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THE E5 ONCOPROTEIN OF BPV-4 DOES NOT INTERFERE WITH THE BIOSYNTHETIC PATHWAY OF NON-CLASSICAL MHC CLASS I

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Abstract

The major histocompatibility complex (MHC) class I region in mammals contains both classical and non-classical MHC class I genes. Classical MHC class I molecules present antigenic peptides to cytotoxic T lymphocytes, whereas non-classical MHC class I molecules have a variety of functions. Both classical and non-classical MHC molecules interact with natural killer cell receptors, and may under some circumstances prevent cell death by natural killer cytotoxicity. The E5 oncoprotein of BPV-4 down-regulates the expression of classical MHC class I on the cell surface and retains the complex in the Golgi apparatus. The inhibition of classical MHC class I to the cell surface results from both the impaired acidification of the Golgi, due to the interaction of E5 with subunit c of the H^+ V-ATPase, and to the physical binding of E5 to the heavy chain of MHC class I. Despite the profound effect of E5 on classical MHC class I, E5 does not retain a non-classical MHC class I in the Golgi, does not inhibit its transport to the cell surface and does not bind its heavy chain. We conclude that, as is the case for HPV-16 E5, BPV-4 E5 does not down-regulate certain non-classical MHC class I, potentially providing a mechanism for the escape of the infected cell from attack by both cytotoxic T lymphocytes and NK cells.

Introduction

Viruses have to evade the innate and adaptive host immune response to establish infection and persist at least until new progeny virus is released. Many viruses have developed the ability of down-regulating the major histocompatibility complex (MHC) class I (Piguet, 2005), thus avoiding lysis by cytotoxic T lymphocytes (CTL; effector cells capable of recognising and destroying transformed or infected cells), while not interfering with expression of some non-classical MHC class I genes. Some non-classical MHC class I molecules, in common with classical class I molecules, engage both inhibitory and activating receptors of natural killer (NK) cells, components of the innate immune response, which may recognise and destroy cells with low levels of MHC class I. The absence of down-regulation of some non-classical MHC class I genes, or their up-

51 regulation, by virus may result in the failure of NK cells to kill virus-infected cells and allows virus
52 to escape both CTL and NK killing (Cohen et al., 1999; Tomasec et al., 2000; Wang et al., 2002).
53 Papillomaviruses (PV) are small oncogenic viruses that infect mucosal and cutaneous epithelia
54 causing benign hyperproliferative lesions (papillomas, warts, condylomas).
55 Infection by PV is under immunological control; this is demonstrated by epidemiological studies
56 showing that in both human and animal hosts with genetic, iatrogenic or acquired cell-mediated
57 immune deficiencies, PV lesions show increased persistence and enhanced neoplastic progression
58 (see O'Brien and Campo, 2002, for review). However, even in immunocompetent individuals,
59 despite expressing abundant viral protein, PV persist for a significant period of time, usually
60 spanning several months, and even years, before activation of the host immune system. This lack of
61 recognition suggests the host immune system is unaware of, and/or disabled by, PV infection.
62 Indeed, the ability of PV to persist has been attributed to several causes: the nature of the virus life
63 cycle, which takes place wholly in the epithelium and therefore prevents high levels of the viral
64 proteins having access to the immune cells; the entry of PV to a latent state with very low levels of
65 expression of viral antigens; and, as demonstrated recently, the ability of the virus to directly
66 subvert and evade the host immune response (Frazer et al., 1999; O'Brien and Campo, 2002; Tindle,
67 2002). It is likely that the contribution of each of these factors results in a delay or prevention of
68 resolution of infection.
69 It is becoming clear that the oncoproteins of PV can interfere with the host immunosurveillance and
70 response (O'Brien and Campo, 2002). We have shown that the E5 oncoprotein of both BPV and
71 HPV inhibits the transport of MHC class I to the cell surface and retains it in the Golgi apparatus
72 (Araibi et al., 2004; Ashrafi et al., 2002, 2005; Marchetti et al., 2002). Additionally we have shown
73 that BPV-4 E5 interacts physically with bovine MHC class I heavy chain (HC) and that both this
74 interaction and the down-regulation of the complex require the C-terminus domain of E5 (Marchetti
75 et al., 2005).

76 Although effectively down-regulating surface classical MHC class I, HPV-16 E5 does not interfere
77 with the transport of non-classical MHC class I to the cell surface (Ashrafi et al., 2005). It is not
78 known if this is the case also for BPV E5.
79 Recently, nine putative non-classical class I sequences (*N*50001-N*50501*, see
80 http://www.ebi.ac.uk/ipd/mhc/bola/) have been identified in cattle cDNAs, most of which have not
81 yet been functionally characterized. *N*50001* (GenBank accession: AY188807) was first identified
82 in 1995 when it was termed HD59 (Ellis et al., 1996) and subsequently was mapped to a region of
83 the cattle MHC containing several classical class I genes (Di Palma et al. , 2002). *N*50001* encodes
84 a HC with a truncated cytoplasmic domain (8 rather than 28 amino acids), and in this sense
85 resembles human HLA-G (Geraghty et al., 1987).
86 Here we show that BPV-4 E5 cannot down-regulate expression of N*50001 MHC class I complex
87 or interact with N*50001 HC, suggesting that, like other viruses, BPV is potentially capable of
88 evading CTL and NK killing of infected cells.
89
90
91 **Results**
92 We have shown that BPV-4 E5 binds to bovine class I HC and have mapped the HC-interacting
93 domain of E5 to its C-terminus end (Marchetti et al., 2005). We have also shown that HPV-16 E5
94 down-regulates classical HLA I (MHC class I) but not non-classical HLA-E (Ashrafi et al., 2005).
95 To determine whether BPV-4 E5, like HPV-16 E5, is incapable of down-regulating certain non-
96 classical MHC genes, we took advantage of a recently described gene in the bovine MHC class I
97 cluster, *N*50001* (Di Palma et al., 2002).
98 *N*50001* has an early stop codon in the sequence encoding the intra-cytoplasmic domain
99 (producing a C-terminal truncated HC), has a number of base pairs substitutions leading to unusual
100 amino acids (see Figure 1), has a large deletion in the 3'UTR, and significant differences in the
101 promoter region compared to classical class I genes (data not shown). It is prone to alternative

102 splicing, in common with some other non-classical class I genes, and the isoform most often found
103 was used in this study (Figure 1). This isoform contains a small section of intron 4 resulting in 4
104 additional amino acids at the end of the alpha 3 domain. N*50001 is recognised by mAb IL-A88, a
105 mAb that recognises a monomorphic determinant on all bovine classical MHC class I molecules
106 (Toye et al., 1990) (Figure 4). mAb IL-A88 was used throughout the present work.

107

108 **Generation of mouse P815 cells expressing bovine MHC class I**. It is not possible to analyse the
109 expression or the function of *N*50001* in bovine cells (*in vivo*) because there is no specific antibody
110 for its protein. To investigate the properties of this gene, its cDNA was introduced into the mouse
111 cell line P815, giving rise to P815-N*50001 cells. P815-N*01301 cells were generated in a similar
112 way. There was no need to co-transfect the bovine β_2 microglobulin (β_2m) gene with the bovine
113 MHC HC genes into P815 cells because the bovine class I HC associates with mouse β_2m, and
114 when the complex reaches the cell surface it rapidly exchanges mouse β_2m with bovine β_2m
115 provided by the foetal calf serum in the culture medium (Ellis et al., 2005). Both *N*01301* and
116 *N*50001* are expressed in P815 cells and transported to the cell surface (see below and Figure 3).

