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The E5 protein of human papillomavirus type 16 selectively down-regulates surface HLA class I

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Abstract

Papillomaviruses have evolved mechanisms that result in escape from host immune surveillance. The E5 protein is expressed early in papillomavirus infection in the deep layers of the infected epithelium. It is localised to the Golgi apparatus (GA) and endoplasmic reticulum. The E5 protein of bovine papillomavirus (BPV) impairs the synthesis and stability of major histocompatibility (MHC) class I complexes and prevents their transport to the cell surface due to retention in the GA. Here we show that human papillomavirus type 16 (HPV-16) E5 also causes the retention of MHC (HLA) class I complexes in the GA and impedes their transport to the cell surface, which is rescued by treatment with interferon. Unlike BPV E5, HPV-16 E5 does not affect the synthesis of HLA class I heavy chains, nor the expression of the transporter associated with antigen processing TAP. These results show that down-regulation of surface MHC class I molecules is common to both BPV and HPV E5 proteins. Moreover, we determined that HPV-16 E5 down-regulates surface expression of HLA-A and HLA-B, which present viral peptides to MHC class I-restricted cytotoxic T lymphocytes (CTL), but not the natural killer (NK) cell inhibitory ligands HLA-C and HLA-E. Selective down-regulation of cell surface HLA class I molecules may allow the virus to establish infection by avoiding immune clearance of virus-infected cells by both CTL and NK cells.

Introduction

Papillomaviruses (PV) are small DNA tumour viruses which infect the epithelia of humans and animals causing benign hyperproliferative lesions. In most cases PV infections are cleared after several months following activation of the host immune system against viral antigen [1]. However, occasionally the lesions do not regress and can progress to cancer. Certain PV are more commonly associated with malignancy, including the human PV (HPV) types 16 and 18, "high risk" viruses for the development of cervical cancer in women [2], and bovine PV (BPV) type 4, associated with carcinomas of the alimentary canal in cattle [3]. Persistent viral infection is required for neoplastic progression and failure of virus clearance is attributed to a poor immunological response.

The PV genome encodes three transforming proteins, E5, E6 and E7. E5 is a small hydrophobic protein ranging in size from 42 amino acid residues in BPV-4 to 83 amino acid residues in HPV-16. E6 and E7 are the main transforming proteins of HPV [4, 5]; E5 is the major transforming protein of BPV and plays a lesser role in transformation by HPV [6]. While E6 and E7 are expressed throughout the course of the disease and are necessary for the maintenance of a transformed phenotype, E5 is expressed only during the early stages of infection and its expression is often, but not always, extinguished as the lesion progresses toward malignancy [6]. These characteristics point to a role of E5 in establishment of PV infection and the initiation of cell transformation.

The E5 protein is localized in the Golgi apparatus (GA), endoplasmic reticulum and occasionally the plasma membrane of the host cell. Its localisation in the endomembrane compartments, where it interacts with the vacuolar ATPase 16k ductin/subunit c [7-9], is deemed responsible for the lack of acidification of the GA and endo-lysosomes and the consequent impaired functions of these organelles [10, 11].

Deleted: epithelial

We have shown that one of the outcomes of BPV E5 expression in primary cells is the retention of major histocompatibility (MHC) class I complexes in the GA and the inhibition of their transport to the cell surface [12, 13]. Furthermore, BPV E5 inhibits both transcription of the MHC class I heavy chain gene and affects the stability of the heavy chain protein [12]. In this study we show that HPV-16 E5 also prevents the transport of MHC (HLA) class I complexes to the cell surface due to retention in the GA. Moreover, we show that HPV-16 E5 selectively down-regulates HLA-A and HLA-B molecules on the cell surface but does not affect the transport of HLA-C and HLA-E. These studies identify a potential novel mechanism by which PV-infected cells may avoid clearance by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, aiding in the establishment and persistence of PV infection.

Materials and Methods

HPV-16 E5 expression constructs.

The E5 ORF was cloned into three expression plasmids: pcDNA3 (Invitrogen, Glasgow, UK), under the transcriptional control of the universal cytomegalovirus (CMV) immediate early promoter (pc-16E5); pL2, under control of the Epstein-Barr virus (EBV) ED-L2 promoter, active only in epithelial cells [14] (pL2-16E5), and the retrovirus expression plasmid pLZRSpBMLZ (Clontech, UK). Amphotropic retrovirus expressing HPV-16 E5 (RT-16E5) was generated by transient transfection of the packaging cell line Phoenix [15] as previously described [12].

Establishment of HPV E5-expressing cell lines

The immortalised human keratinocyte HaCaT cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) without $CaCl_2$ (Invitrogen), supplemented with 1mM sodium pyruvate, 2mM glutamine and 10% fetal calf serum (FCS) at 37°C in 5% CO2. Primary bovine PalF cells [12] and NIH 3T3 cell lines were grown in DMEM, 10% FCS at 37°C in 5% CO_2.

HaCaT cells were stably transfected with 4 µg of pcDNA, pL2, pc-16E5 or pL2-16E5 per $1x10^6$ cells using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. Following transfection, the cells were selected in DMEM containing 500µg/ml G418 (Invitrogen) for 21 days. After this time, G418-resistant colonies were marked, individually picked and expanded into clonal cell lines for analysis.

The retrovirus RT-16E5, or its empty counterpart (generated using wild-type pLZRSpBMLZ plasmid), was used to infect primary PalF cells as described [12] and the cells were analysed two days later.

NIH3T3 cells expressing HPV-6b E5 or HPV-16 E5 under the control of the mouse moloney leukaemia virus (MMLV) LTR in pZip-neo, were a kind gift from Dr Show-Li

Chen (National Defence Medical Center, Taipei, Taiwan) and Prof Richard Schlegel (Georgetown University, Washington, USA), respectively.

Quantitative RT-PCR

Total RNA was isolated from HaCaT cells using the RNeasy Mini kit (Qiagen, Crawley, UK), and residual DNA was removed by DNase I treatment (Invitrogen). Real-time RT-PCR for HPV-16 E5 and β-actin mRNA was carried out using the Taqman EZ RT-PCR kit (Applied Biosystems, USA). Each reaction was performed in triplicate using 100 ng of RNA. Oligonucleotide primers, designed using Primer Express (v1.7, Perkin-Elmer, UK) were as follows: 16E5wt F 5'-TGACAAATCTTGATACTGCATCCA-3'; 16E5wt R 5'-CTGCTGTTATCCACAATAGTAATACCAATA-3'; and a FAM/TAMRA probe 5'-AACATTACTGGCGTGCTTTTTGCTTTGCT-3'. Primers and probe sequences for β-actin quantitation were purchased from Applied Biosystems (UK). PCR reactions were performed using an ABI Prism 7700 Sequencer. Standard curves were generated using 10-fold serial dilutions of each template DNA, which were used to quantitate the relative levels of E5 and β-actin mRNA. E5 mRNA levels were normalized according to the β-actin controls.

