes with p53 for p300 binding (49). Even though the binding domain of E1A shows only little overall homology to the CRPV or HPV38 E6 p300 binding domain, all three proteins contain a hydrophobic residue followed by phenylalanine. One difference between CRPVE6 and the cutaneous HPVE6 proteins is the ability of the latter ones to bind E6AP, which makes them capable of degrading the UV-induced Bak protein to escape induction of apoptosis. For CRPV such a function is not required as the fur of the rabbit protects the skin from UV-irradiation.

It has been described that genital HPV types that inactivate p53 via E6AP-dependent degradation also prevent p300-mediated acetylation of p53. However, these E6 proteins differ from CRPV and HPV38 E6 because they can bind to p53 and form trimeric complexes with p53 and p300 (28). The HPV16E6 protein interacts with the N-terminal CH1 and with C-terminal regions of p300/CBP, including the TAZ2 domain (27, 29). The mechanism of p53

inactivation obviously differs from E1A, as HPV16E6 still requires p53 binding to repress its transcriptional activity. Furthermore the interaction domain described for HPV16E6 with p300 is different from the one we identified for CRPV and HPV38E6. For HPV16E6 residue G134 within the second zinc finger domain was described to be important (28). However, this mutation has not only been described to be impaired for p300 binding, but also for E6AP and p53 binding and degradation (38). The benefit of p300-mediated p53 inactivation for HPV16 and 18 is puzzling as they, in contrast to CRPV and HPV38 E6, predominantly inactivate p53 via degradation, suggesting that repression of p300-mediated p53 acetylation might be necessary for its ubiquitination by E6AP. This was reported for Mdm2-mediated p53 degradation (50). Alternatively, p300-inhibition might be necessary to maintain residual undegraded p53 in an inactive state prior to its degradation.

In summary this is the first report demonstrating that the E6 protein of skin cancer-associated papillomaviruses mainly target p53 acetylation and not degradation of p53 via binding to p300 to immortalize primary keratinocytes and to induce tumors in an *in vivo* situation.

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Table 1: Sequence comparison of HPV16 and CRPV E6.

Type	Position	Sequence
CRPVLE6	132-140	QWRGHCSSC
CRPVSE6	36-43	QWRGHCSSC
HPV16E6	132-139	RWTGRCMSC

Table 2: *In-vivo* papilloma growth *

Time post infection [Days]	Animal	Infected with	Number of injection sites with papillomas >3 mm diameter
39	A	pLA2-CRPV	6/6
		pLA2-CRPV E6Ala195-199	0/6
		pLA2-CRPV E6Ala200-204	0/6
		pLA2-CRPV E6Ala210-214	6/6
38	B	pLA2-CRPV	6/6
		pLA2-CRPV E6Ala195-199	0/6
		pLA2-CRPV E6Ala200-204	0/6
		pLA2-CRPV E6Ala210-214	6/6
55	A	pLA2-CRPV	6/6
		pLA2-CRPV E6Ala195-199	0/6
		pLA2-CRPV E6Ala200-204	0/6
		pLA2-CRPV E6Ala210-214	6/6
54	B	pLA2-CRPV	6/6
		pLA2-CRPV E6Ala195-199	0/6
		pLA2-CRPV E6Ala200-204	0/6
		pLA2-CRPV E6Ala210-214	6/6

* see figure 4A

Figure legends

Figure 1: CRPVE6 represses p53 mediated p21 induction and interacts with p300

A) Cutaneous PV E6 proteins do not reduce p53 stability.

Human HPV16E6/E7-, HPV 38E6/E7- or rabbit CRPV E6/E7-immortalized keratinocytes were treated with cycloheximide (CHX) or MG132 and p53 levels were analyzed at different timepoints by immunoblotting.

B) RNA was isolated 48h post transfection from Saos-2 cells transiently transfected with human hp53, rabbit rp53 and CRPVE6 expression vectors. Induction of p21 mRNA was analyzed by quantitative real time PCR (** = p-value <0.005).

C) E6-MBP fusion proteins purified from *E. coli* were incubated with HEK293T cell lysates. Eluates were analyzed by western blotting.

D) upper panel: MBP-pulldown analysis of CRPVSE6 and the G38V mutant with p300. Lower panel: MBP-fusion protein input determined by Coomassie stain. Aliquots of the purified, immobilized MBP proteins were taken prior to the incubation with HEK293T lysate.