117

118 **Stable introduction and expression of BPV-4 E5 into P815-N*01301 and P815-N*50001 cells**.
119 Any interaction between E5 and N*50001 cannot be studied in bovine cells, such as our well
120 characterised PalF cell lines, as there are no specific antibodies for this HC and the mAb IL-A88,
121 which recognises N*50001, recognises all other MHC class I HC. The P815-N*01301 and P815-
122 N*50001 cells gave us an opportunity to analyse the functional relationship between E5 and
123 N*50001 independently from the interaction between E5 and N*01301. We introduced E5 into
124 these cells, either expressed from the murine MuLV transcriptional promoter in pZipneo (pz4E5),
125 or expressed from the IE promoter of HCMV in pcDNA3 (pc4E5). The corresponding empty
126 vectors were also introduced into the P815-N*01301 and P815-N*50001 cells as controls. We
127 generated the following cell lines: P815-N*01301-pz4E5, P815-N*01301-pc4E5, P815-N*01301-

128 pZip, P815-N*01301-pcDNA, P815-N*50001-pz4E5, P815-N*50001-pc4E5, P815-N*50001-pZip
129 and P815-N*50001-pcDNA.
130 We determined the expression of E5 by quantitative RT-PCR. There was little or no difference in
131 E5 expression whether expressed from the MLV LTR or the CMV IE promoter, either in P815-
132 N*01301 or in P815-N*50001 cells (Figure 2).
133
134 **Expression of bovine MHC class I in P815 cells expressing BPV-4 E5**.
135 The expression of N*01301 or N*50001 was analysed by flow cytometry. As expected, E5 induced
136 a remarkable down-regulation of N*01301-containing MHC class I (Figure 3). There was very little
137 surface MHC class I, with a reduction of more than five fold compared to control cells carrying
138 empty vector, and also the amount of total (surface plus intra-cytoplasmic) MHC class I was less
139 than half of that in control cells. The results shown in Figure 3A were obtained with P815-
140 N*01301-pc4E5 cells, but the results obtained with P815-N*01301-pz4E5 cells were essentially
141 identical (data not shown). These results confirm that 4E5 has a drastic effect on the expression and
142 transport of MHC class I (Ashrafi et al., 2002; Marchetti et al., 2005). The results also show
143 conclusively that 4E5 is capable of down-regulating MHC class I in the absence of any other viral
144 oncogene.
145 In contrast to what was observed with N*01301, N*50001 was not at all affected by E5 and the
146 levels of surface or total MHC were indistinguishable from those in control cells (Figure 3).
147 Identical results were obtained with P815- N*50001-pz4E5 cells (data not shown).
148 The decrease in N*01301 expression but not in N*50001 expression was confirmed by western
149 blotting. There was less N*01301 expressed in P815-N*01301-pc4E5 or P815-N*01301-pzE5 cells
150 than in the control cells (Figure 4A); on the contrary, the expression of N*50001 was unchanged in
151 P815-N*50001-pc4E5 or P815-N*50001-pz4E5 cells, although in control cells it was lower than
152 that of N*01301 (Figure 4A). It has to be noted that mAb IL-A88 did not react with mouse MHC
153 class I in the parental P815 cells (Figure 4A, middle lane), and therefore a possible cross-reaction

154 with bovine and mouse MHC class I can be discounted. Additionally mAb IL-A88 did not react
155 with mouse MHC class I in flow cytometry experiments with parental P815 cells (data not shown)
156 confirming lack of cross-reaction.
157

158 **Turnover of N*01301 and N*50001 in P815-4E5 cells**.
159 To investigate the reasons for the decreased expression of N*01301 in the cells expressing 4E5, the
160 half life of the HC was measured by inhibiting the translation of new proteins with cyclohexamide
161 and by following the turnover of HC by western blotting. In control cells, N*01301 was stable and
162 could still be detected after 24 hours of treatment; in remarkable contrast, in the 4E5-expressing
163 cells N*01301 was unstable and was practically undetectable after 10 hours (Figure 4B). There was
164 no difference between the stability of N*50001 in control or 4E5-expressing cells; in both cases
165 N*50001 HC was still detectable after 24 hours of treatment, comparable to that of N*01301 in
166 control cells (Figure 4B). These results confirm the general inhibition of classical MHC class I
167 expression by E5, from stability of the HC protein to the transport of the class I complex to the cell
168 surface (Araibi et al., 2004; Ashrafi et al., 2002, 2005; Marchetti et al., 2002, 2005). The results also
169 show that E5 has no effect on the expression, half-life and transport of non-classical N*50001-
170 MHC class I.
171

172 **Localisation of classical and non-classical MHC class I in P815-4E5 cells**.
173 In PalF-4E5 cells, E5 retains the MHC class I in the Golgi apparatus (Marchetti et al., 2002). We
174 wondered whether the reduction in surface N*01301-MHC class I was also due to the retention of
175 residual complex in the Golgi. We performed two-colour confocal immunofluorescence in the
176 various P815 cell lines where MHC class I was detected with mAb IL-A88 and a FITC-conjugated
177 secondary antibody, and the Golgi apparatus was detected with Bodipy Ceramide-Texas Red. In all
178 control cells, whether expressing N*01301 or N*50001, MHC was both associated with the Golgi
179 membranes and clearly on the cell surface (Figure 5). In contrast the cellular localisation of

180 N*01301- and N*50001-MHC class I was markedly different in P815 cells expressing c4E5 or
181 z4E5. Classical N*01301-MHC class I was totally associated with Golgi membranes and no
182 complex was detectable on the cell surface with this technique (Figure 5), whereas, in sharp
183 contrast, N*50001-MHC was clearly on the cell surface, in addition to the Golgi membranes
184 (Figure 5), and its localisation pattern was no different from the one in control cells. Again, no
185 reaction was observed in parental P815 cells (data not shown).
186 The localisation of N*50001 was analysed also in bovine PalF control cells (transformed but
187 without E5) and in PalF-4E5 cells (transformed and expressing E5) (Ashrafi et al., 2000; Pennie et
188 al., 1993). FLAG-tagged N*50001 was detected both in the Golgi apparatus and on the cell surface
189 in both control and PalF-4E5 cells, while a GFP fusion form of classical HC B2705 was wholly
190 confined to the misshapen Golgi apparatus in the PalF-4E5 cells, but not in the control cells (data
191 not shown), as previously reported (Marchetti et al., 2002). The results obtained in both P815 and
192 PalF cells show beyond doubt that E5 is incapable of inhibiting the transport of N*50001-MHC
193 complex from the Golgi compartment to the surface of the cell.
194

195 **Physical interaction between E5 and HC**. E5 and N*01301 HC interact physically and we have
196 proposed that this interaction contributes to the down-regulation of the HC and the retention of the
197 complex in the Golgi (Marchetti et al., 2005). Therefore we asked whether E5 would complex with
198 N*50001 HC and used *in vitro* co-precipitation assays as *in vitro* interaction between E5 and HC is
199 a faithful read-out for *in vivo* interaction (Marchetti et al., 2005; Ashrafi et al., submitted). As
200 before, we *in vitro* transcribed/translated E5, N*01301 and N*50001 HC, and precipitated the HC
201 with mAb IL-A88. Whereas E5 co-precipitated with N*01301 HC, it did not do so with N*50001
202 HC (Figure 6A). The lack of interaction between E5 and N*50001 was confirmed by a competition
203 experiment in which unlabelled N*50001 was added to labelled E5 and labelled N*01301.
204 Unlabelled N*50001 failed to prevent the interaction between E5 and N*01301 (Figure 6A),
205 confirming that it cannot bind to E5.