FACS analysis of MHC class I expression

HaCaT, PalF and NIH3T3 cells were grown in T175 cm^2 flasks until sub-confluent. After removal of the medium, the cells were washed once with PBS, then detached from the flask with trypsin/EDTA and pelleted at 200g for 5 min at room temperature (RT). The cell pellet was resuspended in DMEM, 10% FCS and incubated for 1 hour at 37°C to allow surface antigens to be re-expressed. The cells were then washed and resuspended in PBS/1% bovine serum albumin (BSA) (PBS-B) at a concentration of 10^7 cells/ml. For the detection of surface MHC class I molecules, 100μl of cells were aliquoted and incubated for 1 hour at 4C with an equal volume of monoclonal antibody (mAb) as follows: pan anti-human MHC class I W6/32 (1:100; Serotec, UK), anti-bovine MHC class I IL-A19 (1:1000) [16], anti-mouse H-

2L^d CL9011-A (1:50; Cedarlane Laboratories, UK), or anti-HLA-C/E DT9 (1:50; a kind gift from Dr Veronique Braud, Centre National de la Recherche Scientifique, Sophia Antipolis, France) for 30 min at 4°C. Following three washes in PBS-B cells were incubated with 1:100 dilution of anti-mouse IgG-FITC (Sigma, UK) for 30 min at 4°C in the dark. The cells were then washed as above, resuspended in 500µl PBS-B and analysed by flow cytometry. If the flow cytometry analysis was not performed immediately, the cells were resuspended in 500µl of 3% paraformaldehyde in PBS and kept at 4°C. A mouse monoclonal antibody against HPV-16 E2 (TVG261; a kind gift of Dr M. Hibma) was used as negative control (1:50).

For the detection of intracellular MHC class I, the cells were first permeabilised with 0.5% saponin in PBS-B for 30 min at RT. Following a wash in PBS-B, the cells were then incubated with primary antibody as described above.

All samples were examined in a Beckman Coulter EPICS Elite analyser equipped with an ion argon laser with 15 mV of excitation at 488 nm. The data were analysed using Expo 2 software.

Immunofluorescence detection of MHC class I and GA.

In all experiments, HaCaT cells ($1x10^4$) were aliquoted into 24-well plates containing coverslips and grown overnight. After removal of the medium, cells were washed twice with PBS and fixed in 1.85% formaldhehyde in PBS containing 2% sucrose for 10 min at RT. After fixation, cells were washed twice and incubated in permeabilising solution (0.5% NP-40, 10% sucrose in PBS) for 10 min at RT and then washed as above.

For detection of MHC class I, the fixed and permeabilised cells were incubated with 1:50 dilution of W6/32 antibody for 1 hour at RT. Following a further two washes, the cells were incubated with 1:500 dilution of anti-mouse IgG-FITC (Sigma) for 1 hour at 4°C in the dark. For visualisation of the GA, the cells were incubated with mAb 4A3 (1:200) recognising golgin GM130, an integral GA protein [17] for 1 hour at RT. Following two washes

as above, the cells were incubated with 1:1000 dilution of anti-mouse IgG-TRITC (Sigma) for 1 hour at 4°C in the dark.

To analyse the localization of MHC class I in E5-expressing cells, control HaCaT cells (pcDNA and pL2) and E5-expressing cells (pc-16E5 and pL2-16E5) were incubated with mAb 4A3, washed as described above then incubated with anti-mouse IgG-TRITC (1:1000), and FITC-conjugated W6/32 (1:10; Sigma).

In all cases, the cells were washed three times after incubation with secondary antibody, and the coverslips mounted onto slides using Citifluor™. Images were captured using a Leica TCS SP2 true confocal scanner microscope (Leica-microsystems, Heidelberg Germany) and a wavelength of 488nm (MHC class I) or 543nm (GA). The merge between the FITC and TRITC fluorescent signals was achieved using the Leica TCS SP2 accompanying software.

Immunoblotting detection of MHC class I and TAP.

HaCaT cells were removed from the flasks by trypsinisation, washed with PBS, then lysed by sonication in lysis buffer (100mM Tris HCl, pH 7.5, 2% SDS, 20% glycerol) and insoluble material removed by centrifugation at 20,000g for 10 min at 4C. Ten µg of lysate were electrophoresed in 4-12% NuPAGE gels (Invitrogen), and proteins transferred to nitrocellulose membrane using a semidry blotting apparatus at 20V/150A for 1 hour. The membranes were blocked in 5% milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) for 1 hour at RT.

For detection of MHC class I, the membranes were incubated with the following mAb: HC10, specific for HLA class I heavy chain (1:50; a kind gift from Dr Stephen Man, Cardiff University), MEM-E/02 specific for human HLA-E (1:50; Serotec), or AB-1 (1:20,000; Oncogene Research Products, UK) specific for actin. For detection of the transporter associated with antigen processing TAP, the membranes were incubated in 1:1000

dilution of rabbit anti-TAP-1 antibody (Chemicon, UK). After repeated washing with TBST the membranes were incubated with either 1:20,000 dilution of anti-mouse Ig-HRP (Amersham Pharmacia Biotech, UK) for HC10 and MEM-E/02, 1:5000 dilution of anti-mouse IgM-HRP (Oncogene Calbiochem-Novabiochem International, UK) for AB-1, or 1:5000 dilution anti-rabbit IgG-HRP (Sigma), in 5% milk/TBST for 1 hour at RT. The membranes were washed three times with TBST and bound antibody detected by enhanced chemoluminescence (ECL) (Amersham Pharmacia Biotech).

Treatment of cells with interferon

One million parental HaCaT cells, control cells expressing pcDNA3 or pL2, and cells expressing HPV-16 E5 were seeded in T175 cm^2 tissue culture flasks. The following day, the medium was replaced with fresh medium with or without 500 U/ml β-IFN (Sigma). After 48 hours, the cells were harvested for detection of MHC class I by immunofluorescence or flow cytometry analysis as described above.