Figure 2: Identification of the p300 binding site in CRPVE6

A) MBP interaction assay using SE6 fragments, wildtype SE6 proteins and MBP.

B) Alanine scanning mutagenesis of CRPVSE6 identifies residues 195-204 as p300 interaction domain. The amino acids within the mutant proteins were changed to alanine at the positions (Pos.) indicated. The protein amount for the immunoblots was normalized according to protein concentration.

C) Coimmunoprecipitation analysis of untagged, HA-tagged wildtype and mutant E6 with p300.

D) Localization of mutant and wildtype CRPVE6 proteins. Expression vectors for E6 and HA-tagged E6, E6 Ala195-199 and E6Ala200-204 proteins lacking the start codon of SE6 were transfected into C33A cells. Localization of E6 proteins was analysed by indirect immunofluorescence using an HA-antibody.

Figure 3: CRPVE6 prevents apoptosis induction by p53 via p300-dependent acetylation of p53

A) Saos-2 cells were transfected with 4 different p300-siRNA and tested for p300 protein levels (upper graph) by western blotting and the most effective siRNA (#2) was tested for p300 transcript reduction (middle graph) by quantitative real time PCR. Lower graph: analysis of p53-dependent p21 RNA induction in the presence of siRNA-2 for p300.

B) Acetylation of K380 is necessary for transcriptional activity of rp53. Saos-2 cells were transfected with expression plasmids for rp53 or rp53K380R and p21 transcript levels were determined by qRT-PCR (** = p-value <0.005).

C) CRPVE6 prevents hp53 acetylation by p300. Saos-2 cells were transfected with hp53, CRPVE6 and CRPVE6Ala200-204 and total p53 or K382-acetylated p53 protein levels were determined by immunoblotting (upper panel) and quantified (lower panel) relative to Saos-2 cells transfected only with p53 using the Quantity One quantification software.

D) Saos-2 were transfected with pcDNA3.1, hp53, rp53 or rp53K380R expression vectors. In addition, rp53 was cotransfected with E6 or E6Ala200-204. Apoptotic cells were identified by TUNEL staining.

left panel: One representative field of stained cells.

right panel: Quantification of three independent experiments. Highly significant differences (p-value <0.005) were marked with **.

Figure 4: Binding of CRPVE6 to p300 prevents apoptosis and contributes to tumor formation *in vivo*.

A) Two rabbits were infected with pLA2-CRPV, CRPVE6Ala210-214, pLA2-CRPVE6Ala195-199 or pLA2-CRPVE6Ala200-204 and pictures of the tumors were taken 39 and 55 days post infection. The few single very small papilloma that were formed by p300 binding deficient genomes were indicated with arrowheads.

B) Sections of tumors removed after 3 months were TUNEL stained to detect apoptotic cells.

Figure 5: HPV38E6 interaction with p300 in necessary for cell immortalization

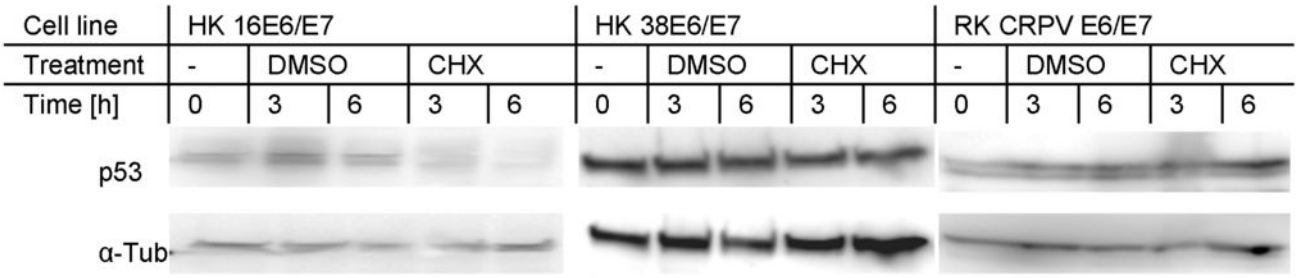
A) HPV38E6 and E6Ala80-84 were tested for p300 binding by MBP pulldown assay.