206 As described above, N*50001 lacks the C-terminus intra-cytoplasmic tail of classical MHC class I
207 HC and is 19 amino acids shorter than N*01301 (Figure 1). Given the absence of interaction
208 between E5 and N*50001 we wondered whether the intra-cytoplasmic domain of class I HC was
209 responsible for binding to E5. We made a C-terminus truncation mutant of N*01301 HC lacking the
210 final 19 amino acid residues and performed *in vitro* co-IP experiments with 4E5. This mutant co-
211 precipitated with 4E5 (data not shown) showing that the interaction between HC and 4E5 does not
212 involve the C-terminus intracytoplasmic domain of HC. Furthermore, N*01301 HC bound E5
213 whether tagged with V5-His at its C-terminus, like N*50001, or not (data not shown),
214 demonstrating that the C-terminus epitope has no effect on the interaction.
215 We corroborated the *in vitro* interaction between E5 and HC in a "pull-down co-
216 immunoprecipitation" experiment. Protein lysates for P815 parental cells, P815-N*01301 and
217 P815-N*50001 cells were incubated with labelled E5 and immunoprecipitated with mAb IL-A88.
218 As expected, no E5 could be precipitated from P815 control cells or P815-N*50001 cells, while E5
219 co-precipitated with N*01301 HC in P815-N*01301 cells (Figure 6B).
220 Taken together, the results presented above lead to the firm conclusion that E5 does not interfere
221 with the biosynthetic pathway of non-classical N*50001 MHC class I.
222
223 **Discussion**
224 *Viruses and classical and non-classical MHC class I.* It is well-documented that many viruses
225 evade the host immune system by interfering with the antigen processing and presentation pathways
226 (Piguet, 2005). These viral mechanisms often target MHC class I, and there are several examples of
227 discrimination between classical and non-classical class I. While classical class I expression is most
228 often down-regulated by viral interference at one or more points in the pathway between
229 transcription and cell-surface expression, expression of non-classical class I genes usually remains
230 normal (Cohen et al., 1999; Tomasec et al., 2000; Wang et al., 2002). It has been proposed that this
231 enables the virus-infected cell to evade cytotoxic T cell responses while at the same time sending

232 inhibitory signals to NK cells via expression of one or more non-classical class I genes. This relates
233 to engagement of inhibitory NK receptors by non-classical class I molecules (Borrego et al., 2005;
234 Hofmeister and Weiss, 2003).
235
236 *Non-classical MHC class I in cattle*. There is considerable evidence that N*50001 and related
237 alleles in cattle are encoded by a non-classical MHC class I gene. The classification of *N*50001*
238 (and related alleles) as non-classical is based on several criteria, namely association with a number
239 of unrelated MHC haplotypes (at least one of this set of alleles is present on all MHC haplotypes
240 examined; Ellis, unpublished data), lack of significant polymorphism, a truncated cytoplasmic
241 domain (8 rather than 28 amino acids), unusual amino acid substitutions throughout, a characteristic
242 motif within the transmembrane domain, a large deletion in the 3' UTR and significant differences
243 in the 5' upstream region in comparison to classical class I genes. These alleles may be widely
244 transcribed, and there is currently no evidence to suggest tissue-specific expression (Davies et al.,
245 2004); Ellis, unpublished data). In addition, a number of alternatively spliced transcripts of
246 *N*50001* can be found (Ellis, unpublished data), a characteristic seen in non-classical class I genes
247 in other species, most notably HLA-G (Riteau et al., 2001). *N*50001* and related alleles are
248 transcribed at relatively low levels in peripheral blood lymphocytes, and at slightly higher levels in
249 trophoblast tissue during pregnancy (Davies et al., 2004), in common with some other putative non-
250 classical cattle MHC class I alleles.
251
252 *BPV-4 E5 and MHC class I.* Because there is no monoclonal antibody available which exclusively
253 recognizes non-classical class I molecules, to study the relationship of BPV-4 E5 with classical and
254 non-classical MHC I, we separately expressed a classical, *N*01301*, and a non-classical, *N*50001*,
255 allele in mouse P815 cells and subsequently introduced E5 in these cells. This proved to be a
256 powerful system which has confirmed that E5 down-regulates classical MHC class I, while it does
257 not interfere with the biosynthetic pathway of this non-classical MHC class I.

258

259 *BPV-4 E5 and classical MHC class I.* As in bovine PalF cells (Marchetti et al., 2005), E5 down-

260 regulates classical MHC class I at more than one level. However, in PalF cells, E5 was co-

261 expressed with E6, E7 and activated ras (Ashrafi et al., 2000), leaving open the possibility, albeit

262 remote, that down-regulation of MHC class I was due to a functional interaction between the viral

263 and cellular oncoproteins. In P815 cells, E5 profoundly inhibits classical MHC class I *in the*

264 *absence of other viral or cellular oncoproteins*. E5 impacts on classical MHC class I at several

265 levels: transcription of the HC gene, expression of protein and transport of the complex to the cell

266 surface (Marchetti et al., 2005). Transcription of the bovine N*01301 HC cannot be analysed in

267 P815 cells: E5 inhibits transcription from the homologous promoter of the HC gene (Marchetti et

268 al., 2005), while in P815 cells the N*01301 HC gene is under the control of the CMV IE promoter,

269 which is not affected by E5 (data not shown). In P815 cells, in addition to blocking MHC class I in

270 the Golgi apparatus, E5 induces the degradation of N*01301 HC. In control cells, the half-life of the

271 HC is approximately 20 hours, whereas in the presence of E5 its half-life is dramatically reduced to

272 less than 5 hours. We do not yet know how E5 induces degradation of HC. However, we have

273 commented before (Marchetti et al., 2005) on the similarities between E5 and HIV 1 Nef, and it is

274 interesting to note that Nef re-directs HC to the lysosomes for degradation through the simultaneous

275 interaction with HC and with the adaptor protein 1 (AP-1) complex (Roeth et al., 2004), and that

276 inhibitors of lysosomes (in conjunction with interferon treatment) restores the expression of HC in

277 PalF-4E5 cells (Marchetti et al., 2005). It is thus possible that E5 too interacts with both HC and

278 AP-1 inducing lysosomal degradation of HC, but this remains to be elucidated.

279

280 *BPV-4 E5 and non-classical MHC class I.* In marked contrast to its inhibitory effect on classical

281 MHC class I, E5 does not affect the non-classical MHC class I N*50001. This conclusion is based

282 on numerous pieces of evidence. N*50001 reaches the cell surface, N*50001 HC is expressed to the

283 same levels as in control cells and its half-life is not affected, and crucially E5 does not interact with