Results

Detection of E5 expression in cell lines

As E5 is expressed at very low levels in cells and there are no reliable antibodies against the protein, it is very difficult to detect its expression by immunoblotting [18]. Therefore we instead confirmed that the E5 ORF was being transcribed using quantitative RT-PCR. Ten clones from each HaCaT cell transfection with pcDNA, pL2, pc-16E5 or pL2-16E5 were picked and expanded into cell lines for analysis. RNA was isolated and the relative level of E5 mRNA expression was determined by comparison to β-actin mRNA. The results of 6 clones expressing HPV-16 E5 (3 as pc-16E5 and 3 as pL2-16E5) are shown in Figure 1. Although low (four orders of magnitude less than that of β-actin), all of the cell lines expressed E5 mRNA. The amount of E5 mRNA was comparable among each cell line, ranging from approximately 0.01-0.02 pg per 100ng of RNA. The levels of E5 mRNA in HaCaT keratinocytes are approximately one hundred-fold lower than those in W12 cells (derived from cervical intraepithelial neoplasia) [19], which express E5 from the resident multi-copy episomal HPV-16 genome (data not shown). This observation therefore excludes the possibility that any effect seen in HaCaT cells is due to E5 over-expression.

Down-regulation of surface HLA class I in cells expressing HPV E5

Given that BPV E5 can inhibit transport of MHC class I molecules to the cell surface [12, 13] we investigated whether HPV-16 E5 could likewise down-regulate surface HLA class I. Using FACS analysis, we determined the levels of HLA class I in HaCaT cell lines expressing HPV-16 E5 under the control of the CMV promoter (pc-16E5) or expressing E5 under the control of the epithelial-specific EBV promoter (pL2-16E5). HaCaT cells harbouring empty plasmids expressed similar levels of HLA class I as parental HaCaT cells, with approximately twice as much total (surface plus intracellular) HLA class I than surface alone (Figure 2A). In contrast, all of the pc-16E5 and pL2-16E5 HaCaT cell lines analysed

had reduced levels of surface HLA class I, approximately half that of the control or parental cells, whereas the level of total HLA class I remained constant (Figure 2A). This effect was highly reproducible, and specific as no signal above background (secondary antibody only) was detected when an anti HPV-16 E2 antibody was used (Figure 2C). Furthermore, we showed a marked reduction of surface MHC class I in NIH 3T3 mouse fibroblasts expressing HPV-6 E5 or HPV-16 E5 under the MMLV LTR, and in primary bovine PalF cells acutely infected with recombinant retrovirus expressing HPV-16 E5 (RV-16E5) (Figure 2B). These results show that down-regulation of MHC class I is stimulated by E5 proteins encoded by both BPV and HPV, including low risk (HPV-6) and high risk (HPV-16) viruses. Moreover, stimulation of MHC class I down-regulation in primary cells (PalF) shows that this effect is not due to the immortalised phenotype of the HaCaT keratinocytes.

<u>HLA class I is retained in the GA in E5-expressing epithelial cells</u>

To ascertain the intracellular localisation of HLA class I, E5-expressing HaCaT cell lines, parental and control cell lines, were co-stained with the antibodies W6/32 (pan MHC class I) and 4A3 (anti-golgin). In the control cells, HLA class I was expressed both on the cell surface and in the GA (Figure 3A). Identical staining patterns were observed in parental HaCaT cells (data not shown). In contrast, in HPV-16 E5-expressing cells HLA class I was detected almost exclusively in the GA (Figure 3B). These results show that HPV-16 E5 prevents the HLA class I complex from reaching the cell surface, and retains it in the GA .

<u>Expression of HLA class I heavy chain is not inhibited by HPV-16 E5</u>

The results above suggested that, in contrast to BPV E5, HPV-16 E5 did not have any effect on the overall levels of the HLA class I heavy chain. To confirm this observation, we determined the relative levels of HLA class I in the control and E5-expressing HaCaT cell lines using mAb HC10, specific for the human HLA class I heavy chain [20]. Although the levels of HLA class I heavy chain were slightly lower in the pL2 and pL2-16E5 cell lines

than in the pcDNA and pc-16E5 cell lines, there were no significant differences between the cells expressing HPV-16 E5 and their respective control cells (Figure 4), confirming that HPV-16 E5 does not down-regulate expression of the HLA class I heavy chain.

E5 has no effect on TAP expression.

Transport of class I complexes to the cell surface is prevented if the transporter associated with antigen processing (TAP) is malfunctioning [21]. Therefore it was important to establish whether E5 was affecting HLA class I transport by inhibiting TAP expression, as reported for HPV-11 E7 [22]. TAP-1 protein was investigated in control and E5-expressing HaCaT cell lines by immunoblotting. There appeared to be a lower level of TAP-1 in the pL2 cells but there was no significant reduction of TAP-1 in cells expressing E5 (Figure 4), indicating that down-regulation of surface HLA class I is not due to an ability of E5 to down-regulate TAP-1. An effect on the functionality of TAP cannot however be ruled out.

Treatment of E5 cells with interferon rescues HLA class I traffic to the cell surface.

β-interferon (β-IFN) increases transcription from the MHC class I heavy chain gene promoter [23] leading to higher expression of heavy chain. To determine if increased synthesis of heavy chain led to an increase in transport of HLA class I complexes to the cell surface, we treated HaCaT parental, control and E5-expressing cells with β-IFN, and analysed HLA class I expression and localisation by FACS and immunofluorescence. Treatment with β-IFN increased the total amount of HLA class I approximately two-fold in all of the cell lines tested (Figure 5A, cf. with Figure 2A). Moreover, we found an approximately two-fold increase in surface HLA class I in the parental and control cells, and an approximately four-fold increase in the E5-expressing cells (Figure 5A, cf. with Figure 2A). This resulted in all of the cell lines, including those expressing HPV-16 E5, as having similar levels of surface HLA class I. β-IFN treatment did not affect expression of E5 (data not shown) and therefore the observed increase in HLA class I levels cannot be attributed to changes in E5 expression.

Unlike in BPV E5-expressing cells [13], β-IFN treatment therefore appeared to overcome the block exerted by E5 on HLA class I transport. To confirm this observation, HaCaT cells carrying empty vector or expressing HPV-16 E5, untreated or treated with IFN, were incubated with mAb W6/32 and analysed for HLA class I localisation. In the untreated E5-expressing cells, HLA class I was mostly detected in the GA, as before (Figure 5B). In contrast, HLA class I was detected on the surface of E5-expressing cells treated with IFN. These experiments show that HPV-16 E5-expressing cells are responsive to IFN and that the E5-induced HLA class I transport inhibition is reversible by IFN.

HLA-C/E expression is not inhibited by HPV-16 E5

While MHC class I molecules HLA-A and -B are the main presenters of antigenic peptides to CTL, HLA-C and non-classical MHC molecules, such as HLA-E, inhibit NK cell-mediated lysis by interacting with inhibitory NK receptors [24-26]. To determine whether HPV-16 E5 could selectively down-regulate HLA class I molecules, we determined the levels of HLA-C/E in parental, control and E5-expressing HaCaT cell lines. Cells were stained with mAb DT9 that recognises both HLA-C and -E, and were analysed by flow cytometry for surface and total HLA-C/E. Although the shift in forward fluorescence was small (Figure 6A,B), in agreement with the observation that human fibroblasts have little HLA-E [27], it was consistently higher than background (secondary antibody only; Figure 6B), and higher than the readings obtained with an unrelated antibody (Figure 2C); Importantly, there were no significant differences between the control and E5-expressing cells (Figure 6A).

