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THE E6E7 ONCOPROTEINS OF CUTANEOUS HUMAN PAPILLOMAVIRUS  
TYPE 38 INTERFERE WITH THE INTERFERON PATHWAY.

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## ABSTRACT

Non-melanoma skin cancer is the most frequent malignancy in Caucasian populations. Evidence suggests the involvement of cutaneous Human Papillomavirus (HPV) of the genus beta ( $\beta$ ) in this disease. The ability of E6 and E7 of mucosal HPV to promote cellular transformation and inhibit immune response-related pathways plays a key role in cervical carcinogenesis.  $\beta$  HPV-38 E6 and E7 display transforming activities in *in vitro* and *in vivo* models, but their impact on immune surveillance is unknown. Here we show that HPV-38 E6 and E7 affect the IFN-induced up-regulation of MHC class I. Expression of the two viral proteins in HaCaT keratinocytes led to a decrease of MHC I levels. This down-regulation is associated with a reduction of expression of MHC I heavy chain, of the peptide chaperone TAP and of the STAT-1 downstream effector IRF-1. The down-regulation of these proteins is ultimately due to the inhibition of STAT-1 expression. Analysis of cells expressing either HPV-38 E6 or E7 suggests that these effects are primarily the result of E6 expression, although a contribution by E7 cannot be excluded. We conclude that HPV-38 encodes oncoproteins that potentially contribute to the evasion of host immune surveillance.

## INTRODUCTION

The E6 and E7 proteins of high risk mucosal  $\alpha$ HPV (principally HPV-16 and HPV-18) inhibit components of the biosynthetic pathway of major histocompatibility class I (MHC I) such as class I heavy chain (HC), TAP-1 (transporter associated with peptide) and LMP-2 (low molecular weight protein) (Georgopoulos et al., 2000), and of the interferon (IFN) network, such as ISGF-3, IRF-1, IRF-3, STAT-1 and TYK-2 (Li et al., 1999; Nees et al., 2001; Park et al., 2000; Perea et al., 2000; Ronco et al., 1998). It appears that the high risk viral oncoproteins can affect these important immunological responses in a more severe way than the corresponding low risk proteins (O'Brien and Campo, 2003), in agreement with the numerous observations that high risk HPV E6 and E7 impact on the cell cycle regulatory machinery and tumour suppressors more than their low risk HPV counterparts.

Non-melanoma skin cancer (NMSC) is the most frequent malignancy in the Caucasian population and several lines of evidence suggest the involvement of  $\beta$  genus cutaneous HPVs in the disease.

A subset of  $\beta$ HPVs was first isolated from patients with Epidermodysplasia Verruciformis (EV); in patients infection with EV-HPV types results in large numbers of warts, which progress to cancer in 30% of cases (IARC Monographs, 2005; Jablonska, 1991).

Highly sensitive polymerase chain reaction (PCR)-based assays for HPV DNA detection have shown that the presence of  $\beta$ HPV DNA is not exclusively restricted to skin lesions of EV patients, but can also be frequently found in NMSCs of immunocompromised and immuno-competent individuals (Akgul et al., 2006; de Villiers et al., 1997; Harwood et al., 2000). In addition,  $\beta$ HPV have been detected in healthy skin (Asgari et al., 2008; Astori et al., 1998; Boxman et al., 1997) and pre-cancer lesions (Harwood et al., 2000; Jong-Tieben et al., 1995) suggesting a possible early role for HPV in the initiation of NMSC.

Given the high multiplicity and wide distribution of  $\beta$ HPVs, and their presence in normal skin, it is not clear if some types can be unequivocally linked to cancer as in the case of  $\alpha$ HPVs.

One of the best characterised  $\beta$ HPVs is HPV-38. HPV-38 has been found in approximately 50% of NMSC (Caldeira et al., 2003), although other authors find this

virus in a much lower percentage of case (see Hazard et al., 2006). HPV-38 E6 and E7 proteins transform primary human keratinocytes (Caldeira et al., 2003) and induce hyperproliferation and neoplasia in transgenic mice (Dong et al., 2005), similar in these respects to HPV-16 E6 and E7. To further characterise HPV-38, we analysed its impact on immune molecules, namely whether expression of HPV-38 E6 and E7 proteins is prejudicial to the integrity of MHC class I and the components of the IFN response, as is the case for the E6 and E7 proteins of high risk HPV-16 and HPV-18 (Ronco et al., 1998; Barnard and McMillan, 1999; Li et al., 1999; Park et al., 2000; Perea et al., 2000; Nees et al., 2001).

## Results

**Generation of HaCaT cell lines expressing HPV-38 oncoproteins.** Stable HaCaT cell lines were obtained after infection with high-titre recombinant retroviruses encoding E6 and/or E7 from HPV-38 (38E6E7, 38E6 or 38E7). HaCaT cells carrying empty retrovirus provided the control. The HPV-38 and control cell lines were pools of infected G418-resistant cells; pooling insured that maximum numbers of cells were analysed and that the effects observed were not due to the characteristics of individual clones. Furthermore, the cells were kept frozen and thawed when needed, thus preventing the out-growth of a dominant clone.

No antibodies are available to detect the HPV-38 proteins and therefore we monitored their expression by quantifying their respective mRNAs by quantitative RT-PCR (Q-RT-PCR). E6 and E7 transcripts were detected in approximately the same amounts in HaCaT 38E6 and HaCaT 38E7 cells respectively, whereas fewer 38E6 transcripts than 38E7 transcripts were consistently detected in HaCaT 38E6E7 cells (Figure 1). This may be due to splicing events in the bicistronic E6E7 mRNA as shown for HPV-16 (Tang et al., 2006). This lower level of 38E6 transcripts did not however result into loss of protein activity (see below).

Since E6 and E7 are expressed as bicistronic mRNA during natural infection, we initially determined the effect of both HPV-38 oncoproteins on cellular proteins crucial to the immune response.

**MHC class I is down-regulated in HaCaT cells expressing E6E7 proteins of HPV-38.** The expression of cell surface MHC class I (MHC I) was assessed by flow cytometry both in basal conditions and after treatment with either type I interferon (IFN $\beta$ ) or type II interferon (IFN $\gamma$ ) using mAb W6/32, which recognises monomorphic determinants in human MHC I.

In 38E6E7 cells there was a consistent decrease in surface MHC I in basal conditions. Although small, between 30% and 40% of the MHC I level in control cells, this decrease was consistent and reproducible ( $p$ : 0.04; Figure 2A, B). Levels of surface MHC I were also lower after treatment with IFN $\beta$  or IFN $\gamma$ : levels of surface MHC I were approximately 60% of those of control cells after IFN $\beta$  treatment in 38E6E7

cells ( $p$ : 0.017) and approximately 75-80% of those of control cells after IFN $\gamma$  treatment ( $p$ : 0.015) (Figure 2A, B).