284 N*50001 HC. This last point is further corroborated by the inability of N*50001 HC to inhibit the
285 formation of the complex between E5 and N*01301 HC *in vitro*. Moreover, the finding that the
286 half-life of N*50001 HC is not affected by E5 supports our hypothesis that binding to HC is needed
287 for HC degradation.
288 A major difference between classical N*01301 and non-classical N*50001 HC is the truncation of
289 the intracytoplasmic domain in the latter, but the interaction with 4E5 does not take place via this
290 domain. This finding contrasts with the binding of Nef to the intracytoplasmic domain of HLA-A
291 and with the observation that the inability of Nef to bind some non-classical HLA resides in amino
292 acid differences in the intracytoplasmic domain of HLA-E and the absence of the domain in HLA-G
293 (Pizzato et al., 2004; Williams et al., 2002). There are several amino acid differences throughout the
294 extracellular domains between classical N*01301 and non-classical N*50001 HC (Figure 1), but
295 which one of these differences is responsible for the differential interaction with E5 remains to be
296 established.
297
298 *Biological significance of the lack of down-regulation of non-classical MHC class I by E5*. The lack
299 of significant polymorphism in the non-classical MHC class I allele described here strongly
300 suggests that this complex is not involved in antigen presentation, and is thus more likely to interact
301 with NK receptors, as has been observed for a number of non-classical class I gene products in
302 human and other species. Cattle have recently been shown to have a family of genes with homology
303 to the human KIRs (McQueen and Parham, 2002; Storset et al., 2003), and several other putative
304 NK receptors have been identified (Storset et al., 2004), but thus far interactions between these
305 putative NK receptors and MHC class I molecules have not been demonstrated.
306 Thus while it is possible to speculate that the lack of interaction of E5 with N*50001 constitutes a
307 viral mechanism for evading NK recognition, this remains to be confirmed. Nevertheless, down-
308 regulation of classical MHC class I and lack of down-regulation of non-classical MHC class I by E5

309 strongly suggest that the viral protein is instrumental for the establishment of viral infection by
310 allowing the infected cell to escape both the adaptive and innate immune response of the host.
311
312
313 **Materials and Methods**
314 **Plasmids**. *N*01301* cDNA, encoding heavy chain (HC) N*01301 (formerly HD6), was cloned both
315 in pcDNA3 (Ellis et al., 1999) and pcDNA6-V5-His (Invitrogen, Paisley, UK). Full-length clones
316 of *N*50001* cDNA (AY 188807), encoding N*50001, were generated by PCR from cattle PBMC
317 cDNA. This class I gene is very prone to alternative spicing, and the most common splice variant
318 was used in this study. Multiple clones were sequenced and a clone containing the consensus
319 sequence was introduced into pcDNA6-V5-His (Figure 1).
320 pz4E5 and pc4E5 are plasmids carrying the ORF of BPV-4 E5 under the transcriptional control of
321 the murine MLV LTR or the IE promoter of HCMV respectively. These plasmids have been
322 described before (Ashrafi et al., 2002; Pennie et al., 1993).
323 **Cells**. P815 cells are a mouse mastocytoma cell line (ATCC). They are grown in suspension in
324 RPMI 1640 supplemented with 10% FCS. Stable transfectants expressing N*01301 were produced
325 by transfection of pcDNA6-V5-His *N*01301*, and maintained under blasticidin selection. These
326 cells are designed P815-N*01301. Stable P815 transfectants expressing N*50001 were generated as
327 described before (Ellis et al., 1999), and positive cells were identified using blasticidin selection,
328 screening by western blotting with an anti-V5 monoclonal antibody (mAb) (Invitrogen), and flow
329 cytometry. These cells are designated P815-N*50001. Both P815-N*01301 and P815-N*50001
330 were transfected with either pz4E5 or pc4E5, or the corresponding empty vectors, with an Amaxa
331 nucleofector (Amaxa GmbH) using the kits and following the instructions supplied by the
332 manufacturers. Cells were selected by G418 resistance and stable transfectants were isolated.
333 **Quantitative RT-PCR (Q-RT-PCR)**

334 RNA was extracted from P815 cells using RNeasy mini Kit (Qiagen, UK) and residual DNA was
335 inactivated using DNase I treatment (Invitrogen). Q-RT-PCR for BPV-4 E5 and mouse β-actin
336 mRNA was carried out using the Taqman EZ RT-PCR Kit (Applied Biosystems, Foster City, CA).
337 Each reaction was performed in triplicate using 100ng of RNA. Oligonucleotide primers, designed
338 using Primer Express (v 1.7, Perkin Elmer, Oak Brook, IL), were as follows:
339 BPV4 E5 F5'-TGTCTTTGTGGCTTATCTATGTTTTGT-3';
340 BPV-4 E5 R5'-CCGAGTAATAGTAGAAATTAACAGAAGGTACAC-3'; and FAM/ TAMRA probe 5'-
341 CTTTTCTGGTGTGCTTTTAATTTTCTTGCACTGTTA-3'.
342 β-actin F5'-GCTCTGGCTCCTAGCACCAT-3';
343 β-actin R5'-GCCACCGATCCACACAGAGT-3' and FAM/ TAMRA probe
344 5'-AAGATCATTGCTCCTCCTGAGCGAAAG-3'.
345 PCR reactions were performed using an ABI prism 7700 sequencer. Standard curves were
346 generated using 10-fold serial dilutions of each template DNA, which were used for quantifying the
347 relative levels of E5 and β-actin RNA.
348 **Flow cytometric analysis of MHC class I expression**
349 P815-N*01301 and P815-N*50001 cells either expressing BPV-4 E5 or carrying empty vector were
350 grown in suspension in T175 cm^2 flasks until subconfluent. Following centrifugation, the cell pellet
351 was washed with PBS and resuspended in PBS/1% bovine serum albumin (PBS-B) at a
352 concentration of 10^6 cells/ml. For the detection of surface MHC class I molecules, aliquot of 10^6
353 cells were incubated for 1h at 4ºC with monoclonal antibody (mAb) IL-A88, specific for bovine
354 MHC class I (Toye et al., 1990) in PBS-B. After washing twice with PBS, cells were incubated with
355 1:100 dilution of anti-mouse IgG-FITC (Sigma, UK) for 30 min at 4ºC in the dark. The cells were
356 washed as above, resuspended in 500 µl PBS and analysed by flow cytometry. If the flow
357 cytometry analysis was not carried out immediately, the cells were resuspended in 500 µl of 3%
358 paraformaldehyde in PBS and kept at 4ºC. P815 cells not expressing bovine MHC class I were used
359 as control, as well as cells incubated without primary antibody. For the detection of intracellular

360 MHC class I, the cells were permeabilised with 0.1% saponin in PBS-B for 30 min at RT and then
361 incubated with primary antibody as described above. All samples were examined in a Beckman
362 coulter EPICS Elite analyzer equipped with an ion argon laser with 15mV of excitation at 488 nm.
363 Data were analyzed using Expo 2 software.