In addition, we determined the cellular localisation of HLA-C/E in the E5-expressing cells by immunofluorescence using mAb DT9. There were no differences between the staining patterns of HLA-C/E in the control cells and in the E5-expressing cells (Figure 6C), and therefore we conclude that expression of E5 does not lead to any appreciable decrease in surface HLA-C/E, in agreement with the FACS data.

Finally, we determined the levels of total HLA-E by immunoblotting with mAb MEM-E/02, specific for HLA-E. Similarly, we did not detect any significant differences between HLA-E levels in the control cells and the E5-expressing cells (Figure 6D).

The ease of HLA-C/E detection by immunofluorescence and immunoblotting compared with flow cytometry is likely attributable to the different affinities of the two antibodies for HLA (mAb DT9 and MEM-E/02) and to the greater sensitivity of mAb DT9 in immunofluorescence.

These results confirm that HPV-16 E5 down-regulates the surface expression of the classical HLA class I molecules HLA-A and -B, but not HLA-C or -E. We are unable to discriminate between HLA-C and HLA-E as mAb DT9 recognises both molecules, and mAb MEM-E/02, specific for HLA-E, does not function in flow cytometry or immunofluorescence.

Discussion

Progression from acute HPV infection to malignancy requires persistence of virus, which in turn appears to depend on several factors, including the genetic background of the host [28,29], environmental co-factors [30] and the ability of the virus to avoid immune clearance[31]. The immune system plays a decisive role in determining the clinical outcome of HPV disease, as demonstrated by the increased persistence and enhanced neoplastic progression of HPV infections in hosts with cell-mediated immune deficiencies [32,33]. However, even in immunocompetent individuals, HPV persist for a significant period of time before activation of the host immune system. This lack of recognition suggests the host immune system is unaware of, or disabled by, HPV infection. HPV can subvert the immune response indirectly via the nature of the virus life cycle [34] and by direct interference with the host anti-viral immune mechanisms, including the IFN response and MHC class I antigen presentation to CTL [1, 35].

MHC class I (HLA class I in humans) plays a pivotal role in the eradication of virally infected and transformed cells. The importance of MHC class I in virus clearance is highlighted by the acquisition of numerous mechanisms of interference with the MHC class I pathway by many viruses [36]. Independently of the molecular nature of these mechanisms, the outcome is failure of the infected cells to effectively present viral peptides to effector CTL, resulting in avoidance of detection and destruction.

We have recently shown that both BPV-4 and BPV-1 interfere with the MHC class I pathway, through the retention of MHC class I complexes in the GA by the oncoprotein E5 [1, 12, 13]. Here we show that these properties are not a peculiarity of BPV E5 but are shared by E5 proteins of mucosal HPV, including E5 encoded by the low risk HPV type 6, the etiological agent of genital warts, and HPV-16, the papillomavirus most frequently associated with cervical carcinoma. Since our initial observations were published, it has also been reported

that HPV2a E5 can inhibit HLA class I transport to the cell surface [37]. Therefore, down-regulation of surface MHC class I appears to be a property of many, if not all, papillomavirus E5 proteins.

We found that HPV-16 E5 promotes the retention of HLA class I in the GA, a salient characteristic of cells expressing BPV-4 E5. It is established that E5 proteins bind 16k subunit c, a component of the V_0 sector of the H^+ V-ATPase [7-9] and that a possible outcome of this interaction is the inhibition of acidification of the GA and endosomes [10, 11]. The retention of MHC class I in the GA by BPV-4 E5 is due, at least in part, to the impeded acidification of the organelle, as retention in the GA and down-regulation of surface MHC class I are also caused by monensin, an inhibitor of V-ATPase and GA acidification [13]. The same mechanism may underpin the retention of HLA class I in the GA by HPV-16 E5. However, given the selective down-regulation of HLA types by HPV-16 E5, the lack of GA acidification cannot be the whole explanation and other mechanisms must come into play. These points are currently under investigation.

Despite the similarities, there are also differences between the extent to which BPV-4 E5 and HPV-16 E5 interfere with the MHC class I pathway. BPV-4 E5 down-regulates transcription of the MHC class I heavy chain gene, promotes degradation of the translated polypeptide and blocks the transport of the MHC class I complex to the cell surface [12, 13]. In contrast, HPV-16 E5 does not inhibit expression of the heavy chain, and reduces the transport of HLA class I to the cell surface without completely abolishing it. Furthermore, contrary to what we observed with BPV E5 [13], inhibition of HLA class I transport by HPV-16 E5 is reversible by IFN treatment. The reason for this latter difference is not known but it can be speculated that the increased production of HLA class I heavy chain by IFN is sufficient to overcome the inhibitory effect mediated by the low levels of HPV-16 E5. In contrast, as BPV E5 also inhibits transcription and promotes degradation of the MHC class I heavy chain [12],

IFN treatment is insufficient to restore MHC class I expression to normal levels. These data are also consistent with our hypothesis that there is a correlation between protein oncogenicity and immune evasion [38]. As discussed earlier, BPV E5 is a more effective transforming protein than HPV-16 E5 [6] and therefore would be predicted to have a greater effect on MHC class I down-regulation (and other immune evasion mechanisms) than HPV-16 E5. In contrast, in oncogenic HPV infections, the two major transforming proteins E6 and E7 would complement the inhibitory effect of the lesser transforming protein E5 on the MHC class I pathway. HPV-16 E7 can repress the MHC class I heavy chain gene promoter [39] thus likely replacing the inhibitory action of BPV on the same promoter, and in addition can bind directly to TAP, thus further contributing to the down-regulation of HLA class I [21]. Furthermore, both HPV E6 and E7 can inhibit the type I IFN pathway, thus preventing the IFN-mediated release of E5-induced blockage in HLA class I trafficking [40-42].

