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Human papillomavirus 16 L2 inhibits the transcriptional activation function, but not the DNA replication function, of HPV16 E2

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Abstract

In this study we analysed the outcome of the interaction between HPV-16 L2 and E2 on the transactivation and DNA replication functions of E2. When E2 was expressed on its own, it transactivated a number of E2-responsive promoters but co-expression of L2 led to the down-regulation of the transcription transactivation activity of the E2 protein. This repression is not mediated by an increased degradation of the E2 protein. In contrast, the expression of L2 had no effect on the ability of E2 to activate DNA replication in association with the viral replication factor E1. Deletion mutagenesis identified L2 domains responsible for binding to E2 (first 50 N-terminus amino acid residues) and down-regulating its transactivation function (residues 301-400). The results demonstrate that L2 selectively inhibits the transcriptional activation property of E2 and that there is a direct interaction between the two proteins, although this is not sufficient to mediate the transcriptional repression. The consequences of the L2-E2 interaction for the viral life cycle are discussed.

Key words: HPV-16, E2, L2, transcription regulation, viral DNA replication.

1. Introduction

Papillomaviruses (PV) are non-enveloped DNA viruses that infect stratified squamous epithelia in a wide spectrum of animals, and induce benign hyperproliferative lesions which occasionally progress to cancer (Shah and Howley, 1995). After infection, the viral genome replicates in low copy number in the basal layers of the epithelium and starts expressing its non structural proteins, while high-copy number productive replication and expression of the structural proteins takes place in the differentiating cells of the more superficial layers, leading to the assembly of infectious viral particles (Taichman and LaPorta, 1987).

Replication of the viral genome is dependent on two non structural viral proteins, E1 and E2, which specifically bind the viral DNA (Chiang et al., 1992), and E1 and E2 are the only viral proteins necessary to initiate replication from ori, the viral origin of replication (Chow and Broker, 1994). Additionally E2 is a major transcriptional regulator of the viral early genes (Swindle et al., 1999; Yang et al., 1991).

E2 functions are mediated by its binding to numerous sites (BS) in the PV long control region (LCR). In the LCR of mucosal PV, including human papillomavirus type 16 (HPV-16), there are four BS (BS1-4) (Desaintes and Demeret, 1996). Two of these sites are immediately upstream from the TATA box (BS1,2), separated from each other and from the TATA box by 3 or 4 base pairs (bp). Of the other two sites (BS3,4), one (BS3) is beside the E1 DNA binding site involved in the regulation of viral DNA replication, and one (BS4) is a further 300-400 bp upstream. In general, binding of E2 to BS1,2 causes transcriptional repression, while E2 binding to BS3,4 can lead to activation (Demeret et al., 1997). However, the precise effect of E2 on transcription, whether activation or repression, varies depending on the E2,

LCR and host cells (Bouvard et al., 1994; Demeret et al., 1994; Demeret et al., 1997; Thierry and Howley, 1991).

In the bovine papillomavirus type 1 (BPV-1) system, co-expression of the minor structural protein L2 and E2 causes the redistribution of E2 into the dynamic nuclear substructures pro-myelocytic nuclear domains (ND10), leading to the suggestion that a major redistribution of viral components occurs during virion assembly (Day et al., 1998; Heino et al., 2000). Studies performed by Day and colleagues (Day et al., 1998) in a hamster fibroblast cell line, latently infected with multiple copies of autonomously replicating BPV genomes, have shown that expression of L2 is followed by a localisation of L2 to ND10 and a shift in the localisation of E2 into these domains. ND10 localisation has been observed also for HPV-11 E1 and E2 in transient DNA replication systems (Swindle et al., 1999), and for HPV-33 L2 (Florin et al., 2002).

Despite the observed co-localisation of PV E2 and L2 to ND10, little is known about how expression of L2 affects E2. Here we report that in the presence of HPV-16 L2, HPV-16 E2 loses its transcriptional transactivation ability but not its origin-dependent DNA replication activity. The loss of transactivation activity is accompanied by L2-induced proteasome-independent post-transcriptional down-regulation of E2 in HaCaT cells but not in C33a cells. The domains of L2 responsible for E2 binding, inhibition of E2 transactivation and E2 degradation have been mapped. The implications of these results for the viral life cycle are discussed.

2. Materials and methods

2.1. Cells. HaCaT cells are spontaneously immortalized keratinocytes and are routinely grown in Dulbecco's modified Eagle medium High Glucose without calcium chloride (Life Technologies, UK), supplemented with 10% foetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine, 100 IU penicillin per ml, and 100 μg streptomycin per ml at 37ºC in 5% CO_2. C33a cells are human cervical carcinoma cells grown in Dulbecco's MEM with GlutaMAX-I 4500 mg/L D-Glucose with sodium pyruvate (Life Technologies), supplemented with 10% foetal calf serum, 100 IU penicillin per ml, and 100 μg streptomycin per ml at 37ºC in 5% CO_2.