**HPV-38 E6E7 proteins down-regulate the heavy chain component of MHC I.**

MHC I is a trimeric complex made up of heavy chain (HC),  $\beta$ 2 microglobulin ( $\beta$ 2m) and peptide. To see if the decrease in surface MHC I in 38E6E7 cells was due to a decrease in the expression of its components, we assessed the levels of the  $\beta$ 2m and HC proteins by immunoblotting. We found that there were no differences in levels of  $\beta$ 2m between control and test cells (data not shown) but levels of HC were lower in HaCaT 38E6E7 after IFN treatment (Figure 3A and B, top panels). We investigated the transcription of the HC gene by Q-RT-PCR and found that, despite the reduction in HC protein, there were no differences in HC RNA between control cells and 38E6E7 cells either in basal conditions or after IFN treatment (Supplementary information, Figure 1).

**HPV-38 E6E7 proteins down-regulate TAP expression.**

The assembly of the MHC I complex needs a multitude of chaperons, such as tapasin and TAP-1 (transporter associated with peptide) required for the loading of peptide onto the forming MHC I complex. If the assembly of MHC I is prevented, free HC is degraded (Hughes et al., 1997). Given the results above, which suggest that the reduction in HC is post-transcriptional, we examined the expression of TAP-1 by immunoblotting in cells expressing 38E6E7. TAP-1 was reduced in 38E6E7 cells after IFN treatment, particularly IFN $\gamma$  (Figure 3A and B, second panels).

To ascertain if, as in the case of MHC I HC, the reduction in TAP-1 level was post-transcriptional, we performed Q-RT-PCR for TAP-1 transcripts in basal condition and after IFN treatment. We found lower levels of TAP-1 transcripts in 38E6E7 cells than in control cells (Figure 4A). It therefore appears that the expression of 38E6E7 affects transcription of the TAP-1 gene.

**HPV-38 E6E7 proteins impact on the IFN network.**

One upstream regulator of the TAP gene promoter is the interferon responsive factor 1 (IRF-1) (White et al., 1996) and it has been shown that HPV-16 E7 binds to and inhibits IRF-1 (Perea et al., 2000). We therefore examined the expression of IRF-1 in 38E6E7 cells. IRF-1 was

down-regulated in 38E6E7 cells after IFN $\beta$  treatment while treatment with IFN $\gamma$  rescued its expression to a large degree (Figure 3A and B, third panels), in agreement with the fact that IFN $\gamma$  is the strongest IRF-1 inducer known (Kroger et al., 2002).

As for TAP-1, we looked at the levels of IRF-1 transcripts by Q-RT-PCR and IRF-1 RNA was lower in 38E6E7 cells than in control cells in basal conditions and after IFN treatment, particularly IFN $\beta$  (Figure 4B).

The apparent discrepancy between the immunoblotting and the Q-RT-PCR results (cf Figure 3A,B with Figure 4A,B) can be explained by the different sensitivities of the two techniques. Q-RT-PCR is exquisitely sensitive to differences in RNA levels, whereas immunoblotting detects larger differences in protein levels.

**HPV-38 E6E7 proteins affect the transcriptional activities of the TAP-1, LMP-2 and IRF-1 promoters.** We next investigated how 38E6E7 affect the RNA levels of TAP-1 and IRF-1 by the use of their respective transcriptional promoters. The TAP promoter is a bi-directional one controlling the transcription of both TAP and the proteasome subunit LMP-2 (Wright et al., 1995). The promoters for TAP-1, LMP-2 and IRF-1, cloned upstream of the luciferase reporter gene, were introduced into control and 38E6E7 cells. All three promoters were greatly inhibited in the 38E6E7 cells (Figure 4D), indicating that the reduction in the respective RNA and proteins was due to an inhibition of their promoters by the viral proteins. The TAP-1 and IRF-1 promoters were assayed also after IFN treatment and also in this case they showed decrease activity in 38E6E7 cells (Figure 4D).

**HPV-38 E6E7 proteins affect the levels of STAT-1.** 38E6E7 appear to impact on both IFN $\beta$  and IFN $\gamma$  pathways. Although type I and type II IFNs trigger two parallel signal cascades, their pathways intersect at several points and one component common to both pathways is STAT-1 (Goodbourn et al., 2000). Upon activation by IFN, STAT-1 either heterodimerises with STAT-2 (IFN $\beta$ ), or homodimerises with another molecule of STAT-1 (IFN $\gamma$ ). The dimers are phosphorylated and translocated to the nucleus where they engage their respective targets. One of the targets of STAT-1 is IRF-1 (Ohmori et al., 1997). Given the pivotal role of STAT-1 in the IFN networks, and the reduction of IRF-1 in 38E6E7 cells, we investigated if STAT-1 or



its activity would be inhibited by HPV-38 proteins. We found that the levels of STAT-1 in the 38E6E7 cells were lower than those in control cells both in basal conditions and in IFN stimulated cells; there was accordingly less phosphorylated STAT-1 (pSTAT-1) overall, but the residual STAT-1 appeared to be slightly more phosphorylated than in control cells (Figure 3A and B, bottom panels). As pSTAT-1 is translocated to the nucleus, we analysed its translocation in control and 38E6E7 cells but found no difference between the two cell lines (Figure 5). As there were no changes between control and 38E6E7 cells in either TYK-2, the proximal effectors of the IFN receptors, or STAT-2 (Supplementary information, Figure 2), we conclude that 38E6E7 target STAT-1.

**HPV-38E6E7 proteins inhibit STAT-1 in immortalised primary human keratinocytes.** HaCaT cells have been extensively and fruitfully used in the study of HPV. However, the possibility existed, albeit remote, that the observed effects were due, at least partly, to the nature of the HaCaT cells. Therefore we investigated the levels of STAT-1 in two independently derived lines of primary human keratinocytes which had been immortalised and transformed by HPV-38 E6E7 (Caldeira et al., 2003; Gabet et al., 2008). Also in these cells, 38E6E7 caused a marked decrease of STAT-1 and a corresponding decrease of pSTAT-1 both in basal conditions and after IFN $\beta$  treatment (Figure 6). The results obtained with IFN $\gamma$  were inconsistent and require further investigation.

**HPV-38 E6E7 proteins reduce the levels of STAT-1 mRNA.** HPV-38 E6 does not degrade p53 (Caldeira et al., 2004); however the possibility that the lower levels of STAT-1 in 38E6E7 cells were due to E6-induced degradation could not be discounted. Thus, control and 38E6E7 cells were treated with either the proteasome inhibitor MG132, to see whether STAT-1 was degraded, or with the protein synthesis inhibitor cyclohexamide (CHX) to investigate STAT-1 half life. Neither inhibitor had a major effect on STAT-1 in 38E6E7 cells compared to control cells, although it was clear in both experiments that the levels of STAT-1 were lower in 38E6E7 than in control cells. There was no significant difference in STAT-1 after MG132 treatment in either control or 38E6E7 cells (Figure 7A and B); STAT-1 had a similar half-life in both control and 38E6E7 cells (Figure 7C and D), although small differences in decay

kinetics cannot be ruled out. We conclude that the decrease in STAT-1 levels in 38E6E7 cells is unlikely to be due to degradation or higher turnover of STAT-1. Therefore, to see if lower levels of STAT-1 in cells expressing 38E6E7 might be due to a decrease in STAT-1 mRNA, we performed Q-RT-PCR for STAT-1 mRNA in control and 38E6E7 cells. The amount of STAT-1 mRNA was decreased in the test cells compared to control cells after IFN treatment (Figure 4C), indicating that 38E6E7 decrease levels of STAT-1 primarily by inhibiting STAT-1 gene transcription.