Although an efficient mechanism to avoid CTL-mediated immune clearance, the total absence of surface MHC class I renders cells more susceptible to NK cell attack. Human NK cells express multiple receptors that interact with HLA class I molecules, including killer cell immunoglobulin-like receptors (KIR) that predominantly recognise classical HLA class I including HLA-C, and the C type lectin superfamily of receptors that specifically interact with the non-classical class I molecule HLA-E [24-26]. Recognition of the class I molecules by their inhibitory receptors inhibits NK-mediated cell lysis, which would occur in the absence of HLA-C/E. Accordingly, certain viral proteins, including HIV Nef and the US3/UL40 proteins of CMV, have evolved to selectively down-regulate HLA-A and -B, the main presenters of peptides to CTL, but not HLA-C or –E [27,44,45], and are therefore capable of avoiding both CTL and NK cell killing [46]. We show here for the first time that papillomaviruses, in particular HPV-16, may also employ a similar immune evasion strategy via expression of E5. Our experiments show that neither synthesis nor transport to the cell

surface of HLA-C/E is affected by E5 expression, leading to the conclusion that E5 selectively inhibits surface expression of HLA-A and HLA-B. NK cells of patients with HPV-induced anogenital lesions are incapable of specific killing HPV-16-infected cells [47], although the mechanism by which this occurs is as yet unknown. Moreover, concordant with loss of MHC class I molecules that present viral peptides, there is a very low frequency of HPV-specific HLA-A-restricted CTL in patients infected with HPV-16, an order of magnitude lower than those found in other viral infections including influenza A and EBV [48]. It is not yet known whether these observations are the consequence of E5 expression, however experiments to establish the functional outcome of E5 expression on CTL and NK cell recognition of HPV-transfected cells are in progress.

Regardless, our results support the hypothesis that E5 plays a major role in immune evasion by HPV. To this end, it is interesting to note that HPV-16 E5 has also been reported to inhibit both Fas ligand- and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in HaCaT cells [49], and, consistent with its role in the alkalinisation of endosomes, prevents the endosomal breakdown of the invariant chain [50], a chaperone important in the maturation of HLA class II, leading to inhibition of expression of surface HLA class II [50]. Therefore E5 can disrupt several critical components of the cell-mediated immune response to viruses, which may contribute to the establishment and persistence of HPV infection.

It remains to be seen if E5 expression causes HLA class I down-regulation also *in vivo*. This appears to be the case in bovine papillomas (our unpublished observations); HLA class I down-regulation has been observed in CIN [51] and in cervical carcinomas [52]. However the down-regulation of HLA class I in cervical carcinomas, which often do not express E5, is common to other cancer types, and therefore unlikely to be due to E5. No correlation was

made between HLA class I down-regulation and E5 expression in CIN, and this point warrants further investigation.

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Figure Legends

Figure 1. Expression of E5 RNA in transfected cell lines. HaCaT cells were transfected with pcDNA3 (pcDNA), pcDNA3 expressing the HPV-16 E5 ORF (pc-16E5), pL2, or pL2 expressing the E5 ORF (pL2-16E5). Quantitative RT-PCR was used to determine the relative amount of HPV-16 E5 and β-actin mRNA in three of each cell line. The results of six representative cell lines are shown. Relative RNA values are expressed as the mean of three independent experiments +/- standard deviation.

Figure 2. HPV E5 down-regulates surface HLA class I. **A,** Parental HaCaT cells (three lines), cells harbouring empty vectors (pcDNA3, three lines; pL2, four lines), or expressing HPV-16 E5 (pc-16E5, four lines; pL2-16E5, three lines) were analysed for expression of total and surface HLA class I by FACS with mAb W6/32. The average mean fluorescence for each expression vector was calculated from the flow cytometric analyses. A background of 0.4 (the reading of cells stained with no primary antibody and only secondary antibody) was subtracted in all cases. Standard deviation (+/-) is shown. **B,** FACS analysis of surface and total MHC class I in NIH 3T3 cells carrying empty vector (pZip), expressing HPV-6b E5 (pZ-6E5), or HPV-16 E5 (pZ-16E5), and in PalF cells carrying empty retrovirus (empty RV), or expressing HPV-16 E5 (RV-16E5). One cell line of each was analysed. **C**, FACS analysis with an unrelated antibody (anti HPV-16 E2) showing no reaction above background.

Figure 3. HLA class I is retained in the Golgi apparatus in HPV-16 E5-expressing cells. HaCaT cells carrying empty vectors or expressing E5 (at least three lines of each) were stained with mAb W6/32 (anti-HLA class I) and mAb 4A3 (anti-golgin GM130) and analysed using confocal microscopy. N, nucleus. Representative cells are shown.

Figure 4. HPV-16 E5 does not inhibit expression of HLA heavy chain or TAP. Equal amounts (10μg) of protein lysates from one line each of HaCaT cells carrying empty vectors

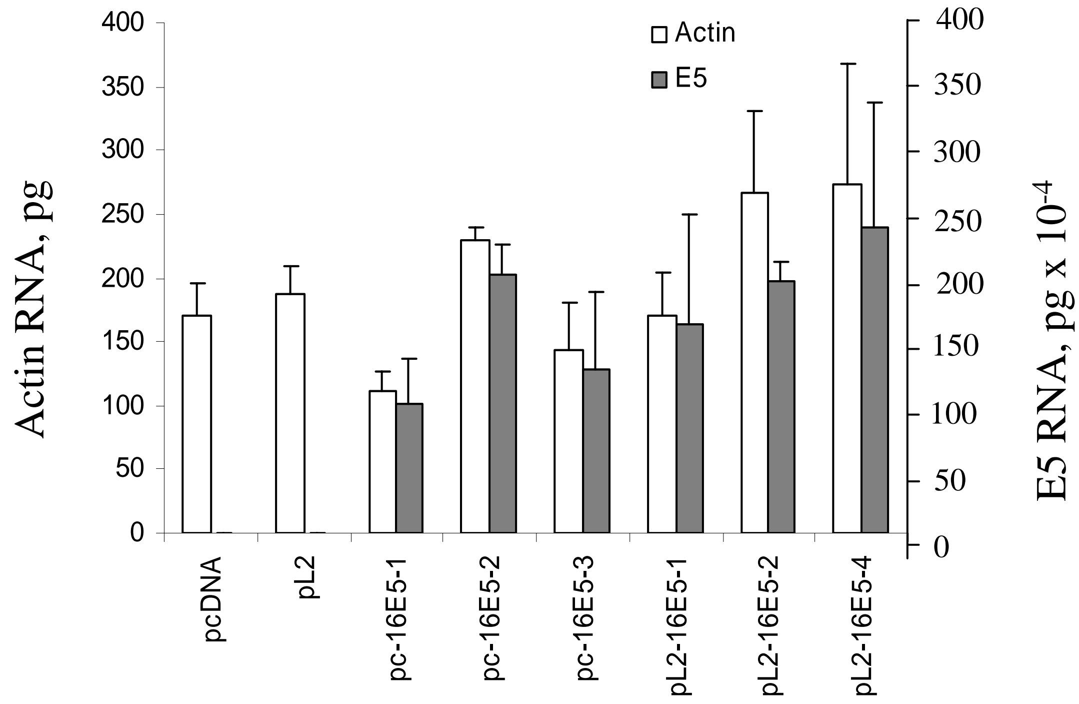
(pcDNA, pL2) or expressing HPV-16 E5 (pc-16E5, pL2-16E5) were analysed by immunoblotting with mAb HC10 (anti-heavy chain, hc), anti-TAP-1 antiserum, or mAb AB-1 (anti-actin).