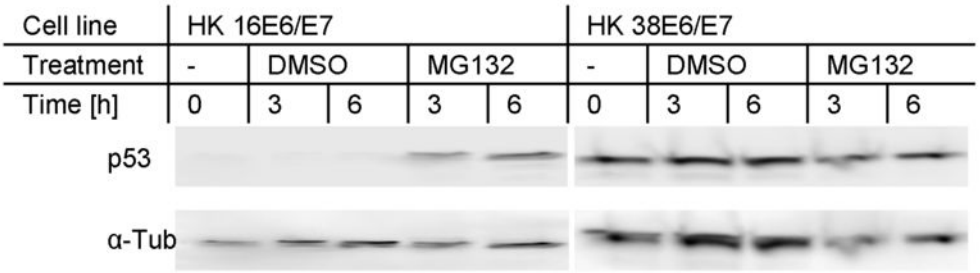
B) Saos-2 cells were transfected with hp53, HPV38E6 or HPV38E6Ala80-84 and total p53 or K382-acetylated p53 protein levels were determined by immunoblotting. Lower panel shows quantification of acetylated p53 relative to total p53 levels.

C) Expression vectors for hp53 and for HPV38 E6 or HPV 38E6 Ala80-84 were cotransfected and p21 RNA levels were determined by qRT-PCR.

D) Immortalization of normal human keratinocytes by HPV38E6/E7. Cells were infected with HPV38E6 and E7, HPV38E6Ala80-84 together with E7 or E7 alone. After selection, cells were passaged and total cell number was counted and extrapolated.

Fig 1 A





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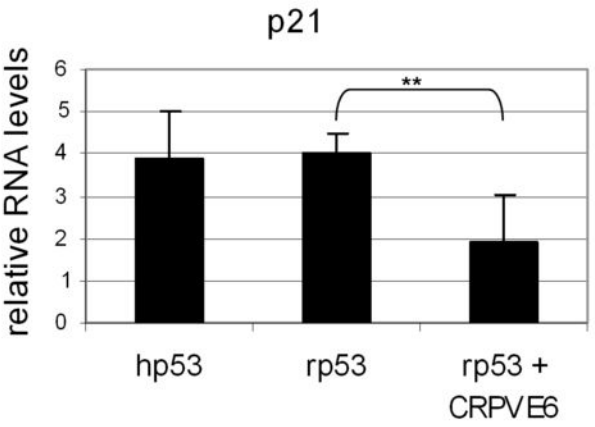

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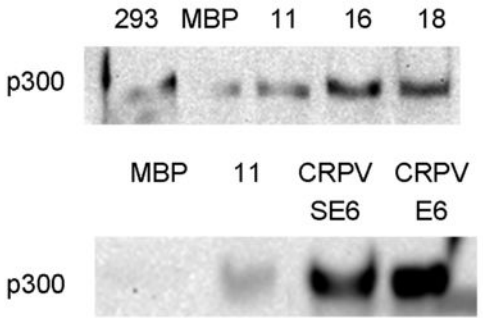

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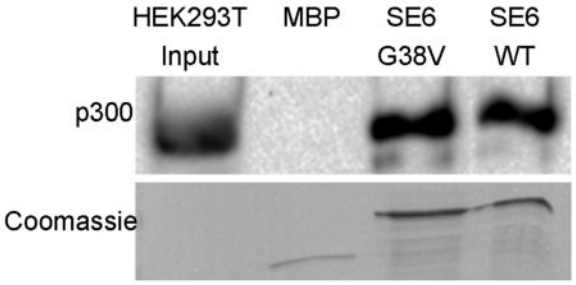