**The effects on IFN pathway are due to HPV-38 E6.** To try to ascertain whether HPV-38 E6 or E7 had the greater impact on the IFN pathway, we analysed STAT-1, IRF-1, TAP-1 and MHC I in cells expressing either E6 or E7. STAT-1, TAP-1, MHC I HC and surface MHC I were all down-regulated in 38E6 cells (Figure 8A and B). However, the levels of IRF-1 did not seem to change between the two cell lines (Figure 8A, second panel). It could be argued that the lack of discernible effect in the 38E7 cells was due to lack of E7 expression, notwithstanding the presence of equivalent amounts of E6 and E7 mRNA in the two cell lines (Figure 1). 38E7 degrades pRb (Caldeira et al., 2003); therefore the status of pRb is a read out for E7 expression. pRb was clearly reduced in 38E7 and 38E6E7 cells, but not in 38E6 cells as expected (Supplementary information, Figure 3), showing that E7 is indeed expressed in those cell lines. Thus it appears that 38E6 has the greater impact on the immune molecules analysed, but a contribution by 38E7, particularly in the down-regulation of IRF-1 in 38E6E7 cells, cannot be ruled out.

## DISCUSSION

Viruses need to escape the host immune response to establish a productive infection. HPV-16 E5 down-regulates surface MHC class I (Ashrafi et al., 2005), thus potentially contributing to the evasion of the host immune response by the viral infected cell. Although all E5 proteins tested so far down-regulate surface MHC I (Ashrafi et al., 2006),  $\beta$ HPVs do not have an E5 ORF and it is reasonable to assume that other viral proteins would achieve a similar effect but using different mechanisms.

One of the downstream effects of both IFN $\beta$  and IFN $\gamma$  is an increase in expression of MHC I. HPV-16 and -18 E6 and E7 both interfere with components of the IFN pathway (Li et al., 1999; Nees et al., 2001; Park et al., 2000; Perea et al., 2000; Ronco et al., 1998), thus potentially reducing the amount of MHC I on the cell surface.

Given the precedents for interference with the IFN pathways by mucosal HPV E6 and E7, we investigated whether  $\beta$ HPV-38 E6 and E7 can likewise affect the interferon signalling cascade and one of its final steps, MHC I expression.

Indeed, we found that 38E6E7 inhibit STAT-1 and several of its down-stream signal transduction steps, including IRF-1, TAP-1 and ultimately MHC I.

**HPV-38 E6E7 proteins down-regulate STAT-1.** Upon IFN treatment, STAT-1 either heterodimerises with STAT-2 (IFN $\beta$ ), or homodimerises (IFN $\gamma$ ) and becomes phosphorylated. The phosphorylated complexes are translocated to the nucleus where they target their respective promoters. One of the targets of STAT-1 is the IRF-1 promoter. The IRF-1 promoter is inhibited in cells expressing 38E6E7 and this inhibition is not due to the failure to activate (phosphorylate) STAT-1 or to translocate activated pSTAT-1 to the nucleus, but to a decrease in STAT-1 levels and therefore pSTAT-1 (Figure 3 bottom panels and Figure 5). HPV-16 E6 binds to and promotes the degradation of numerous cellular proteins (Mantovani and Banks, 2001). The down-regulation of STAT-1 in 38E6E7 cells, however, does not appear to be due to degradation or to modification of its half life (Figure 7). Rather, the decrease in STAT-1 levels is a consequence of a decrease in STAT-1 mRNA (Figure 4). Although we have not analysed specifically the activity of the transcriptional promoter of the STAT-1 gene, we believe the reduction in STAT-1 transcripts is due to an inhibition of the promoter, as found for the promoters of IRF-1, TAP-1 and

LMP-2 (see below). How 38E6E7 affects transcription of the STAT-1 gene is not yet known and this point deserves investigation. Preliminary results suggest that down-regulation of STAT-1 is not due to gene hypermethylation (Friedrich et al., 2005), or to up-regulation of the STAT-1 negative controller STRA-13 (Ivanov et al., 2006).

It has to be pointed out that down-regulation of STAT-1 takes place also in primary human keratinocytes immortalised by HPV38 E6E7 (Figure 6), ruling out an artefact due to the established nature of HaCaT cells. STAT-1 is down-regulated in keratinocytes expressing 38E6E7 also after IFN $\beta$  treatment. No conclusions could be made for IFN $\gamma$  as its effect was erratic and unreliable.

**HPV-38 E6E7 proteins down-regulate IRF-1 and TAP-1.** STAT-1 binds to and activates the transcription of the IRF-1 gene (Ronco et al., 1998). IRF-1 transcriptional promoter is down-regulated in 38E6E7 cells with low amounts of IRF-1 transcripts (Figure 4B, D). This is in agreement with the inhibition of STAT-1 by 38E6E7 (Figure 3).

IRF-1 positively regulates the transcriptional promoter of the TAP-1 gene (Chatterjee-Kishore et al., 1998). Consistent with the down-regulation of IRF-1, the TAP-1 promoter is also inhibited in 38E6E7 cells (Figure 4A and D). The transcriptional inhibition of TAP by 38E6E7 is in agreement with previous results showing that oncogenic viruses such as HPV-16 and adenovirus 12 inhibit TAP transcription (Georgopoulos et al., 2000; Proffitt and Blair, 1997).

**HPV-38 E6E7 proteins down-regulate MHC I.** TAP-1 chaperones peptides to the MHC I complex, and the complex needs peptide to be stable. A decrease in TAP leads to unstable MHC I and degradation of HC. Indeed loss of TAP-1 is highly correlated with loss of MHC I in cervical carcinomas (Cromme et al., 1994).

38E6E7 cells have consistent lower levels of surface MHC I than control cells, down by approximately 20-40% (Figure 2). Although small, and less than the one found in HaCaT cells expressing HPV-16 E5 (Ashrafi et al., 2005), this decrease is comparable to the one reported in cells expressing the pre-S2 antigen of woodchuck hepatitis B

virus or the full genome of HBV (Chen et al., 2006; Wang and Michalak, 2006). It remains to be seen if this extent of MHC I down-regulation leads to escape from CTL recognition. These experiments cannot be performed in HaCaT cells as they express primarily HLA-A3 and to our knowledge no HLA-A3-restricted CTL lines exist.