Figure 5. IFN treatment rescues transport of HLA class I to the cell surface. **A**, Parental HaCaT cells (three lines), cells carrying empty vectors (pcDNA and pL2, three lines each) or cells expressing HPV-16 E5 (pc-16E5, pL2-1E5, three lines each) were treated with 500U/ml β-IFN for 48 h and analysed for expression of total and surface HLA class I by FACS analysis with mAb W6/32. The average mean fluorescence for each expression vector was calculated from the flow cytometric analyses. A background of 0.4 (the reading of cells stained with no primary antibody and only secondary antibody) was subtracted in all cases. Standard deviation (+/-) is shown. **B**, Immunofluorescence detection of HLA class I with MAb W6/32 in at least three cell lines carrying empty vector (pcDNA) or expressing HPV-16 E5 (pc-16E5), untreated or treated with β-IFN as in **A**. N, nucleus. Representative lines are shown.

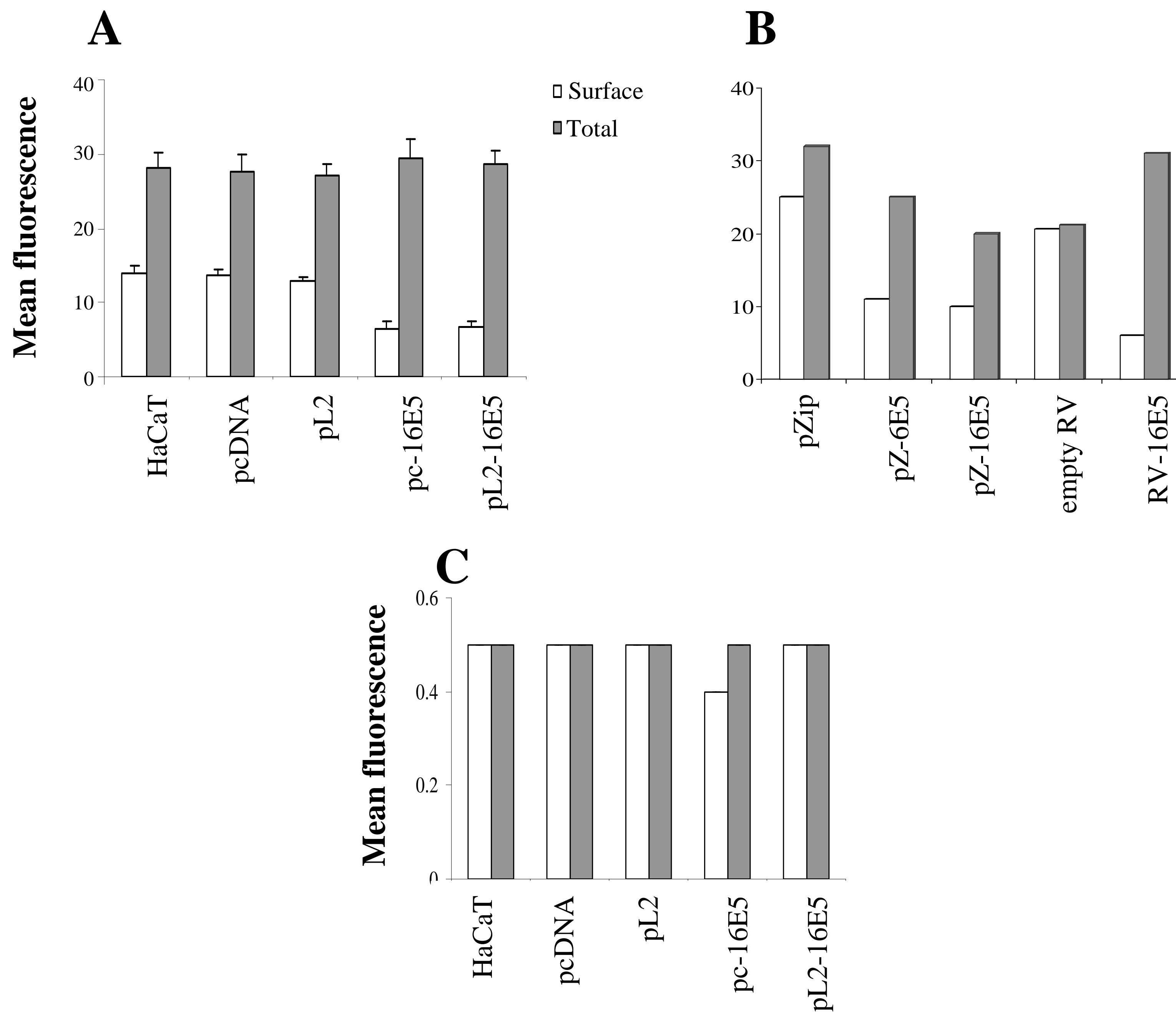
Figure 6. HPV-16 E5 does not down-regulate HLA-C/E. **A**, FACS analysis of surface and total HLA-C/E with mAb DT9 in parental HaCaT cells (three cell lines), cells carrying empty vectors (pcDNA and pL2, three lines for each vector) or expressing HPV-16 E5 (three lines of pc-16E5 and four of pL2-16E5). The average mean fluorescence for each expression vector was calculated from the flow cytometric analyses (example in panel B). A background of 0.4 (the reading of cells stained with no primary antibody and only secondary antibody) was subtracted in all cases. Standard deviation (+/-) is shown. **B**, Representative FACS profiles of pc-16E5 and pL2-16E5 cells (one line of each). Dotted line and open histogram: forward fluorescence with secondary antibody only; solid histogram: forward fluorescence with primary and secondary antibody. **C**, Immunofluorescence detection of HLA-C/E with mAb DT9 in two lines each of cells carrying empty vector (pL2) or expressing HPV-16 E5

(pL2-16E5). N, nucleus. **D**, Detection of HLA-E heavy chain by immunoblotting. Protein lysates from three lines each of parental HaCaT cells, cells carrying empty vector (pcDNA3 or pL2) or expressing E5 (pc-16E5 or pL2-16E5) were analysed by immunoblotting with mAb MEM-E/02 (anti-HLA-E) or mAb AB-1 (anti-actin). Representative lines are shown.