364 **Localisation of MHC class I in P815 cells by immunofluorescence**

365 An eight-well slide (Chamber Slides Lab-tek, VWR International) was washed with alcohol, air-
366 dried and treated with 40µl/well poly-L lysine hydrobromide (Sigma) for 10 minutes to allow
367 adhesion of the cells. After removal of poly-L lysine, the slide was washed with distilled water and
368 air-dried. P815 cells were pelleted, washed twice in PBS and resuspended in fixing solution (5%
369 formaldehyde in PBS containing 2% sucrose) for 10 minutes. After two washes, the cells were
370 plated in PBS containing 1% FCS, at $1x10^2$ cells/well, and left for 15 minutes to settle down. After
371 removal of PBS, the cells were treated with permeabilising solution (0.01% Tween-20 in PBS) for
372 10 minutes. The Golgi apparatus was visualised with 1:1000 dilution of BODIPY Ceramide Texas
373 Red (TR) (in 2.5% Hepes in serum-free 1640 RPM medium) for 30 min at 4ºC. BODIPY Ceramide
374 specifically locates to the Golgi apparatus. After removal of BODIPY Ceramide, cells were
375 incubated in 2.5% Hepes in serum-free 1640 RPM medium for 30 min at 37ºC. For detection of
376 MHC class I, cells were first incubated with IL-A88 antibody for 1h at RT followed by incubation
377 with 1:1000 dilution of anti-mouse IgG-FITC for 1h in dark with two washes in between. The cells
378 were washed three times and the slide was mounted with a 60mm coverslip using citifluor.

379 **Imaging**

380 Images were captured using a Leica TCS SP2 true confocal scanner microscope (Leica
381 Microsystems, Heidelberg, Germany) at a wavelength of 488 nm (MHC class I: FITC) or 543 nm
382 (Golgi apparatus: Texas Red). The merge between the fluorescent signals was achieved using the
383 accompanying software.

384 **Immunoblotting**

385 P815 cells were lysed in 300µl of lysis buffer (0.5% NP40, 50mM Tris PH 7.8, 150mM NaCl)
386 containing a protease inhibitor cocktail (Roche, Lewes, UK). Following incubation on ice for 30
387 min, the cell debris was eliminated by centrifugation and the protein concentration was measured
388 using the BCA/$CuSO_4$ assay. To determine the stability of classical and non-classical bovine MHC
389 class I heavy chain in P815 cells, aliquots of 2x 10^6 cells were treated with 100µg/ml
390 cyclohexamide (Sigma) or the solvent DMSO (Sigma) for different periods of time at 37ºC. The
391 cells were washed with ice cold PBS, pelleted and resuspended in 100µl of lysis buffer as described
392 above.

393 The protein samples were mixed with 4x NuPAGE® SDS sample buffer (40% Glycerol, 500mM
394 Tris-HCl (pH 6.8), 8% SDS, 0.075% Serva blue G250, 0.025% phenol red), and 10x NuPAGE®
395 Reducing Agent (0.5M DTT). The samples were heated at 70ºC for 10 minutes before loading in
396 NuPAGE® 4-12% Bis-Tris-HCl (pH 6.4) poly-acrylamide gels (Invitrogen Ltd). Electrophoresis
397 was performed in MES SDS Running buffer (Invitrogen Ltd) at a constant voltage 200V for 60
398 minutes.

399 Separated protein samples were transferred to nitrocellulose membranes by wet electrophoretic
400 transfer using a Xcell II™ blotting apparatus (Invitrogen Ltd) as per manufacturer's instructions.
401 The blotting was performed at a constant voltage of 30V for 80 minutes in 1x NuPAGE® transfer
402 buffer containing 10% methanol. Transfer was checked by staining the nitrocellulose membrane
403 with Ponceau S solution (Sigma).

404 Membranes were blocked in 5% milk in Tris buffered saline (TBS) containing 0.05% Tween 20 (T-
405 TBS) for 1h at RT or overnight at 4ºC. For detection of MHC class I, the membranes were
406 incubated with mAb IL-A88 in 5% milk in T-TBS for 2 hours at RT. After two washes in T-TBS,
407 the membranes were incubated with 1:5000 dilution of horseradish peroxidase-linked sheep anti-
408 mouse IgG whole antibody (Amersham Biosciences) in 5% milk in T-TBS for 1 hour at RT. Bound
409 antibody was detected by incubating the membrane for 5 minutes at RT with ECL plus western
410 blotting detection system (Amersham Biosciences) according to the manufacturer's instructions.

411 After exposure, bound antibody was removed from the membranes with stripping buffer (100mM 2-
412 Mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH6.7) at 50ºC for 30 minutes. After washing in T-
413 TBS, the membranes were blocked in 5% milk in T-TBS for 1 hour at RT prior to re-probing with
414 1:20,000 dilution of anti-actin antibody (mAb Ab-1; CALBIOCHEM). After incubation with mAb
415 Ab-1, the membranes were washed two times in T-TBS and incubated with anti-mouse IgG linked
416 to horseradish peroxidase as described above.

417 **Co-immunoprecipitation.**

418 **a. In vitro transcription/translation and co-immunoprecipitation.**

419 In vitro transcription/translation reactions were performed using the TNT® T7 Quick Coupled
420 Transcription/Translation System (Promega, UK) in presence of Redivue L-[^{35}S] Methionine
421 (Amersham Pharmacia Biotech, UK) following the manufacturer instructions. Briefly, 1 μg of
422 pc4E5, pcDNA3-*N*01301* or pcDNA6-V5-His-*N*01301*, or pcDNA6-V5-His-*N*50001* was mixed
423 in a 50μl reaction containing TNT® mix (TNT® lysate with energy generating system, T7 RNA
424 polymerase, nucleotides, salts, recombinant RNasin® ribonuclease inhibitors) in presence of canine
425 microsomal membranes (CMM) (Promega, UK) at 30°C for 1.5 h. Half of each
426 transcription/translation reaction product was immunoprecipitated with either 10 μl rabbit antiserum
427 Ab 274, raised against the C-terminus of the BPV-4 E5 protein (Anderson et al., 1997; Pennie et al.,
428 1993) or with 3 μl mAb IL-A88. The other half of each reaction was left without antibody as a
429 negative control. For co-immunoprecipitation experiments, the individual transcription/translation
430 products were mixed in equivalent amounts and immunoprecipitated with double the amount of
431 either antibody. For competition experiments, no [^{35}S] Methionine was added to the reaction
432 containing pcDNA3-*N*50001* and the unlabelled N*50001 was added to labelled E5 for an hour
433 before the addition of labelled N*01301 as above. After incubation overnight at 4°C, protein G-
434 sepharose bead suspension (Sigma) was added for 1 h at 4°C. Following two washes in a high salt
435 buffer (50mM Tris HCl pH 7.5, 500mM NaCl, 1% NonidetP-40, 0.05% NaDoc) and one wash in a
436 low salt buffer (50mM Tris HCl pH 7.5, 1% NonidetP-400, 0.05% NaDoc) the sepharose beads

437 were resuspended in 20 µl of SDS loading buffer, heated at 75°C for 10 min, and then were
438 electrophoresed in 4-12% NuPAGE gels (Invitrogen). Gels were fixed with glacial acetic acid and
439 methanol, incubated for 15 min in Amplify™ Fluorographic reagent (Amersham, UK), dried and
440 exposed for autoradiography at –70°C overnight or exposed on a screen for quantification on a
441 Storm 840 apparatus using a ImageQuant v5.2 software.

442 **b. "Pull-down co-immunoprecipitation"**.