The changes in immune molecules observed in 38E6E7 cells appear to be due primarily to 38E6, although we cannot rule out a more subtle contribution by 38E7 particularly to the down-regulation of IRF-1.

It has been reported that HPV-16 E7 interferes with IRF-1, because of a direct binding between 16E7 and IRF-1 (Park et al., 2000; Perea et al., 2000), and with TAP-1, partly because of the inhibition of the TAP-1 gene promoter (Georgopoulos et al., 2000). In contrast to what found with HPV-16, HPV-38 E6 appears to have a greater impact on TAP-1 down-regulation than 38E7 (cf Figure 3 with Figure 8), and may cooperate with 38E7 in IRF-1 down-regulation, as IRF-1 levels are lower in 38E6E7 cells than in either 38E6 or 38E7 cells (cf Figure 3 with Figure 8).

The targeting of STAT-1 by 38E6E7 and particularly by 38E6 is in agreement with the previous observation that STAT-1 is strongly down-regulated by high risk mucosal HPV (Barnard and McMillan, 1999; Chang and Laimins, 2000; Li et al., 1999; Nees et al., 2001), although the mechanisms appear to be different. For instance HPV-18 E6 associates with TYK-2 – the upstream regulator of STAT-1 – and impairs the activation of the JAK-STAT cascade (Li et al., 1999). In contrast, in HPV-38 cells, TYK-2 is not affected, and the down-regulation of STAT-1 is at mRNA level. STAT-1 is part of both IFN type I and IFN type II pathway so its inhibition would affect both pathways, without need for 38E6E7 to down-regulate STAT-2, as is indeed the case (Supplementary information, Figure 2).

Most of the cell transforming activity of  $\beta$  HPVs appears to be encoded by their E6 genes (Massimi et al., 2007). In agreement, the present results concur with this observation suggesting that the E6 proteins of  $\beta$ HVPs may be more potent in their action than the corresponding E7.

## MATERIALS AND METHODS

**Cells and transfection.** pLXSN38E6E7 has been previously described (Caldeira et al., 2003). The HPV-38 E6 and E7 genes were each amplified from the viral genome by PCR and subcloned into the retroviral pLXSN vector (Clontech, USA) between EcoRI and XhoI sites to generate pLXSN38E6 or pLXSN38E7 vectors. The constructs were then transfected into Phoenix cells (Pear et al., 1993) using CalPhos™ Mammalian Transfection Kit (Clontech, USA), and high-titre retroviral supernatants were obtained by culturing the cells for an extra 24h. Supernatants were passed twice through 0.45µm filters to remove all virus-producing cells and used to infect HaCaT cells.

HaCaT cells were grown in 25cm<sup>3</sup> flask in Dulbecco's modified Eagle medium High Glucose without calcium chloride (Life Technologies, UK), supplemented with 10% foetal calf serum, 4 mM L-glutamine, 100 IU penicillin per ml, and 100 µg streptomycin per ml until approximately 70% confluent ( $1 \times 10^6$ ). HaCaT cells were infected with high titre retroviral supernatants (pLXSN38E6E7, pLXSN38E6 or pLXSN38E7) and 24h after infection were washed, trypsinised, placed in 75cm<sup>3</sup> flasks and selected in growth medium containing 800µg/ml G418. Selection continued until all the non-infected control cells had died (approximately two weeks). Due to the high-titre virus we did not observe significant levels of death in infected HaCaT cells. The infected cells were then pooled and frozen, and maintained in growth medium containing 400µg/ml G418 when needed.

Primary human foreskin keratinocytes were transduced with empty retrovirus pLXSN (control), or with pLXSN38E6E7 (38E6E7i) as described by Caldeira et al., 2003. The keratinocytes line 38E6E7.1 is derived from human embryonic keratinocytes (Cascade, USA) as described by Gabet et al., 2008.

**Interferon treatment.** Cells were left to set for 24h after seeding and then treated with either IFNβ (500U/ml) (R&D Systems, UK) or IFNγ (0.075µg/ml) (Sigma-Aldrich, UK) for 30 min for the study of STAT-1. This timing was decided based upon published observations (Haspel et al., 1996) and our own (data not shown) that the appearance of phosphorylated STAT-1 (pSTAT-1) in the nucleus was maximal within 20-30 minutes after treatment.

For the study of MHC I, the treatment has to be longer to allow time for maturation of the complex, and it was established that optimal treatment time was 48h (data not shown). The analysis of IRF-1 and TAP-1 was also done after 48h IFN treatment.

**Detection of MHC class I by flow cytometry.** HaCaT cells expressing E6 and/or E7 of HPV-38 and control cells were incubated with anti-human HLA class I mouse monoclonal antibody W6/32 (1/100) (Serotec, UK) at 4°C for 30 min, and then with antimouse IgG-FITC (1/100) (Sigma-Aldrich, UK) at 4°C for 30 min in the dark. After washing, the cells were re-suspended in 500 ml of 3.4% paraformaldehyde in PBS and kept at 4°C. 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.

**Antibodies for Western blot.** The following primary antibodies were used: anti-STAT-1 (C-terminus) mouse monoclonal antibody (BD Biosciences, UK); anti-phospho-STAT-1 (Tyr 701) rabbit polyclonal antibody (New England Biolabs, UK); anti-TAP-1 (753-748) rabbit polyclonal antibody (Merck Biosciences, UK); anti-IRF-1 mouse monoclonal antibody (BD Biosciences, UK); anti-STAT-2 mouse monoclonal antibody (BD Biosciences, UK); anti-Tyk2 mouse monoclonal antibody (BD Biosciences, UK); anti-tapasin rabbit polyclonal antibody (Abcam, UK); anti  $\beta$ 2 microglobulin (BBM.1) mouse monoclonal antibody (Cancer Research UK Technologies [CRT], UK); anti-MHC I heavy chain (HC10) mouse monoclonal antibody (CRT); anti-lamin B1 rabbit polyclonal (Abcam, UK); mouse monoclonal anti-GAPDH (Abcam, UK); anti-actin mouse monoclonal antibody Ab-1 (Oncogene™, UK), anti-pRb (clone G3-245) mouse monoclonal antibody (PharMingen, UK). The secondary antibodies used were either GPR, a sheep anti-mouse Ig, linked to horseradish peroxidase (GE Healthcare, UK) or goat anti-rabbit IgG (whole molecule)-peroxidase antibody (Sigma-Aldrich, UK).

**Western blot analysis.** HaCaT cells expressing HPV-38 oncoproteins and control cells were treated with IFN for either 30 min or for 48h. Cells were lysed in SDS-lysis buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 2% glycerol), clarified by centrifugation for 10 min at 4°C and the supernatant was recovered. Protein concentration was

determined by absorbance measurement at 562 nm. 10 µg of each sample were electrophoresed on Nu PAGE™ 4-12% Bis-Tris gel (Invitrogen, UK) under denaturing conditions and transferred onto a nitrocellulose membrane (Invitrogen, UK). Membranes were incubated with varying primary antibodies (for STAT-1, pSTAT-1, STAT-2, Tyk2, TAP-1, IRF-1, β2m, MHC I heavy chain, tapasin) followed by incubation with the corresponding secondary antibodies HRP-conjugated. Western blots were developed by enhanced chemiluminescence (ECL Plus, GE Healthcare, UK).