Ashrafi et al. Figure 1



Ashrafi et al. Figure 2



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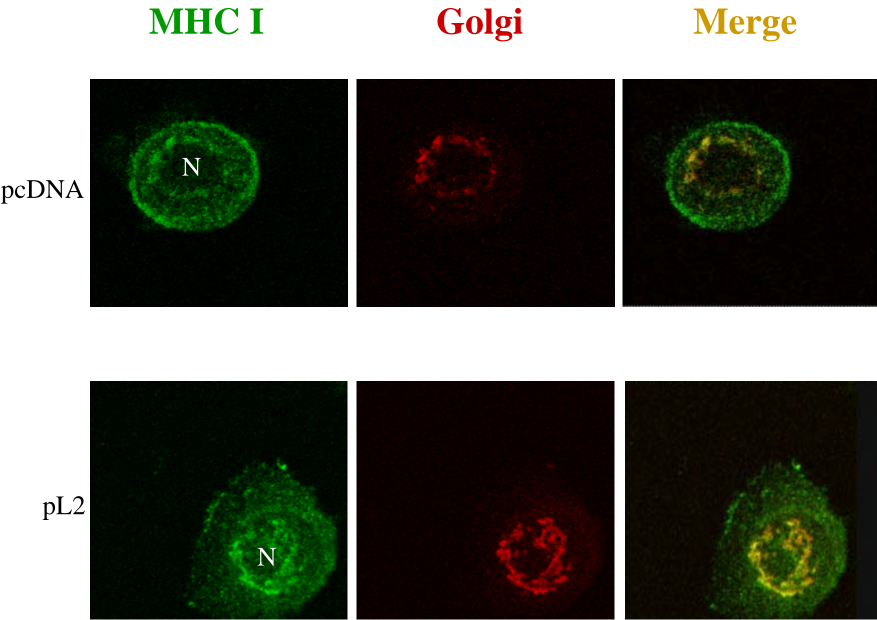

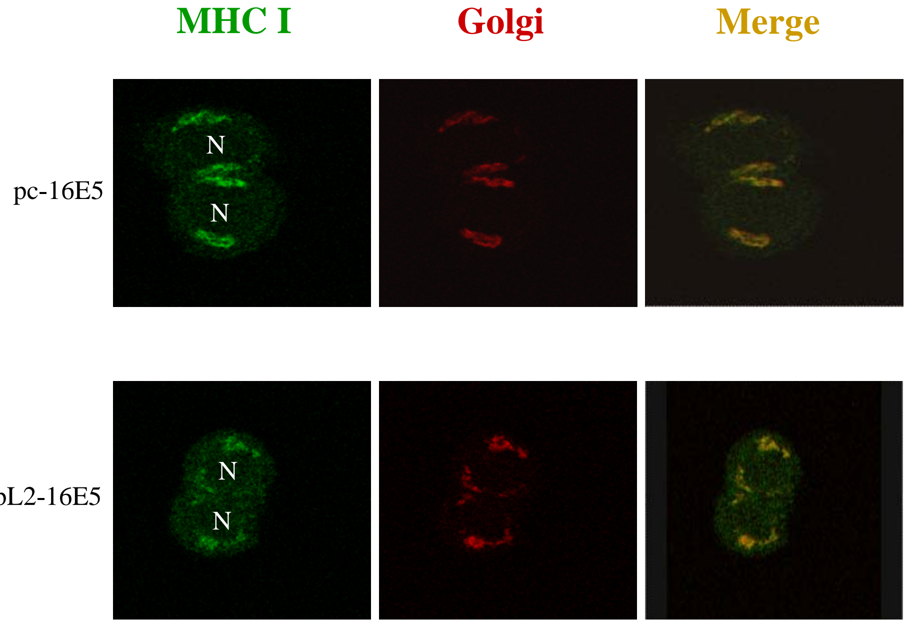

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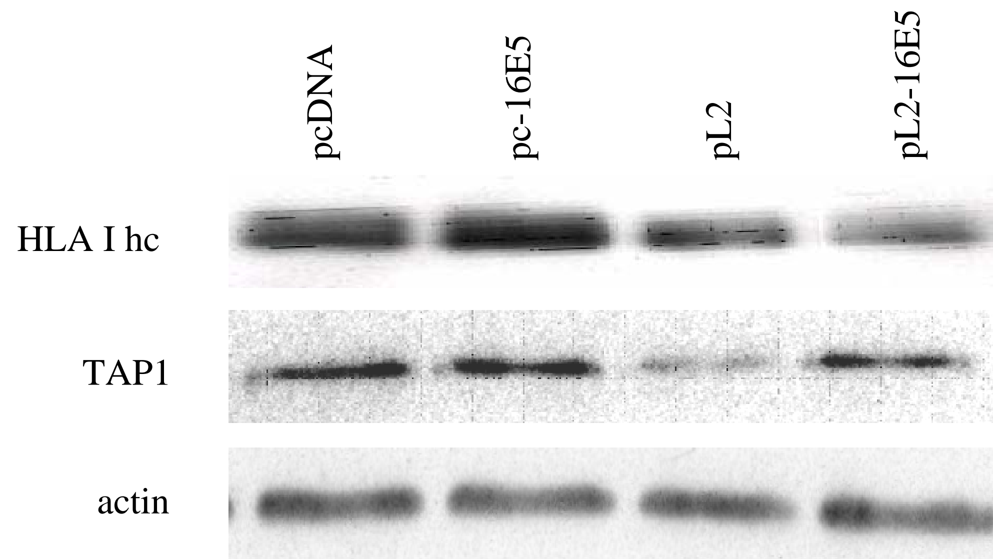
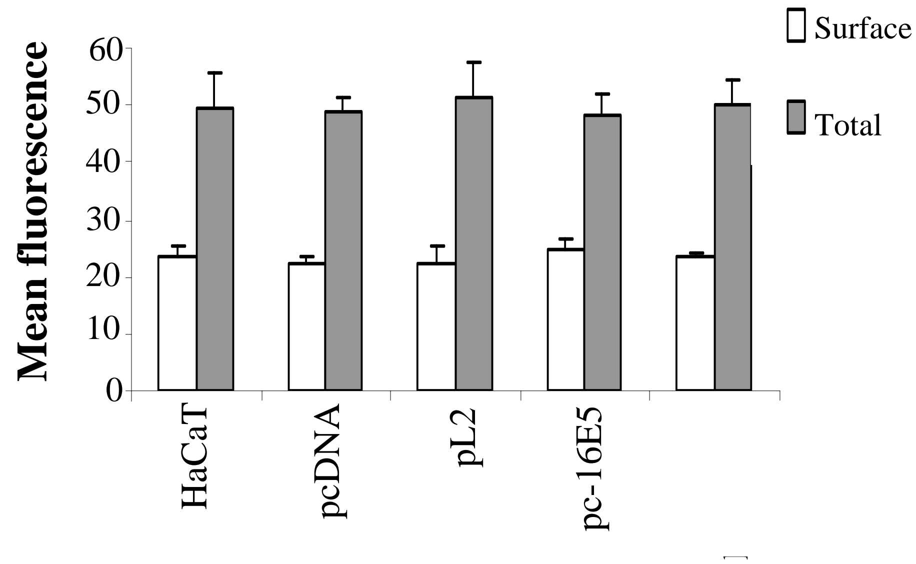