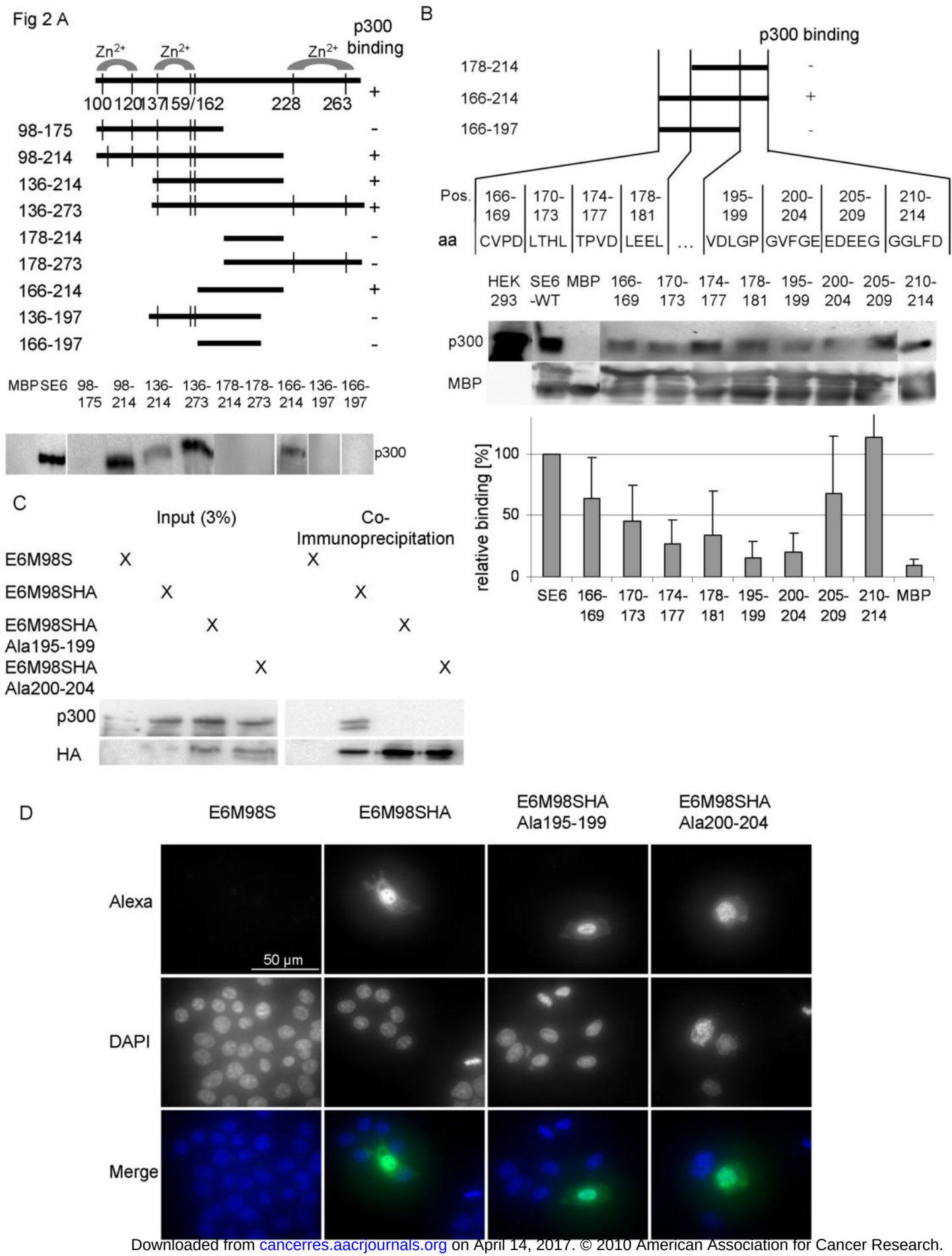
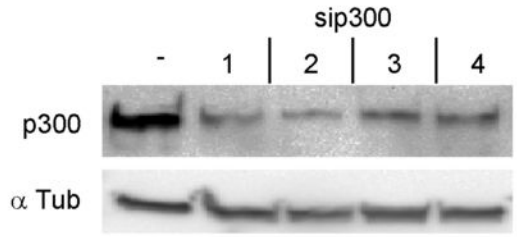
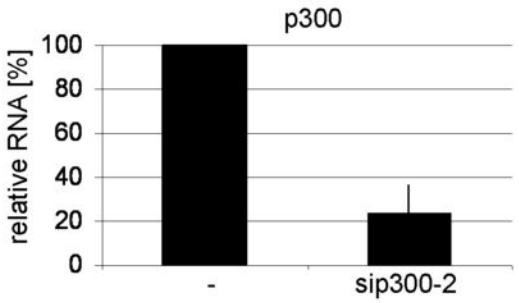
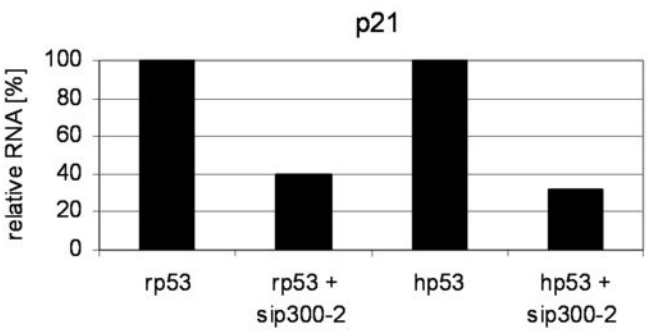