443 Protein lysates were obtained from P815, P815-N*01301 and P815-N*50001 cells. Cells were lysed
444 in RIPA buffer containing a cocktail of protease inhibitors (Roche, Lewes, UK). Protein lysates
445 (100 µg) were incubated with *in vitro* ^{35}S-labelled E5 overnight at 4°C and immunoprecipitated
446 with mAb IL-A88 as described above. Immunopreciptates were electrophoresed in 4–12%
447 NuPAGE gels (Invitrogen). Gels were fixed with glacial acetic acid and methanol, incubated for 15
448 min in Amplify Fluorographic reagent (Amersham, UK), dried and exposed for autoradiography at
449 -70°C overnight.

450

451

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549 **Figure Legends**

550 Figure 1. Alignment of the predicted amino acid sequences of N*01301 and N*50001 (splice
551 variant). Dots indicate identity, * indicates stop codon. Divergent amino acids (bold) are seen only
552 in N*50001 (or other cattle non-classical class I alleles) and are not seen in cattle classical class I
553 alleles (www.ebi.ac.uk/ipd/mhc/bola/). Four additional amino acids at the end of the alpha 3 domain
554 of N*50001 are the result of incorrect splicing of intron 4.

555 Figure 2. BPV-4 E5 ORF is transcribed at comparable levels in all P815 cells. E5 and β-actin RNA
556 was measured by Q-RT-PCR. The histograms represent the average with standard deviation of six
557 independent measurements. Note that the levels of E5 RNA are three orders of magnitude lower
558 than those of β-actin RNA.

559 Figure 3. BPV-4 E5 down-regulates surface and total classical N*01301 MHC class I but not non-
560 classical N*50001 MHC class I. **A**, example of flow cytometry profiles of surface and total classical
561 N*01301 and non-classical N*50001 MHC class I in P815 control cells or cells expressing E5. **B**,
562 mean forward fluorescence with standard deviation calculated from the flow cytometry profiles of
563 at least three independent experiments in duplicate.

564 Figure 4. BPV-4 E5 induces degradation of classical N*01301 MHC class I heavy chain but not
565 non-classical N*50001 heavy chain. **A**, steady state levels of classical N*01301 and non-classical
566 N*50001 heavy chain in P815 control cells or cells expressing E5. **B**, half-life measurement of
567 classical N*01301 and non-classical N*50001 heavy chain in cycloexamide-treated P815 control
568 cells and cells expressing E5. HC, heavy chain.

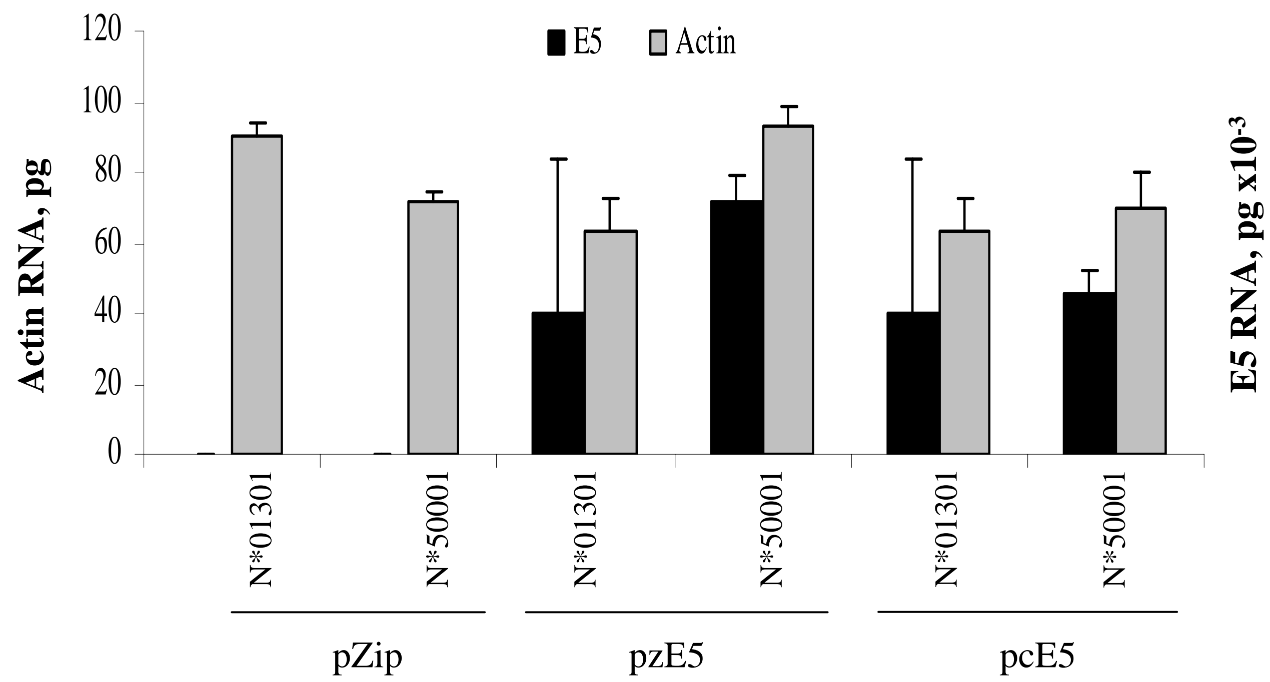
569 Figure 5. BPV-4 E5 induces retention of classical, but not non-classical, MHC class I in the Golgi
570 apparatus. **A**, immunofluorescent detection of classical and non-classical MHC class I in P815
571 control cells or cells expressing E5.

572 Figure 6. BPV-4 E5 interacts with classical N*01301 heavy chain but fails to interact with non-
573 classical N*50001 heavy chain. **A**, ^{35}S-labelled *in vitro* transcribed/translated E5 and N*01301 HC
574 or N*50001 HC were immunoprecipitated with mAb IL-A88, against MHC class I heavy chain; the

575 precipitate was run in a NuPAGE gel and the dried gel exposed on a screen on a Storm 840
576 apparatus. Classical N*01301 HC interacts with E5 (lane 3), while non-classical N*50001 HC fails
577 to interact with E5 (lane 5) and to compete with classical N*01301 HC for binding to E5 (lane 9).
578 Several additional bands are visible which derive from the translation of the proteins (see input
579 lanes) which may be due to dimers or multimers of E5 or to degradation products of HC. **B**, "Pull-
580 down co-immunoprecipitation" between E5 and N*01301 HC. Protein extracts from P815, P815-
581 N*01301 and P815- N*50001 cells were incubated with ^{35}S-labelled *in vitro* transcribed/translated
582 E5 and precipitated with mAb IL-A88. E5 is precipitated from P815-N*01301 cells (lane 2) but not
583 from P815 parental or P815-N*50001 cells (lanes 3 and 4).
584
585