2.2. Plasmids: pCMV-$E2_{16}$ (Bouvard et al., 1994) contains the HPV16 DNA fragment from nt 2725 to 3852) cloned into the *Xba*I-*Sma*I sites of the cytomegalovirus immediate-early promoter/enhancer-based expression vector $pCMV_4$ (Del Vecchio et al., 1992). p16-L2 contains the HPV-16 L2 DNA sequence from nt 4135 to 5656 inserted into pcDNA3 (Invitrogen, UK), and p16-HAL2 is p16-L2 with three copies of HA1 epitope (Lee et al., 1998) at the N-terminus of L2. p18LCR-BS1 is a pGL3 luciferase reporter plasmid that contains a HPV-18 LCR with four point mutations in the E2 binding site 1 introduced by PCR. p18LCR-BS1-3 is a pGL3 luciferase reporter plasmid that contains the HPV-18 LCR with mutations in the E2 binding sites 1 to 3. p18LCR-BS1-3 was derived from a CAT reporter plasmid, generously provided by F. Thierry (Thierry and Howley, 1991). Plasmid p18-6E2 (kindly provided by K. Vance) is a luciferase reporter plasmid with six E2 binding sites, separated by 4 base pairs, linked to the HPV-18 promoter, ptk is a plasmid with the luciferase gene under the control of the tk promoter, and ptk6E2 is the same plasmid with the six E2 binding sites linked to the tk promoter (Vance et al., 1999). p16ori-m is a modification of plasmid p16ori (Del Vecchio et al., 1992) in which a

base change was introduced at nt 115 (T instead of C) to generate a Dpn I site (Boner et al., 2002).

pGEX-4T-2 (Amersham Pharmacia Biotech, UK) encodes glutathione S-transferase (GST). Full length L2 and 5' or 3' deleted ORFs were inserted into pGEX-4T-2 to create GST-L2 fusion proteins with the GST moiety at the N-terminus of L2. Deletion mutagenesis of the L2 ORF was done by PCR.

pEGFP-C1 (Clontech, UK) encodes the Green Fluorescent Protein (GFP). It was used to clone full length L2 and 5' or 3' deleted ORFs to generate GFP-L2 fusion proteins with the L2 moiety at the C-terminus of GFP.

2.3. Antibodies for Western blot. The following primary antibodies were used: anti HPV-16 E2 mouse monoclonal antibody TVG261 (Hibma et al., 1995); mouse anti-HA monoclonal antibody HA.11 (Cambridge Biosciences, UK); anti HPV-16 L2 polyclonal rabbit serum (Gornemann et al., 2002) and anti GFP polyclonal rabbit serum SC-8334 (Santa Cruz Biotechnology, UK). The secondary antibodies used were GPR, a sheep anti-mouse Ig (Amersham Pharmacia Biotech) and anti rabbit IgG (Sigma, UK), both linked to horseradish peroxidase. For the detection of actin, mouse monoclonal antibody Ab-1 (Oncogene™, UK) and goat anti-mouse IgM-HRP (Santa Cruz Biotechnology) were used.

2.4. Western blot analysis. Cells were seeded onto 100mm cell culture dishes at a density of 10^6 cells/dish and cultured overnight. The cells were transfected with 4μg pCMV-$E2_{16}$ and/or 4μg p16-HAL2 using Lipofectamine Plus™ Reagent (Life Technologies) or calcium phosphate as above. HaCaT-16E2 cells were identically transfected with p16-HAL2. Cells were harvested 24 h post-transfection, washed twice with cold PBS and then lysed on ice in 250 μl of SDS- lysis buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 2% glycerol), clarified by centrifugation for 10 min at 4ºC

and the supernatant was recovered. Protein concentration was determined by absorbance measurement at 280 nm. 10 μg of each sample were electrophoresed on Nu PAGE™ 4-12% Bis-Tris gel under denaturing conditions and transferred onto a nitrocellulose membrane (Invitrogen, UK). Membranes were either incubated with mAb TVG261 (dilution 1:250), anti-HA mAb HA.11 (dilution 1:1,000), anti HPV-16 L2 serum (1:3,000), anti GFP serum (1:5,000) or mAb Ab-1 (dilution 1:20,000), followed by incubation with GPR or anti-rabbit IgG at a dilution 1:5,000. Western blots were developed by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

For proteasomes inhibition studies, 16 hours after transfection, the cells were treated for 8 h with 5μM MG132, 12.5μM Lactacystin, 100μM ALLM or 100μM ALLN (Calbiochem, UK) before further processing.