Fig 3 A







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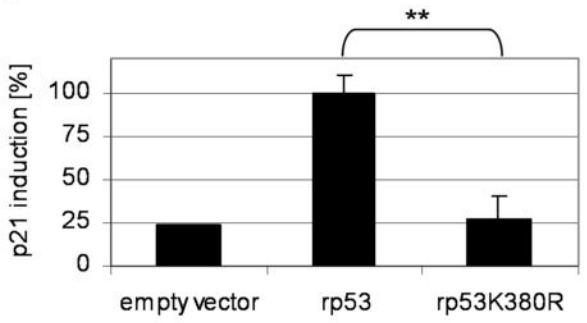

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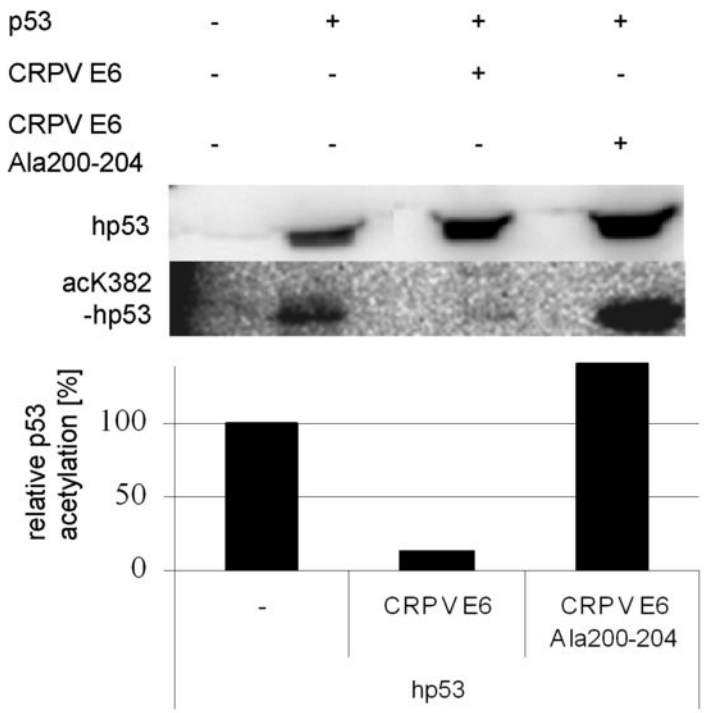

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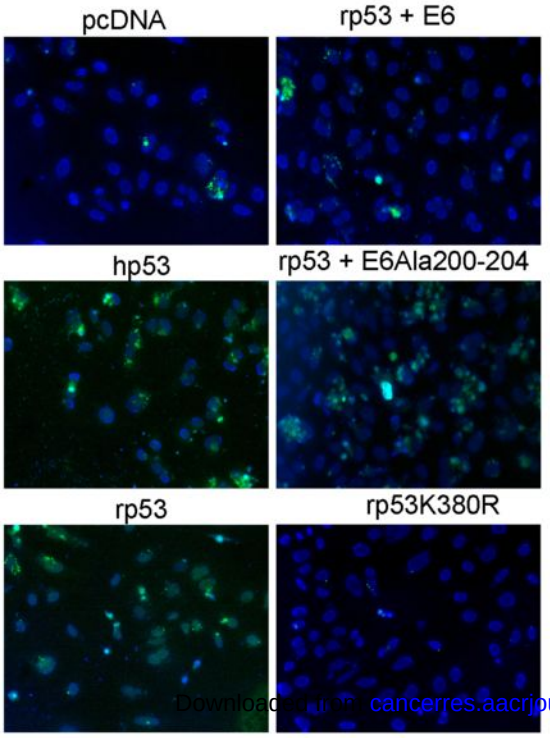