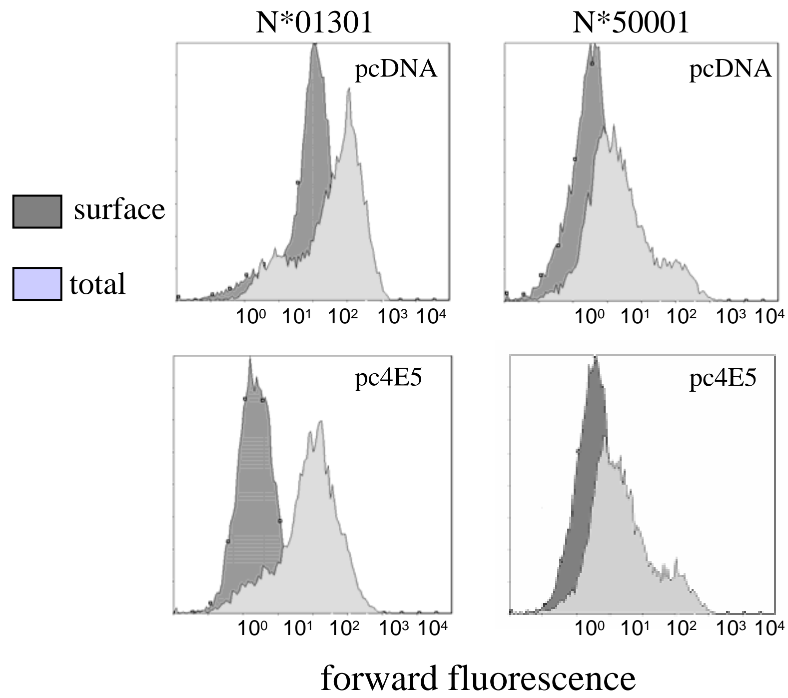
```
                            Alpha 1
                   *         20         *         40         *
N*01301 : LFMLLLGALVLIETRA|GSHSLRYFHTAVSRPGLREPLFITVGYVDDTQFVRFDSDAR
N*50001 : .LL....V..PRD...|.P..M...L........G..R..................RP

                                                          Alpha 2
             60         *         80         *        100        *
N*01301 : DPRTEPRQPWMEKEGPEYWDRETQISKENALWYREALNNLRGYYNQSEA|GSHTLQEM
N*50001 : ...M...AR.V.D.......Q..R.Q...TQTF.AN..T.L........|...I..W.

              120        *         140        *        160        *
N*01301 : YGCDVGSDGRLRRGYEQYGYDGRDYLALNEDLRSWTAADTAAQISKRKMEAAGAAER
N*50001 : H..G......L...N.HA...K.....................T...W..T......

                                       Alpha 3
                 180        *         200        *        220
N*01301 : FRNYLEGTCVEWLRRYLENGKDTLLRA|DPPKAHVTRHPSSEHEVTLRCWALGFYPEE
N*50001 : .......K..KL...H...........|...M....H..I..R...............

           *        240        *         260        *        280
N*01301 : ISLTWQRNGEDQTQDMELVETRPSGDGNFQKWAALVVPSGEEQRYTCRVQHEGLQEP
N*50001 : ...........................T.......A.....................

              Transmembrane domain                  Cytoplasmic domain
             *        300        *         320        *        340
N*01301 : LTLRW----|EPPQTSFLTMGIIVGLVLLVVAVVAGAVIWMKKHS|GEKRRTYTQAASNDSAQ
N*50001 : .....DKGG|....P.VPI....LV...............S..R.|...G.I..*

                 *
N*01301 : GSDVSLTVPK*
N*50001 :
```