2.5. Luciferase reporter assays. Cells were seeded in 60mm plates and were transfected with 0.1μg of the reporter plasmids p18LCR-BS1and p18LCR-BS1-3, p18-6E2 and ptk6E2, using Lipofectamine Plus™ Reagent for HaCaT cells, and the calcium phosphate precipitation method for C33a cells. E2 function was determined by co-transfecting the reporter plasmids with increasing amounts of pCMV-$E2_{16}$ (up to 10ng) or with a fixed amount (1ng) of pCMV-$E2_{16}$ and increasing amounts of p16-HAL2, p16-L2 or p16-L2 deletion mutants (0.01μg – 1μg). Total amount of DNA was adjusted to 2μg with $pCMV_4$. Cells were harvested 48 hours post-transfection and luciferase assays were performed according to the manufacturer's instructions (Promega, UK).

2.6. Transient DNA replication assays. Transient DNA replication assays were performed in C33a cells, as first described by Sakai and colleagues (Sakai et al., 1996) and as modified by Boner *et al.* (Boner et al., 2002). Cells were set up in

100mm plates at $6x10^5$ per plate and transfected with 1μg p16ori-m, 5μg pCMV-$E1_{16}$, 10ng or 100ng pCMV-$E2_{16}$ with or without 1μg or 2μg p16-HAL2 using calcium phosphate precipitation for C33a cells or Lipofectamine Plus™ Reagent method for HaCaT cells, respectively. Low molecular weight DNA was extracted using the Hirt protocol (Hirt, 1967) modified as described below. Three days after transfection, cells were washed twice with PBS and lysed in Hirt solution (0.6% SDS, 10mM EDTA). NaCl was added to 1M final concentration, and the lysates were left overnight at 4^oC. Samples were centrifuged at 15000g for 30minutes at 4^oC, the supernatant, containing low molecular weight DNA, was retained, extracted with phenol-chloroform and DNA precipitated with ethanol. To create linearised plasmid, 25μl of each sample were digested with 20U Xmn I for 3 hours at 37^oC. Linearised plasmid was further digested with 20U Dpn1 overnight at 37^oC. As Xmn I linearises p16ori-m while Dpn I distinguishes replicated from unreplicated DNA, replication was assayed after Xmn I and Dpn I digestion. DNA was electrophoresed in a 1% agarose gel in 0.5x TBE and transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech) by Southern blotting. Hybridisation was done using QuikHyb hybridisation solution (Stratagene Europe) according to the manufacturer's instructions. A Molecular Dynamics Storm 840 (Amersham Pharmacia Biotech) phosphorimager was used to scan the Southern blot and quantify hybridised signals. The extent of replication was calculated by measuring the ratio of double cut/single cut bands. This method of measurement controls for variation in transfection efficiency between experiments.

2.7. Probe for hybridisation. The p16ori-m (2μg) plasmid was digested with Pvu II. The digest was run down in a 1% agarose gel and a band of ~700bp, corresponding to the sequence of HPV16-ori, was cut out. It was purified using the QIAquick™ Gel Extraction kit (Qiagen, UK) and eluted in 60μl dH_2O. A Prime-it II Random labelling

kit (Stratagene Europe) with α^{32}PdCTP was used to generate the probe according to the manufacturer's instruction and purified using a NICK Column (Amersham Pharmacia Biotech, UK).

2.8. Real Time Quantitative PCR. HaCaT cells were seeded and transfected with 4μg pCMV-$E2_{16}$ and/or 4μg p16-HAL2 as described above for Western blot, and total RNA was isolated with RNeasy®MiniKit (Qiagen, UK) and resuspended in RNase-free water following the instructions of the supplier. Thirty-two ng total RNA was digested with 0.5U DNAse I (Invitrogen™, UK) according to the manufacturer's protocol. The reverse transcription was primed in duplicate with Random Primers (Promega, UK) at a concentration of 0.5μg in a 25μl reaction mixture with or without 3U AMV-RT and 0.5mM each dNTP, 20U rRNAsin, and 1X AMV-RT buffer (all from Promega) following the instructions of the supplier. As a preliminary screening, the products of the reactions with or without AMV-RT were amplified by PCR with 2.5U Taq Polymerase (Gibco BRL®, UK) in a final volume of 50μl containing 1X PCR buffer (Gibco BRL®, UK), 125μM of each dNTP (Promega, UK) and 0.5μM of each primer (sense: 5'CGA TGG AGA CTC TTT GCC AA; antisense: 5'TAT AGA CAT AAA TCC AGT) for 30 cycles at 94ºC 1 min, 50ºC 1 min and 72ºC for 2 min and 1 cycle at 72ºC for 10 min and the PCR products were electrophoresed in a 1% agarose gel. Once confirmed that the RNA was free from DNA contamination, cDNA was amplified in triplicate with the primers Tq16E2f 5'CCT GAA ATT ATT AGG CAG CAC TTG and Tq16E2r 5'GCG ACG GCT TTG GTA TGG at a concentration of 300mM each in a 50μl reaction that contained in final concentration: 1X PCR Buffer II, 200μM dATP, dCTP, dGTP, and 400μM dUTP, 5.5mM $MgCl_2$ and 1.25U AmpliTaq® DNA Polymerase with GeneAmp®. The reaction also contained the detection probe TaqMan® Probe: 5' CAA CCA CCC CGC CGC GA, at a