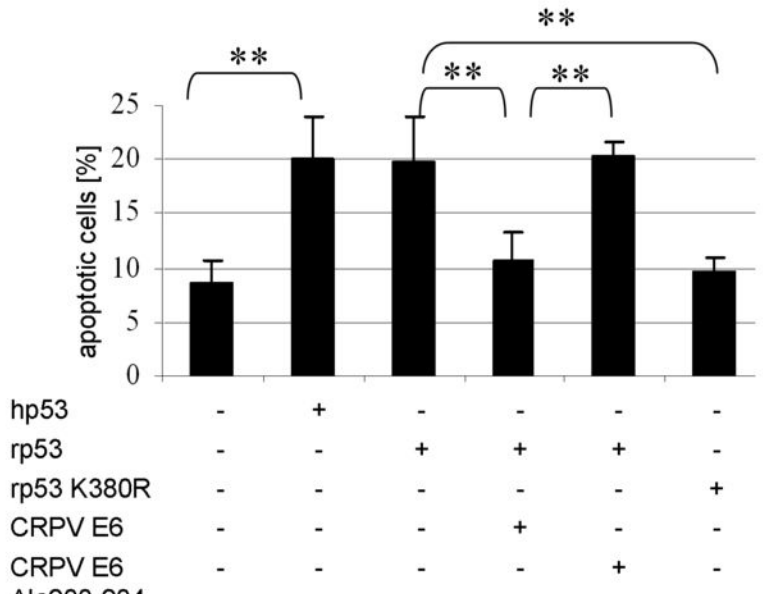
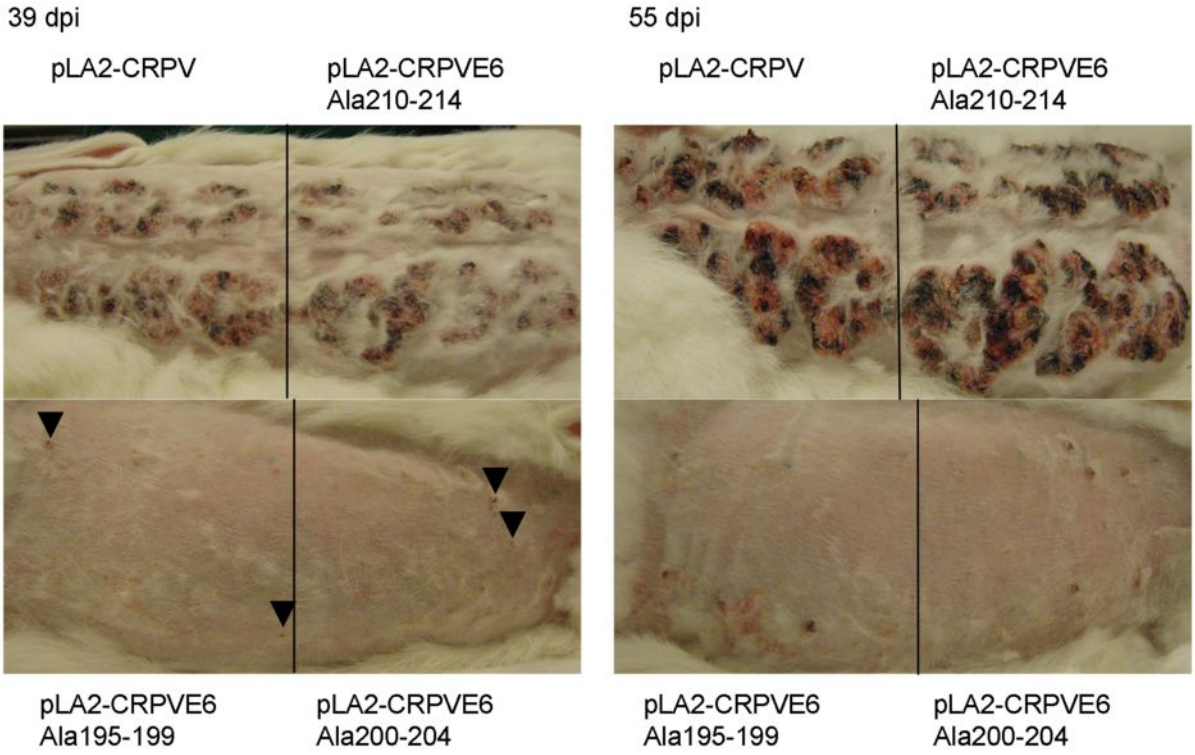