Araibi et al., Figure 1



Araibi et al., Figure 2

A



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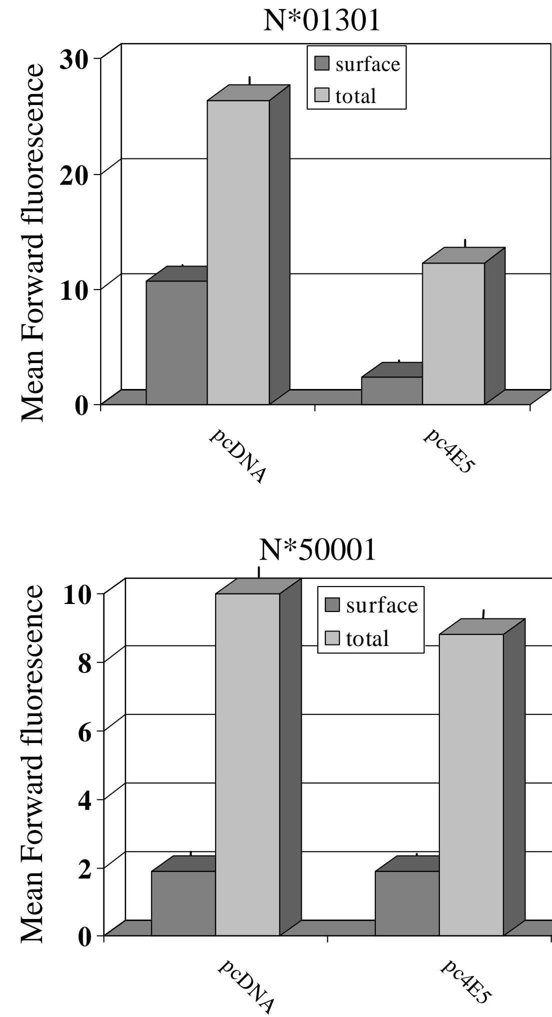
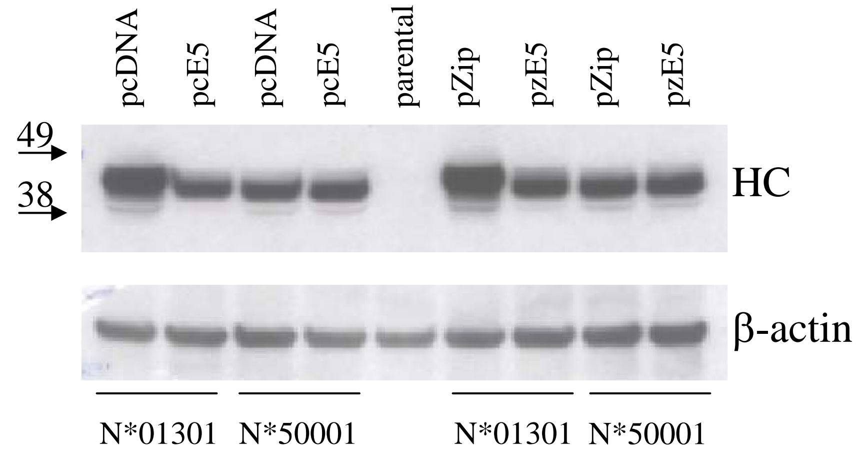
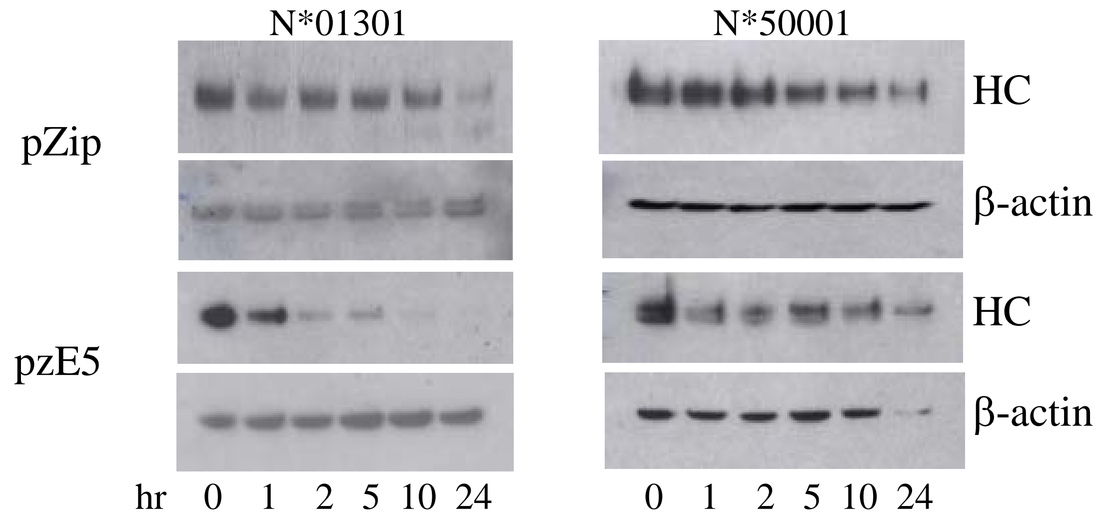
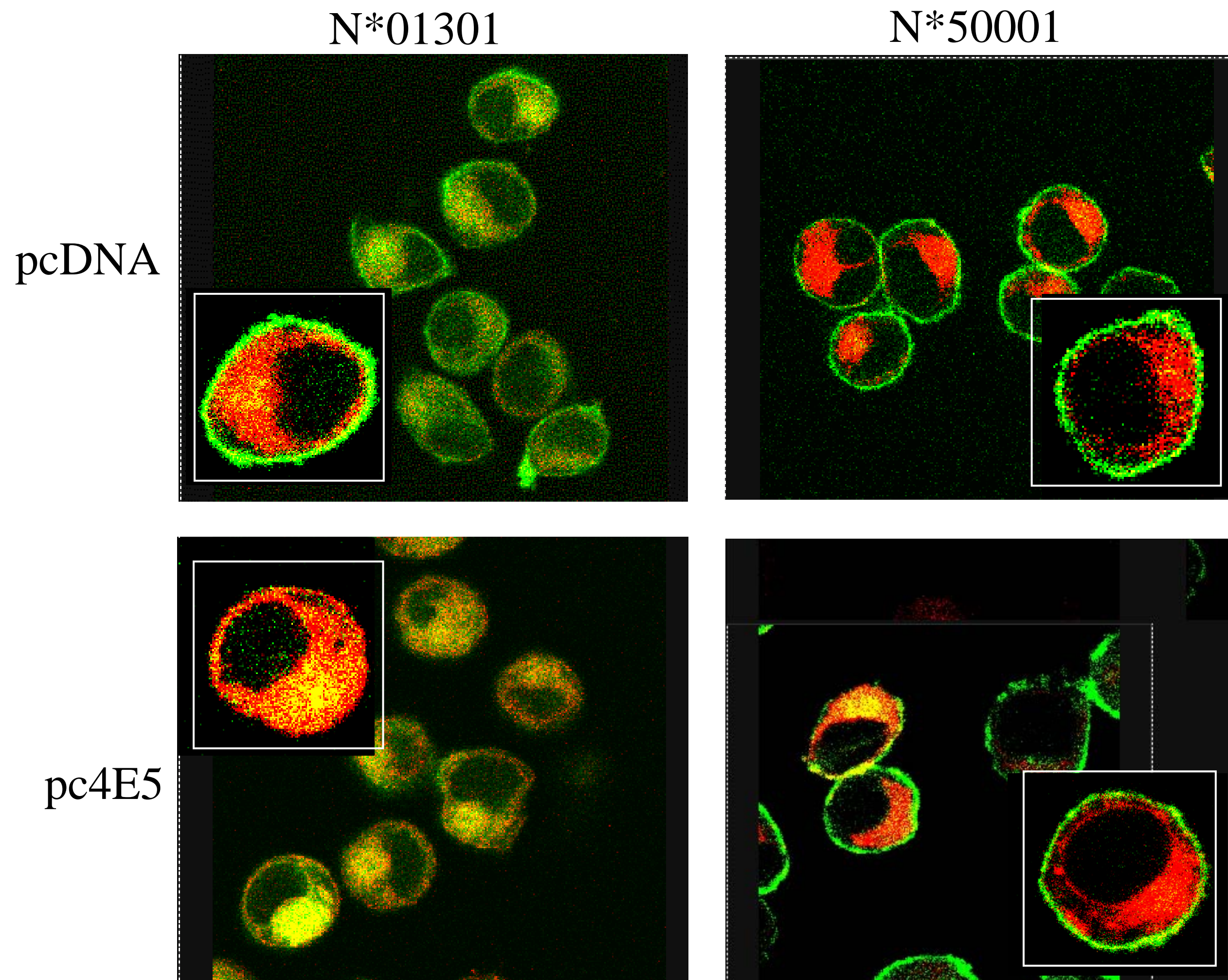


Araibi et al., Figure 3



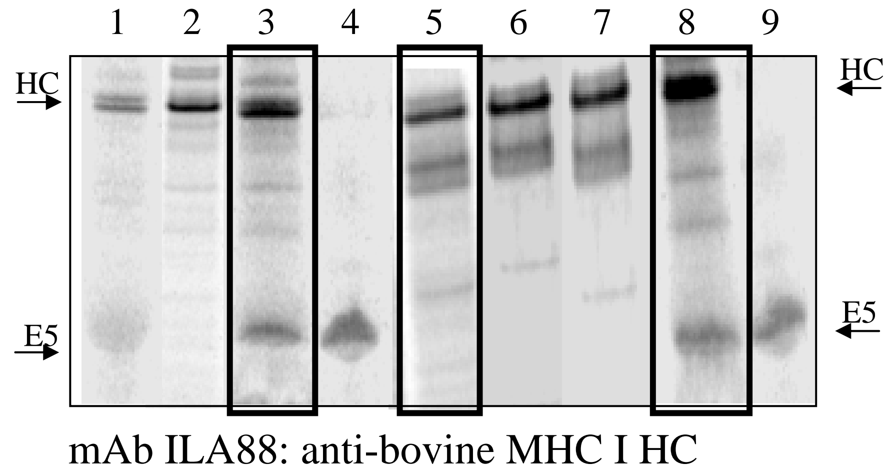


Araibi et al., Figure 4



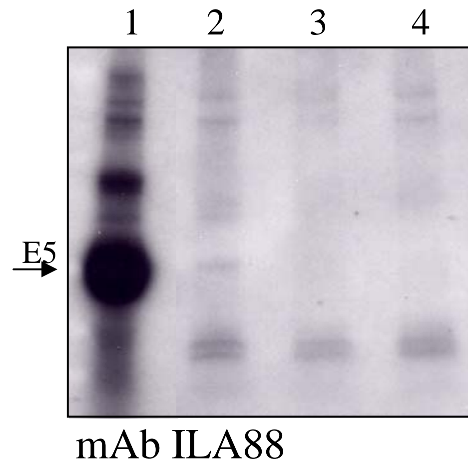
Araibi et al., Figure 5

A



1: input N*01301
2: IP N*01301
3: IP E5+ N*01301
4: input E5
5: IP E5+N*50001
6: IP N*50001
7: input N*50001
8: IP E5+N*01301+N*50001
9: input E5

B



1: input E5
2: P815-N*01301+E5
3: P815+E5
4: P815-N*50001+E5

Araibi et al., Figure 6