concentration of 300nM. The thermal cycling conditions were 2 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 7700 Sequence Detector (PE Applied Biosystems, UK), which contains a GeneAmp PCR System 9600. As control cDNA was amplified with primers for actin: β-actin Forward Primer and β-actin Reverse Primer (PE Applied Biosystems, UK) at a concentration of 60nM each in a reaction mix as described for E2 and with the same cycling conditions, with β-actin Probe (PE Applied Biosystems, UK) at a concentration of 40nM.

2.9. Detection of GFP fusion proteins. HaCaT cells were seeded onto nº 01 coverslips in 24-well plates at a density of 10^5 cells/well and cultured overnight. When 80% confluent, cells were transfected with 0.1μg of either pEGFP-L2 or pEGFP-L2 deletion mutants, or with control empty plasmids (pEGFP-C1), using Lipofectamine Plus™ Reagent according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were washed twice with PBS and fixed by 10-min incubation at room temperature with 1.85% formaldehyde diluted in PBS containing 2% sucrose, and washed three times with PBS. Cells were incubated with 4'6'-Diamino-2-phenylindole (DAPI) for 10mins, to stain the nucleus, washed in PBS-FCS and then distilled water, dried and mounted in AF1 (Citifluor, UK). Cells were analysed with a Leica TCS SP2 true confocal scanner microscope (Leica-microsystems, Heidelberg Germany). Images were merged and acquired using Leica confocal software.

2.10. Expression and purification of GST fusion proteins. Colonies of E. coli BL21 containing recombinant pGEX-GST-L2 fusion expression vectors were grown at 37^{o}C with shaking until an absorbance of 0.6-0.8 at 600nm was reached. Expression was induced by the addition of IPTG (final conc. 0.4-0.1mM). Bacteria

were pelleted and resuspended in 5ml of BugBuster™ Protein Extraction Reagent (Novagen, Merck, UK) containing 1 tablet protease inhibitor cocktail per 10ml reagent, and incubated for 30min. The cell extracts were centrifuged at 2500g for 30min. 1ml aliquots of the supernatant were transferred to fresh tubes and stored at –70°C. Fusion proteins were purified on glutathione-Sepharose beads by incubating 1ml supernatant with 50µl beads for 30 min at room temperature with rotation. The beads were pelleted in a microfuge (14000 rpm, 20 sec) and washed three times with 0.5 ml NETN (20mM Tris-HCL, pH 8.0), 100mM NaCl, 1mM EDTA, 0.5% Nonidet P-40 (NP-40) containing 1 tablet protease inhibitor cocktail per 10ml buffer. The beads were resuspended in 50µl NETN and purified proteins were analysed by 10% SDS-PAGE before subsequent manipulations.

2.11. In vitro transcription/translation of E2. HPV-16 E2 ORF was subcloned from pCMV-HPV16 E2 into the BamHI site of pBluescript SKII under control of the T7 promoter using standard molecular biology techniques. HPV-16 E2 was in-vitro transcribed-translated using TNT Quick Coupled Transcription/Translation System (Promega, UK) as instructed by the manufacturer to produce ^{35}S-methionine labelled E2. The efficiency of transcription-translation was checked using a luciferase control plasmid construct to produce ^{35}S labelled luciferase. 5µl of each reaction was analysed by SDS-PAGE and the gel was fixed in 7% methanol and 7% glacial acetic acid for 15min shaking and 10minutes with Amplify™ Fluorographic reagent (Amersham Pharmacia Biotech, UK). The gel was dried and exposed for autoradiography at –70°C overnight.

2.12. GST pull-down assays for L2-E2 interaction. GST pull-down assays were performed as follows: pGEX, pGEX-L2 and pGEX-L2 deletion mutant proteins were expressed and purified as described above. The proteins immobilised on beads were

pre-washed three times in pull-down buffer (PDB: 50mM Tris pH 7.9, 100mM NaCl, 1mM DTT, 0.5mM EGTA, 0.5% NP-40, 1mM PMSF). 7.5µl ^{35}S labelled E2 was incubated with approximately 1µl bead-immobilised fusion protein in a total volume of 200µl fresh PDB for 30 min at 4^{o}C with rotation. The beads were pelleted in a microfuge (14000 rpm, 10sec) and washed four times in PDB. Bound proteins were separated by 10% SDS-PAGE, fixed, the gel was dried and exposed for autoradiography at –70^{o}C overnight. Bands were then analysed by densitometry using a UMAX Powerlook III Flatbed scanner and ImageQuant v5.2 software.