Fig 4 A



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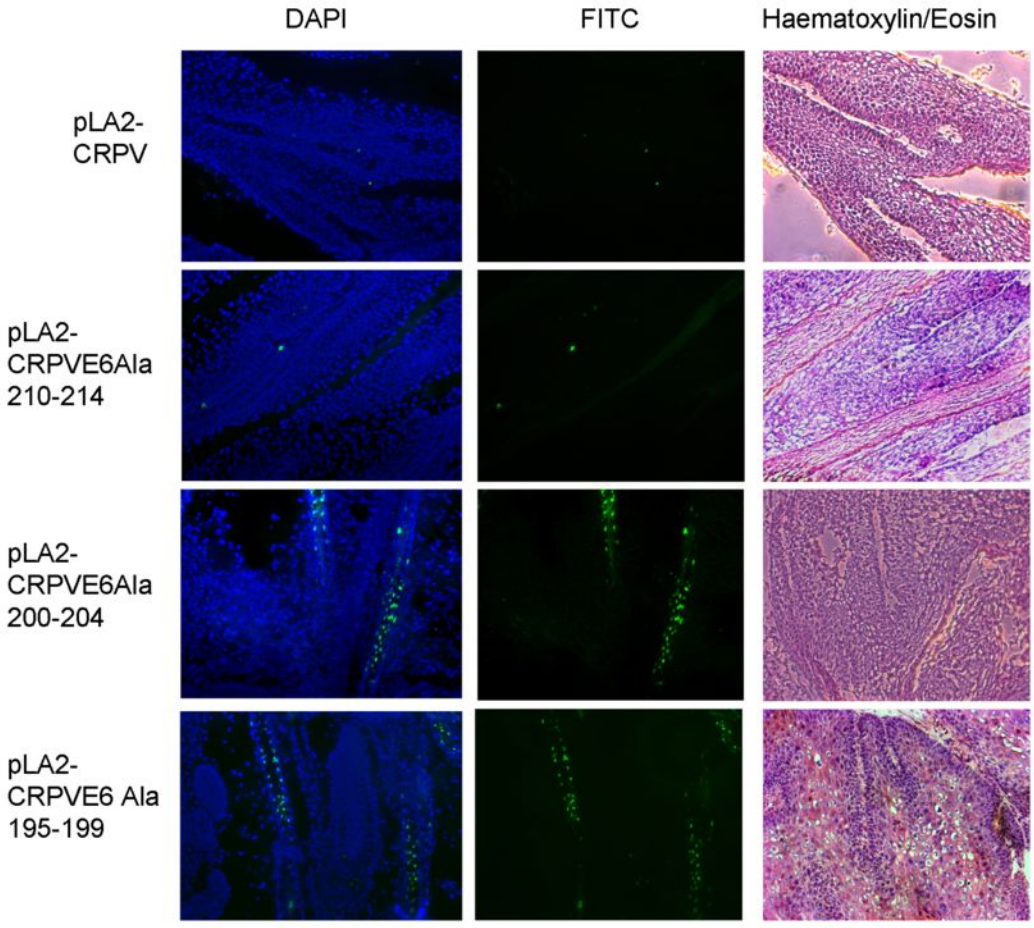
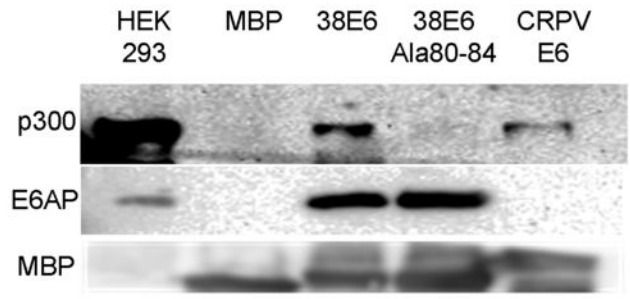


Fig 5 A



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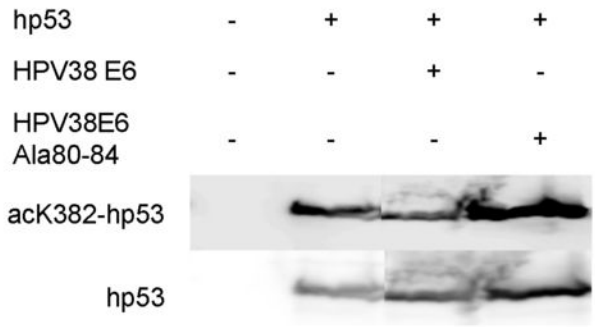