To study possible degradation of STAT-1, 38E6E7 cells and control cells were treated for 8h with 5µM MG132 (Calbiochem, UK) before further processing, whereas to investigate STAT-1 half life the cells were treated with 0.1mg/ml of the protein synthesis inhibitor cyclohexamide (CXH) (Sigma-Aldrich, UK) for several hours (1, 2, 5, 10, 24h).

Bands were analysed by densitometry using a STORM860 scanner (molecular Dynamics, UK) and ImageQuant TL version 2005 software (Amersham Biosciences, UK). In all cases, the density of the bands was adjusted to the density of the corresponding actin bands, and the bands of control cells in basal conditions were arbitrarily taken as 100. All the other bands were referred to the control bands.

**Nuclear-cytoplasm fractionation.** Cells were grown and treated as explained for Western blot and the nuclear and cytoplasmic fractions were separated using a Nuclear Extraction Kit (Imgemex, Cambridge Bioscience, UK) following the instructions of the supplier. One µg only of each sample (to avoid precipitation with loading buffer) was electrophoresed as indicated for Western Blot. To control the efficiency of fractionation the blots were stained with either rabbit polyclonal antiserum to Lamin B1 (Abcam, UK) for the nuclear fraction, or GAPDH (Abcam, UK) for the cytoplasmic fraction.

**Quantitative RT-PCR.** Quantitative RT-PCR was employed to monitor the expression of HPV-38 oncoproteins, the heavy chain of MHC class I, TAP-1, IRF-1 and STAT-1. Cells were seeded and treated as described for Western blot, and total RNA was extracted using RNeasy®Mini Kit (QIAGEN, UK) and treated with DNase I, Amplification Grade kit (Invitrogen, UK) prior to RT-PCR. The reverse



transcription was primed with Random Primers (Promega, UK) at a concentration of 0.2µg in a 12µl reaction mixture that was then reverse-transcribed using the MMoLV RT kit (Invitrogen, UK) in a volume of 20µl containing 1x First-Strand Buffer, 11mM DTT, 40U RNaseOUT™ Recombinant Ribonuclease Inhibitor and 200U MMoLV RT following the instructions of the supplier.

cDNA was amplified in triplicate either for HPV-38 E6 with the primers 5' ttttgcattgttagcagtggtg and 5'cacggccaagacagttaattca or for HPV-38 E7 with the primers 5' tgcattgccacaggagtt and 5'tggtcctcctctaccactga, at a concentration of 600mM each in a 25µl reaction that contained 12.5 µl TaqMan®Universal PCR Master Mix, No Amperase® UNG (Applied Biosystems, UK). The reactions also contained the detection TaqMan® probes: 6FAM -5' ttatgctctgctcaatatgaatgcagcagttt - TAMRA for HPV-38 E6 and 6FAM -5' cctgatctccagaggatattgaagc – TAMRA for HPV-38 E7 respectively, both at a concentration of 4µM.

cDNA was amplified in triplicate for STAT-1 with the primers 5' ccagcctggttgtaattga and 5' gctggtgacgttgagatc at a concentration of 600mM each in a 25µl reaction that contained 12.5 µl TaqMan®Universal PCR Master Mix, No Amperase® UNG (Applied Biosystems, UK). The reaction also contained the detection probe TaqMan® Probe: 6FAM - agacgacctctctgcccgttggtg - TAMRA, at a concentration of 4µM.

cDNA were amplified in triplicate for TAP-1 and IRF-1 using the corresponding Pre-Developed TaqMan® Gene Expression Assays (Applied Biosystems, UK).

As control cDNA were amplified with the Pre-Developed Assay Reagents for Eukaryotic 18S rRNA Endogenous Control (FAM™ Dye / MGB Probe, Non-Primer Limited) (Applied Biosystems, UK).

The thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All reactions were performed in the model 7500 Sequence Detector (Applied Biosystems, UK).

The results were plotted in a graph as percentage of  $2^{-\Delta\Delta Ct}$  according to published methods (Livak and Schmittgen, 2001).

**Luciferase reporter assays.** Cells were seeded in 60mm plates and were transfected with 1µg of the luciferase reporter plasmids pIRF-1, pTAP-1 or pLMP2 (kind gifts of Y. Ohmori and E. Blair respectively), using LIPOFECTAMINE PLUS™ Reagent

(Life Technologies, UK) according to the manufacturer's instructions. Forty-eight hours post-transfection the cells were harvested and the luciferase assays were performed according to the instructions of the supplier (Promega, UK).

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## FIGURE LEGENDS

Figure 1. HPV-38 E6 and E7 transcription in HaCaT cells. Quantitative RT-PCR (Q-RT-PCR) for 38E6 or 38E7 in cells expressing either or both proteins. The mRNAs were measured relative to 18S rRNA. The bars represent the adjusted error.

Figure 2. Flow cytometry analysis of HaCaT cells expressing HPV-38 E6E7 proteins. Mean forward fluorescence of cell surface MHC class I in basal conditions and after exposure to IFN $\beta$  or IFN $\gamma$  (30 min). The graph shows the results from at least three experiments in duplicate for control cells and 38E6E7 cells (A). The bars represent standard deviation (SD). A representative flow cytometry profile for control and 38E6E7 cells is shown, the numbers next to the peaks represent the mean forward fluorescence of the respective profiles (B).

Figure 3. HPV-38 E6E7 down-regulate immune molecules. (A) Western blot analysis of HaCaT cells expressing HPV-38 E6E7 proteins. Protein lysates from control or 38E6E7 cells grown in basal conditions or in the presence of IFN $\beta$  or IFN $\gamma$  were immunoblotted for: MHC I heavy chain with anti-HC10 mouse monoclonal antibody (CRT); TAP-1 with anti-TAP-1 (753-748) rabbit polyclonal antibody (Merck Biosciences, UK); IRF-1 with anti-IRF-1 mouse monoclonal antibody (BD Biosciences, UK); STAT-1 with anti-STAT-1 (C-terminus) mouse monoclonal antibody (BD Biosciences, UK), and phosphorylated STAT-1 (pSTAT-1) with anti-phospho-STAT-1 (Tyr 701) rabbit polyclonal antibody (New England Biolabs, UK). Actin was detected with anti-actin mouse monoclonal antibody Ab-1 (Oncogene<sup>TM</sup>, UK) as loading control. The MHC I heavy chain (HC) blot was under-exposed to better show the differences in HC after IFN treatment. IFN treatment was 48 hr for MHC I heavy chain, TAP-1 and IRF-1, and 30 min for STAT-1 and pSTAT-1. (B) Densitometric analysis of at least three blots similar to the ones in A. The density (volume) of the bands in control cells in basal condition was adjusted for the density of the corresponding actin bands and taken as 100. The bands from the test cells were referred to the control band. The measurements were repeated at least three times with similar results. The bars represent SD.