3. Results

It has been reported that L2 localises to ND10 in the BPV-1, HPV-11 and HPV-33 systems (Day et al., 1998; Florin et al., 2002; Heino et al., 2000) and, in BPV-1, directs E2 to ND10 (Day et al., 1998; Heino et al., 2000). We asked how HPV-16 E2-L2 interaction would affect E2 functions and investigated it in HaCaT and C33a cells. HaCaT cells are spontaneously immortalised keratinocytes containing no HPV genes, while C33a cells are derived from an HPV-negative cervical cancer.

3.1. HPV-16 L2 down-regulates the transactivation function of E2. To investigate whether the HPV-16 E2 and L2 proteins interact, and what effect this interaction would have on the transcription transactivation function of E2, we used the luciferase reporter plasmids p18-LCR-BS1 and p18-LCR-BS1-3 in which either BS1 or BS1-3 are mutated (Figure 1A). These mutant forms of the HPV18 LCR were chosen as they are transactivated by E2 in contrast to wild type LCR which is repressed by E2 (Demeret et al., 1997). We also used p18-6E2 and ptk6E2, two minimal promoter constructs, containing six E2 binding sites, expressing luciferase under the control of the HPV-18 promoter or the tk promoter, respectively (Figure 1A), which are

transactivated by E2 (Vance et al., 1999). Each reporter plasmid was co-transfected with increasing amounts of pCMV-$E2_{16}$ in HaCaT and C33a cells. In all cases, luciferase expression increased with increasing amounts of E2 and there was no trans-repression even at the highest amounts of E2 used (Figure 1B).

The effect of E2-L2 interactions on the transactivation functions of E2 was investigated by co-transfecting increasing amounts (up to 0.5μg) of p16HAL2 or p16L2 with constant amounts of luciferase reporter (0.1μg) and of pCMV-$E2_{16}$ (1ng) in either HaCaT or C33a cells. pCMV-$E2_{16}$ was kept constant at 1ng as this amount is sub-optimal for E2-mediated trans-activation thus allowing the detection of any effect of L2. The addition of L2 led to an inhibition of E2-mediated transactivation in a dose-dependent manner in both cell lines (Figures 1C). This was observed for all reporter plasmids, although the kinetics of inhibition varied slightly from reporter to reporter. All reporters were however fully inhibited at the highest amounts of L2 used. The addition of L2 in the absence of E2 did not affect the constitutive activity of the reporters (Figure 1C), nor did it affect promoters not responsive to E2 (data not shown). We conclude that L2 inhibits the transactivation function of E2. Co-expression of L2 and E2 did not relieve trans-repression of the wild type LCR by E2 (data not shown).

3.2. HPV-16 L2 does not down-regulate the viral replication function of E2. E2 binds to E1 and promotes origin-dependent viral DNA replication. We examined the effect of L2 on the ability of E2 to co-operate with E1 in a transient DNA replication assay. The DNA replication assays were performed in C33a; replication assays did not work in HaCaT cells, perhaps due to a decrease in cdk2/cyclin E activity as described for HeLa cells (Lin et al., 2000). A representative replication assay is shown in Figure 2A. DNA replication was dependent on the

presence of p16ori-m, E1 and E2. In the absence of E2, no replication took place, while at 10ng of E2 a replicated band was detected, increasing with increasing E2 concentrations (Figure 2A, cf. lanes 6 and 12). There was no replication in the presence of L2 and absence of E2 (Figure 2B). To compare different experiments, the blots were quantified by scanning the membranes and the ratio between replicated and input DNA was measured for three experiments. Replication in absence of L2 was taken as 1; the expression of L2 did not appreciably affect replication and the small difference was not statistically significant (Figure 2B). These results demonstrate that, whereas L2 had little effect on the replication activity of E2 as shown for BPV-1 (Heino et al., 2000), it drastically inhibited the transcriptional function of E2.

3.3. Co-transfection with L2 induces down-regulation of E2. Co-expression of the L2 and E2 genes in HaCaT cells transiently co-transfected with both E2 and L2 plasmids was ascertained independently by detection of L2 and E2 RNA by RT-PCR (L2, data not shown; E2, Figure 4B) and expression of L2 and E2 proteins by Western blots (Figure 3A). E2 levels were greatly reduced when co-expressed with L2, compared to E2 levels when expressed on its own (Figure 3A).