Ashrafi et al. Figure 5

A



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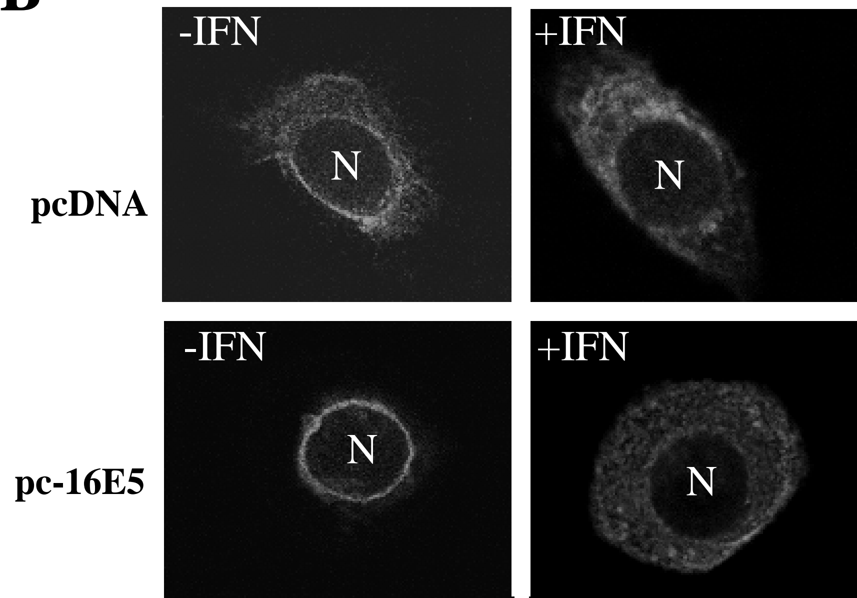
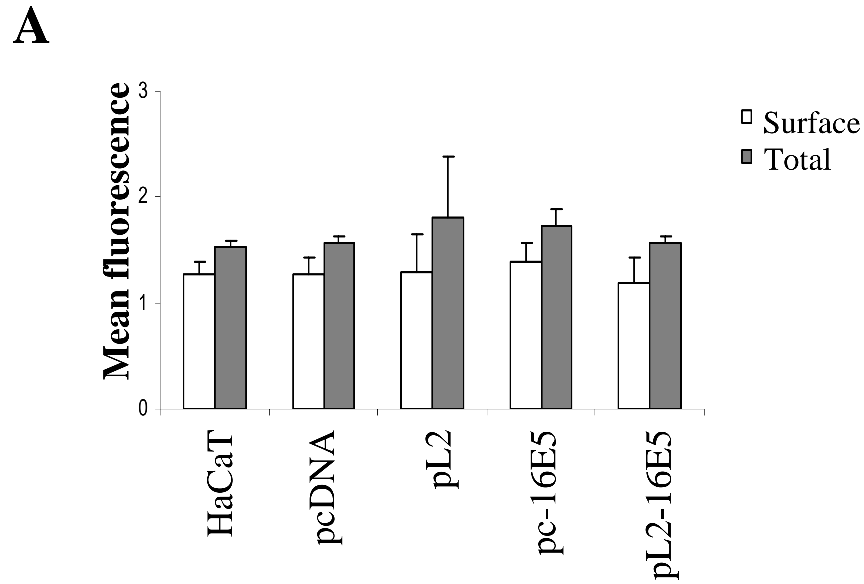


Ashrafi et al. Figure 6

A



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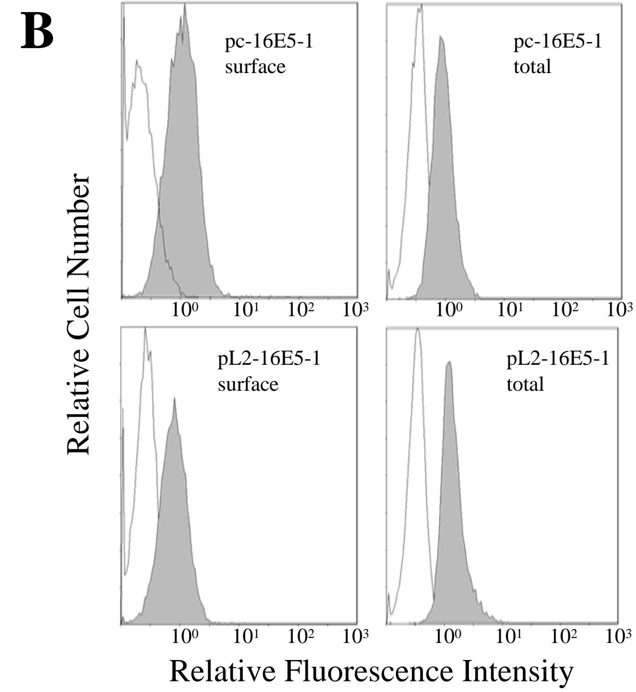

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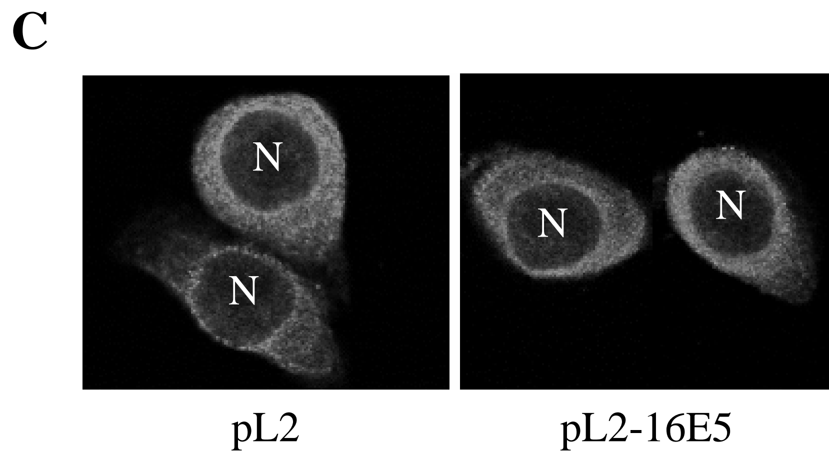

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