Figure 4. HPV-38 E6E7 down-regulate transcription of mRNA encoding immune proteins. Quantitative RT-PCR (Q-RT-PCR) for TAP-1 (A), IRF-1 (B) and STAT-1 (C) mRNA in control and 38E6E7 cells grown in basal conditions or treated with IFN. The mRNAs were measured relative to 18S rRNA. The bars represent the adjusted error. Please note that the determination of STAT-1 mRNA after IFN $\beta$  or IFN $\gamma$  has been done at different times. (D), HPV-38 E6 down-regulates the transcriptional promoters of TAP-1, LMP-2 and IRF-1. Luciferase transcription assays with TAP-1, LMP-2 and IRF-1 promoters in control and 38E6E7 cells in basal condition and after IFN treatment. Promoter activity is expressed as fold increase over that of control cells. The bars represent SD.

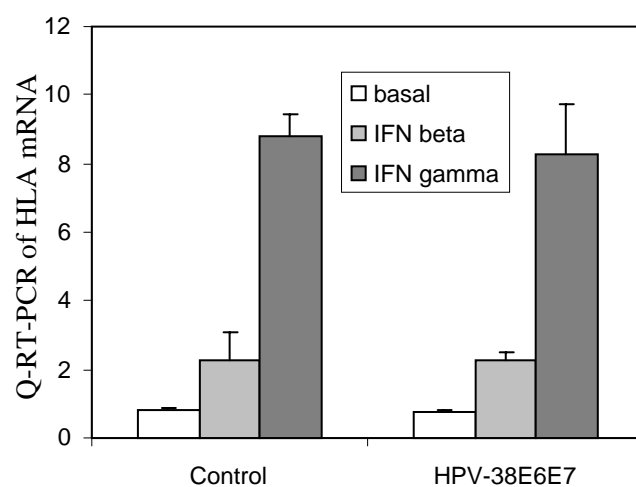
Figure 5. HPV-38 E6E7 down-regulate STAT-1 but do not inhibit its translocation to the nucleus. A, western blot of cytoplasmic and nuclear STAT-1 and pSTAT-1 probed with anti-STAT-1 (C-terminus) mouse monoclonal antibody (BD Biosciences, UK) and anti-pSTAT-1 (Tyr 701) rabbit polyclonal antibody (New England Biolabs, UK). GAPDH and Lamin were chosen as cytoplasmic and nuclear markers respectively. B, quantitation of nuclear/cytoplasmic ratio of pSTAT-1 after IFN treatment. Quantitation in basal conditions is not possible as pSTAT-1 is translocated to the nucleus in detectable amounts only after IFN treatment. Quantitation was done as in Figure 3.

Figure 6. HPV-38 E6E7 down-regulate STAT-1 in immortalised primary human keratinocytes. (A), Control primary human keratinocytes, HPV-38i and HPV-38.1 keratinocytes (Caldeira et al., 2003; Gabet et al., 2008) were grown either in basal conditions or in the presence of IFN $\beta$  for 30 min, and protein lysates were immunoblotted with anti-STAT-1 (C-terminus) mouse monoclonal antibody (BD Biosciences, UK) and anti-pSTAT-1 (Tyr 701) rabbit polyclonal antibody (New England Biolabs, UK). Actin was detected with anti-actin mouse monoclonal antibody Ab-1 (Oncogene<sup>TM</sup>, UK) as loading control. (B) Densitometric analysis of at least three blots for STAT-1, and one blot for pSTAT-1. The density of the STAT-1 bands was calculated as in Figure 3. The measurements were repeated several times with similar results.

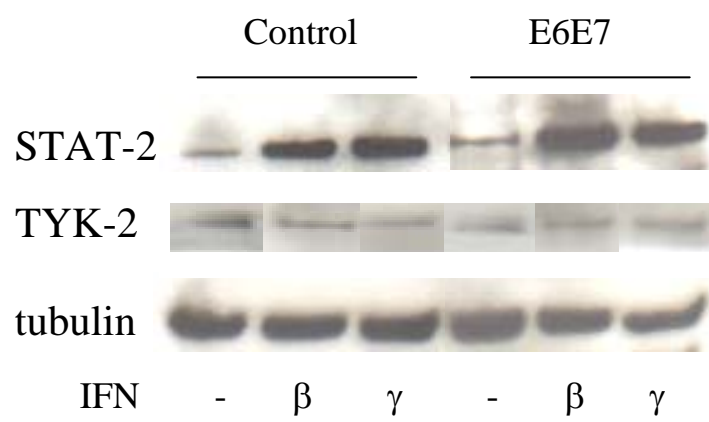
Figure 7. STAT-1 is not degraded by HPV-38 E6E7. (A) Control cells or 38E6E7 cells were treated with MG132 and protein lysates were immunoblotted for STAT-1 with anti-STAT-1 (C-terminus) mouse monoclonal antibody (BD Biosciences, UK). (B) Densitometric analysis of at least three blots similar to the one in A. The density of the STAT-1 bands was adjusted for the density of the actin bands. The bars represent SD. (C) Control cells or 38E6E7 cells were treated with cyclohexamide (CHX) for varying hr (1, 2, 5, 10 and 24) and protein lysates were immunoblotted for STAT-1 with anti-STAT-1 (C-terminus) mouse monoclonal antibody (BD Biosciences, UK). Actin was detected with anti-actin mouse monoclonal antibody Ab-1 (Oncogene™, UK) as loading control in A and B. (D) Densitometric analysis of the blots in C. The density of the STAT-1 bands was calculated as in Figure 3.

Figure 8. HPV-38 E6 down-regulates immune molecules. A, Western blot analysis of STAT-1, pSTAT-1, IRF-1, TAP-1 and MHC I HC in HaCaT cells expressing either 38E6 or 38E7. The cells were grown and lysed as described in Figure 3. The protein lysates were run on gel and probed as described in Figure 3. The blots were quantified as in Figure 3. B, Flow cytometry analysis of MHC I in HaCaT cells expressing either 38E6 or 38E7. Mean forward fluorescence of cell surface MHC class I in basal conditions and after exposure to IFN $\beta$  or IFN $\gamma$ , as in Figure 2. The graph shows the results from at least three experiments in duplicate for control cells, 38E6 or 38E7 cells. Although the differences in MHC I levels between 38E6 cells and control cells were reproducible, they were not statistically significant because of variations in forward fluorescence between experiments.