However, when the same experiment was repeated in C33a cells transiently co-transfected with E2 and L2 plasmids, no decrease in E2 levels was observed in cells expressing L2 (Figure 3B). Therefore, L2-induced E2 down-regulation seems to be cell type-dependent.

These results allow us to conclude that L2-induced inhibition of E2 transcription transactivation function is <u>not</u> due to the marked decrease in E2 levels, as a similar degree of transcriptional transactivation inhibition was observed in HaCaT and C33a cells independently of the amount of E2.

3.4. HPV-16 L2 down-regulates E2 in a proteasome independent manner. To investigate whether the reasons for L2-induced loss of E2 in HaCaT cells was due to protein degradation, transiently transfected HaCaT cells were treated with a panel of proteasome/protease inhibitors. While treatment with the inhibitors increased the amount of E2, it failed to restore E2 levels to those of control cells without L2 (Figure 4A). Thus, although E2 levels are controlled by degradation through the proteasome, in agreement with previous results (Penrose and McBride, 2000), the decrease in E2 amounts in L2-expressing HaCaT cells appears to be due to additional mechanisms. In contrast, in C33a cells the inhibitors increased the amount of E2 to approximately the same extent, whether L2 was present or not (Figure 4A). The reasons why there was no increase in E2 levels after ALLM treatment are not clear, but again E2 levels were constant irrespective of L2 expression.

3.5. HPV-16 L2 does not alter HPV-16 E2 mRNA level. To ascertain whether the decrease in E2 protein in HaCaT cells expressing L2 was due, at least in part, to inhibition of E2 mRNA synthesis, HaCaT cells were transfected with 4μg pCMV-$E2_{16}$ and/or 4μg p16-HAL2 as described above, and total RNA was isolated. The RNA preparations were analysed for E2 mRNA levels by Real Time Quantitative PCR. There was no difference in E2 mRNA levels in cells transfected with E2, or E2 and L2 (Figure 4B), as all the amplification curves were coincident. Therefore we conclude that E2 mRNA level was not altered by the presence of L2, indicating that the observed L2-induced decrease in E2 takes place post-transcriptionally.

3.6. Delineation of L2 domains responsible for inhibition and degradation of E2. To identify the domains of L2 responsible for the observed effects on E2 function and stability, we generated mutants with either the C-terminus or the N-terminus of L2 deleted (Figure 5A). The corresponding deleted L2 ORFs were cloned in pGEX for

the production of GST-L2 fusion proteins to be used in mutant L2-E2 interaction studies, in pEGFP for the production of GFP-L2 fusion proteins to analyse mutant L2 localisation studies and stability, and in pcDNA3 for expression in mammalian cells for transcriptional transactivation studies.

GST-L2 proteins were expressed in E. coli and confirmed to be of the expected mobility (not shown). They were bound to glutathione-Sepharose beads and incubated with *in vitro* transcribed/translated ^{35}S-E2. All the C-terminus deletion mutant of L2 bound E2, including L2(1-50) which contains only the first 50 amino acid residues of the protein (Figure 5B). In contrast, the N-terminus deletion mutants did not bind E2 to any appreciable extent over the background seen with GST alone (Figure 5B). We conclude that L2-E2 interaction is mediated by the very N-terminus of L2. No binding between E2 and luciferase was observed (data not shown), confirming the specificity of the interaction between L2 and E2.

To investigate the effects of L2 deletion mutants on E2 functions, it was necessary to ascertain that L2 mutants localise in the nucleus and have similar expression levels. Nuclear localisation was investigated by using the GFP-L2 fusion proteins expressed in HaCaT keratinocytes. While GFP on its own localised both in the nucleus and the cytoplasm (Figure 6A), GFP-wt L2 (with or without the HA1 epitope at its N-terminus) localised in the nucleus (Figure 6B). All the GFP-L2 C-terminus deletion mutants had a predominantly nuclear localisation (shown for L2(1-200) and L2(1-50) in Figure 6C and D respectively). In contrast, the GFP-L2 N-terminus deletion mutants had a cytoplasmic and nuclear localisation not dissimilar from that of GFP alone (Figure 6E for L2(250-473)).

Next we analysed the expression of GFP-L2 and GFP-L2 mutants in HaCaT cells by western blots using an anti HPV-16 L2 polyclonal rabbit serum (Gornemann

et al., 2002). Although degradation bands were present, the C-terminus deletion mutants L2(1-400), L2(1-300) and L2(1-200) showed similar levels of expression, higher than that of GFP-L2 (Figure 7A). The antibody did not however detect L2(1-50) and was poor at detecting L2(1-100) perhaps because of the loss of reactive epitopes. Therefore the stability of these two proteins was established using the anti GFP polyclonal rabbit serum SC-8334 (Santa Cruz Biotechnology). Both fusion proteins were detected and appeared to be expressed at similar levels (Figure 7B). The L2 N-terminus deletion mutants did not appear to be stable as judged by the poor detection with both antisera. Given their inability to bind E2, disperse cellular localisation and instability, these mutants were not analysed further.