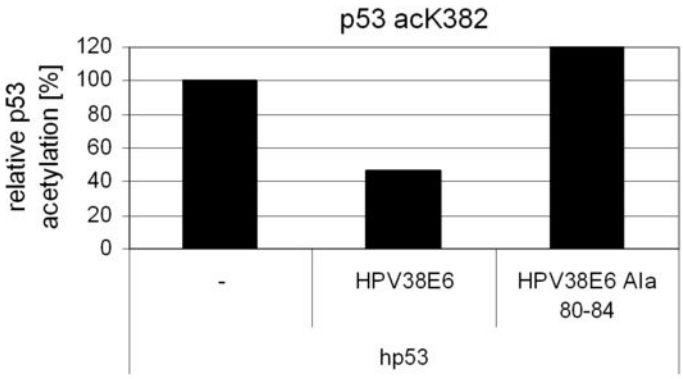

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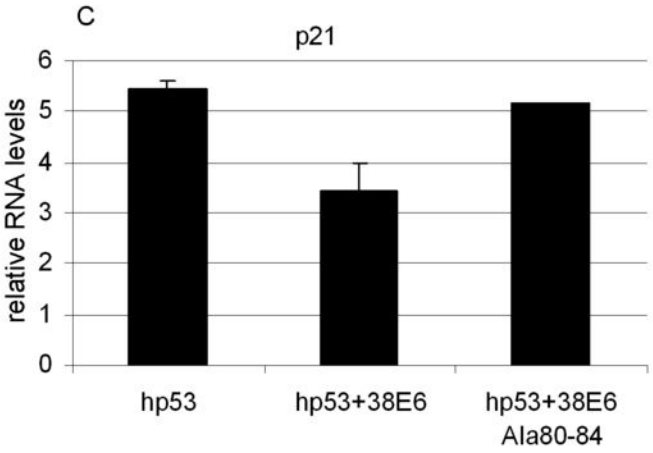

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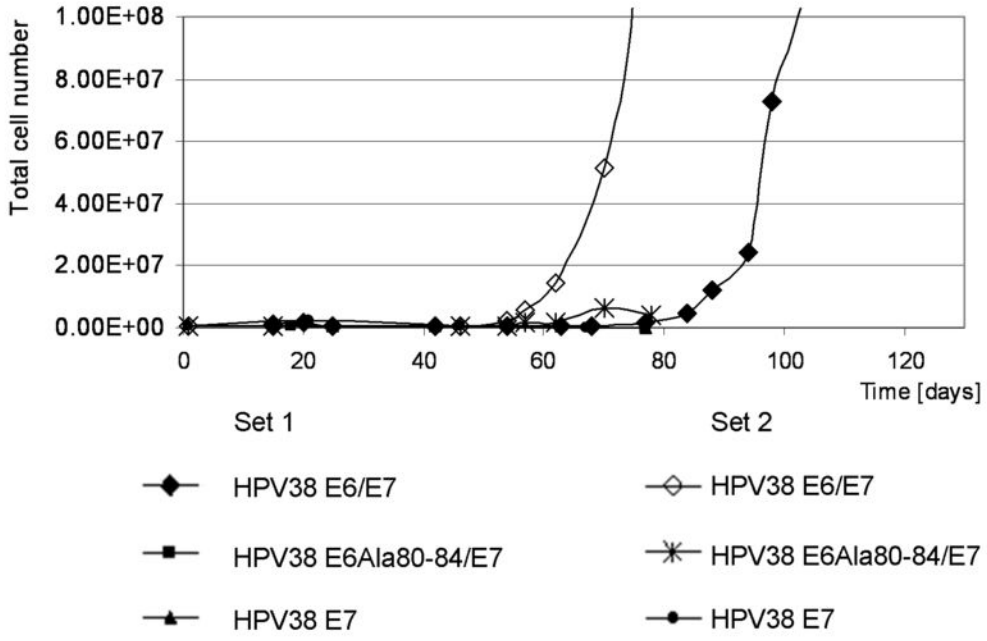

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Cutaneous papillomavirus E6 proteins must interact with p300 and block p53-mediated apoptosis for cellular immortalization and tumorigenesis

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