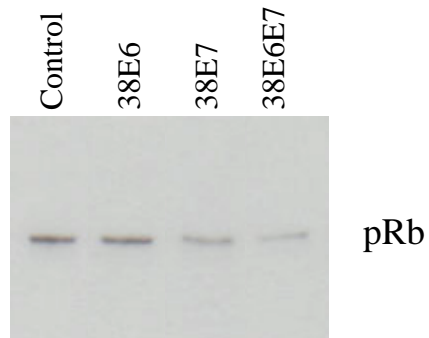
## Cordano et al., Supplementary Information



**Figure 1.** HPV-38 E6E7 do not down-regulate HLA heavy chain mRNA. Quantitative RT-PCR of heavy chain mRNA relative to actin.

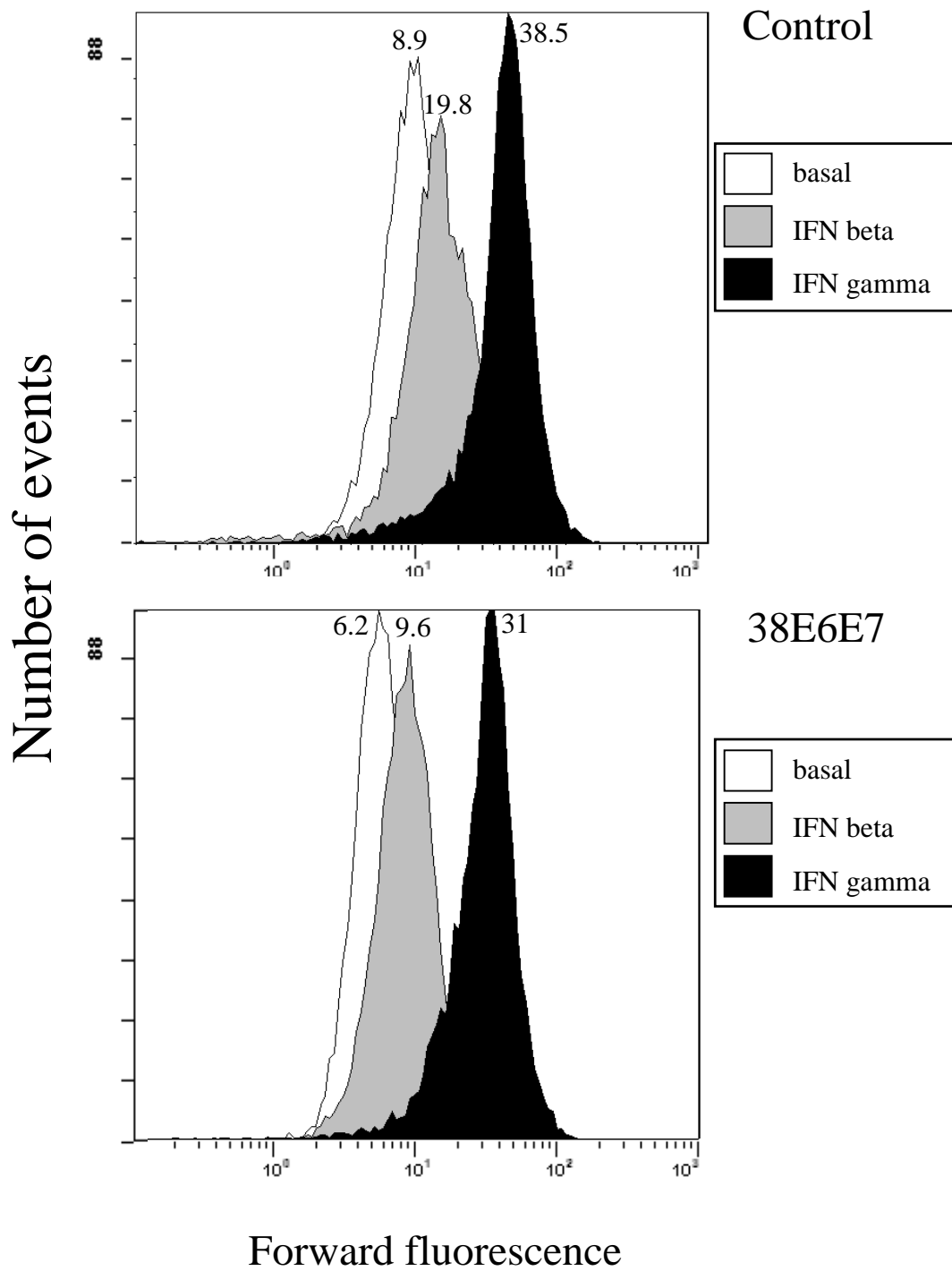


**Figure 2.** HPV-38 E6E7 do not down-regulate STAT-2 or Tyk-2.

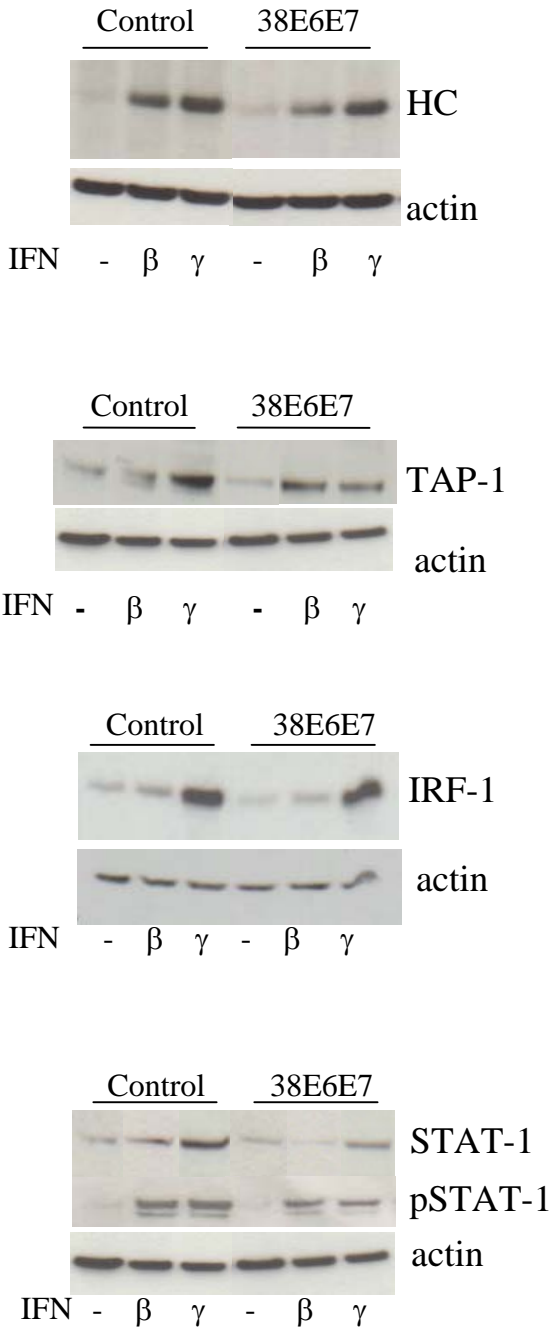