Next we tested the effects of L2 C-terminus deletion mutants on the ability of E2 to trans-activate ptk6E2 in HaCaT cells. The transactivation activity of E2 was inhibited by wt L2 and by L2(1-400) (Figure 8A). The remaining L2 mutants did not inhibit E2 activity to any significant extent (Figure 8A). We conclude that the L2 domain responsible for inhibition of E2 transactivation resides between residue 301 and 400. Thus, although all the L2 C-terminus deletion mutants can bind E2 (Figure 5B), binding is not sufficient for E2 inhibition.

Finally, we investigated the ability of L2 mutants to induce E2 degradation. It was clear that only full length L2 reduced E2 levels and none of the L2 mutants retained this ability (Figure 8B). Thus binding of E2 by L2 is not sufficient to induce E2 degradation.

4. Discussion

4.1. HPV-16 L2 inhibits the transcription transactivation function but not the DNA replication function of E2 . The results presented here demonstrate, for the first time, that HPV-16 L2 can repress the transcriptional activation properties of the HPV-16 transcription/replication factor E2 (Figure 1). At the same time they also show that the L2 protein has little effect on the ability of E2 to activate DNA replication in association with E1, the viral DNA replication factor (Figure 2). Similar conclusions have been drawn previously in the BPV-1 system (Heino et al., 2000). However there are clear differences between these two sets of results. Firstly, in the BPV-1 transcription assays the levels of L2 protein required to repress E2 transactivation function also repressed the E2-responsive promoter, even in the absence of E2. Therefore it is difficult to conclude with certainty that L2 was repressing E2 function; any down-regulation of E2 activity could have been due to repression of the promoter itself. On the contrary, in our HPV-16 system the levels of L2 repressing E2 trans-activation (up to 0.5μg of transfected plasmid) had no effect on any of the E2-responsive promoters in the absence of E2. Therefore we can conclude with certainty that the presence of L2 is repressing the E2 transactivation function. Secondly, in the BPV-1 system L2 repressed the ability of E1 to activate replication whether E2 was present or not, and thus the results are not clearly interpretable. In our HPV-16 system, although E1 cannot activate DNA replication in the absence of E2, in the presence of L2 there is no repression of E1 and E2-mediated DNA replication. In conclusion, we have demonstrated clearly that HPV-16 L2 inhibits the ability of HPV-16 E2 to activate transcription while having no effect on the ability of E2 to activate DNA replication in association with E1.

4.2. L2 inhibition of E2 transcriptional transactivation function is not due to L2-induced degradation of E2. We next investigated a possible mechanism for the inhibition of E2 transcriptional activation property. It seemed possible that L2 expression could result in a decrease of E2 protein levels thus repressing the ability of E2 to activate transcription but allowing residual E2 to efficiently activate DNA replication. In HaCaT cells, L2 does indeed promote a noticeable reduction of E2 levels (Figure 3A). However DNA replication assays do not work in these cells and therefore we could not determine whether the decrease of E2 protein levels resulted in repression of DNA replication. The mechanism of L2-induced reduction of E2 levels in HaCaT cells is not clear although it does not seem to be proteasome-dependent as treatment with proteasome inhibitors resulted in an elevation of E2 levels, as demonstrated previously (Penrose and McBride, 2000), but failed to restore them to normal amounts in the presence of L2 (Figure 4). In contrast, in C33a cells L2 expression does not result in degradation of the E2 protein (Figure 3B) although L2 can still repress the transcriptional transactivation function of E2. The difference between the two cell lines allows the conclusion that L2 can directly repress E2 transcriptional activation function without necessarily degrading E2. This is also confirmed in HaCaT cells where the deletion mutant L2(1-400) can still repress E2 transactivation function without degrading the E2 protein (Figure 8). The lack of any effect by L2 on the DNA replication function of E2 in C33a cells also demonstrates that the effect of L2 on E2 functions is selective.