**Figure 3.** HPV-38 E7 degrades pRb.

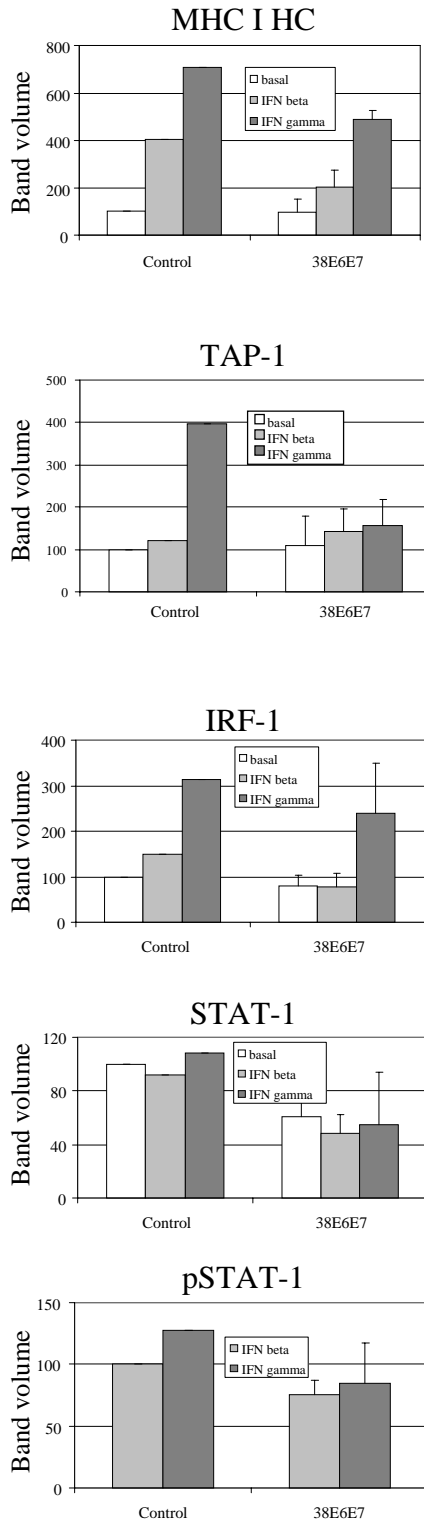
**B**



**A**

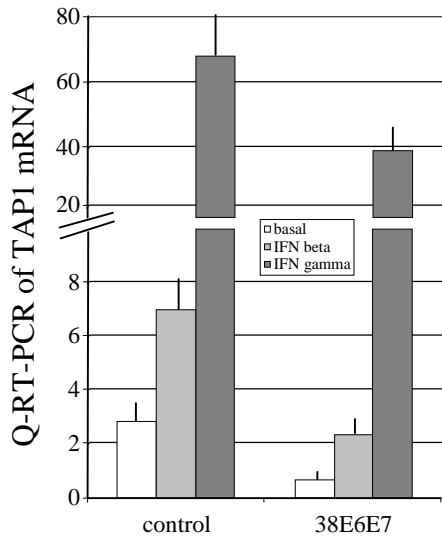


**B**

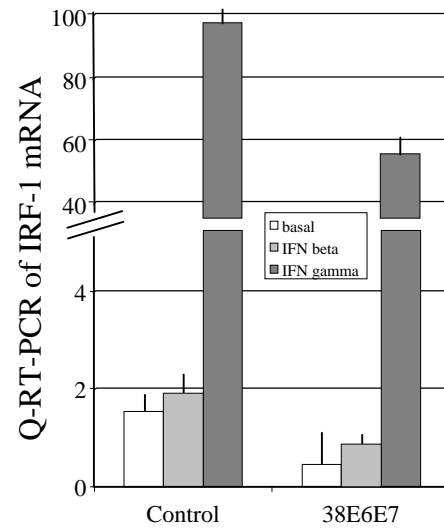




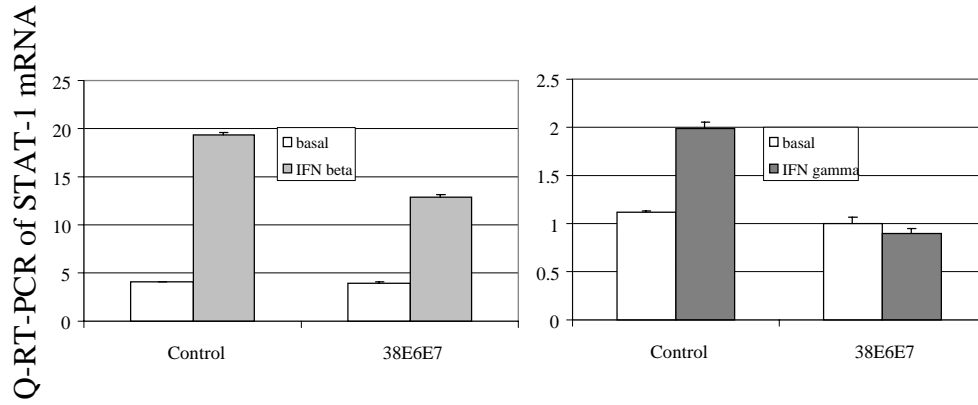
**A**



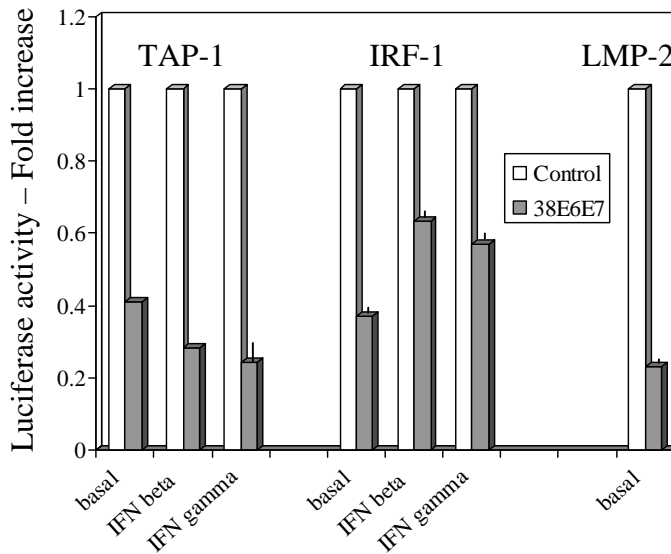
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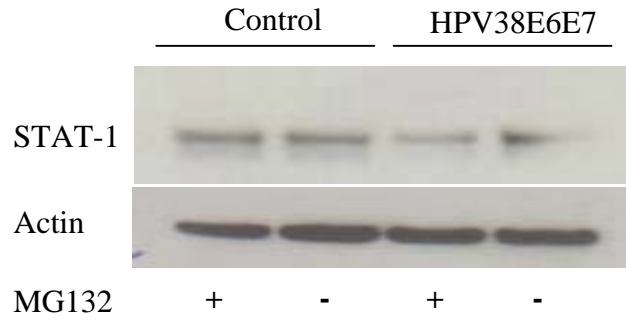
**C**



**D**



**A**



**B**