4.3. Binding of L2 to E2 is not sufficient for functional or physical down-regulation of E2. To investigate whether a direct interaction between E2 and L2 is responsible for repressing the E2 transactivation function, and to identify the responsible domains, a series of L2 deletion mutants were made. Amino acids 1-50

were all that was required for interaction with E2 i*n vitro* (Figure 5). This portion of L2 includes the domain required for DNA binding (Zhou et al., 1994) and nuclear transport (Sun et al., 1995). In agreement, L2(1-50) was sufficient to direct the GFP protein into the nucleus (Figure 6). L2(1-50) however could not repress the ability of E2 to activate transcription, nor did it reduce the levels of E2 (Figure 8), showing that L2 binding of E2 in not sufficient for inhibition of E2 transcription transactivation function or for its physical down-regulation. Deletion of amino acids 301-400 removed the ability of L2 to repress E2 transactivation function, suggesting that this domain interacts with proteins responsible for the inhibition of E2 transactivation function. This suggestion is supported by the observations that this region of L2 overlaps with the ND10 localisation signal (390-420 aa; Becker et al., 2003) and that L2 interacts with the transcriptional repressor PATZ in ND10 (Gornemann et al., 2002). It is therefore possible that the interaction of L2 with both E2 and PATZ (or other transcription repressors) in ND10 is responsible for the inhibition of E2 transcriptional activation function. These points however require further investigation.

5. Conclusions.

The conclusion from these experiments is that L2 can directly interact with E2 and while this may be necessary for the repression of E2 transactivation function, it is not sufficient for either the functional or physical down-regulation of E2 (Figure 8). Functional down-regulation of E2 is brought about by a C-terminal domain of L2 between amino acid residues 301 and 400, whereas the decrease in E2 protein levels appears to require full length L2.

We can also conclude that whatever the nature of the mechanism used by L2 to repress E2 transcriptional function, this has little effect on DNA replication. It will

be of interest to identify the L2 interacting protein(s) responsible for mediating the repression of E2 transcriptional activation and this investigation is currently underway.

The effect of L2 on E2 also has the potential to be crucial for regulation of the viral life cycle. Following L2 expression, E2 transcriptional functions would be repressed, allowing the protein to focus on DNA amplification in association with E1. Most of the DNA amplification occurs in the upper layers of the epithelium and is co-incident with expression of the L2 protein. Whether viral DNA replication occurs in ND10 domains, to which L2 recruits E2, is currently under investigation. The L2-E2 interaction requires further characterisation and is likely to be a target in anti-viral strategies if it is demonstrated to be essential for the viral life cycle.

Aknowledgements

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References

Becker,K.A., Florin,L., Sapp,C. and Sapp,M. (2003) Dissection of human papillomavirus type 33 L2 domains involved in nuclear domains (ND) 10 homing and reorganization. Virology, 314, 161-167.

Boner,W., Taylor,E.R., Tsirimonaki,E., Yamane,K., Campo,M.S. and Morgan,I.M. (2002) A Functional interaction between the human papillomavirus 16 transcription/replication factor E2 and the DNA damage response protein TopBP1. J.Biol.Chem., 277, 22297-22303.

Bouvard,V., Storey,A., Pim,D. and Banks,L. (1994) Characterization of the human papillomavirus E2 protein: evidence of trans-activation and trans-repression in cervical keratinocytes. EMBO J., 13, 5451-5459.

Chiang,C.M., Ustav,M., Stenlund,A., Ho,T.F., Broker,T.R. and Chow,L.T. (1992) Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. Proc.Natl.Acad.Sci.U.S.A, 89, 5799-5803.

Chow,L.T. and Broker,T.R. (1994) Papillomavirus DNA replication. Intervirology, 37, 150-158.

Day,P.M., Roden,R.B., Lowy,D.R. and Schiller,J.T. (1998) The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. The Journal of Virology, 72, 142-150.

Del Vecchio,A.M., Romanczuk,H., Howley,P.M. and Baker,C.C. (1992) Transient replication of human papillomavirus DNAs. The Journal of Virology, 66, 5949-5958.

Demeret,C., Desaintes,C., Yaniv,M. and Thierry,F. (1997) Different mechanisms contribute to the E2-mediated transcriptional repression of human papillomavirus type 18 viral oncogenes. The Journal of Virology, 71, 9343-9349.

Demeret,C., Yaniv,M. and Thierry,F. (1994) The E2 transcriptional repressor can compensate for Sp1 activation of the human papillomavirus type 18 early promoter. The Journal of Virology, 68, 7075-7082.

Desaintes,C. and Demeret,C. (1996) Control of papillomavirus DNA replication and transcription. Semin.Cancer Biol., 7, 339-347.

Florin,L., Schafer,F., Sotlar,K., Streeck,R.E. and Sapp,M. (2002) Reorganization of nuclear domain 10 induced by papillomavirus capsid protein L2. Virology, 295, 97-107.

Gornemann,J., Hofmann,T.G., Will,H. and Muller,M. (2002) Interaction of human papillomavirus type 16 l2 with cellular proteins: identification of novel nuclear body-associated proteins. Virology, 303, 69-78.

Heino,P., Zhou,J. and Lambert,P.F. (2000) Interaction of the papillomavirus transcription/replication factor, E2 and the viral capsid protein, L2. Virology, 276, 304-314.

Hibma,M.H., Raj,K., Ely,S.J., Stanley,M. and Crawford,L. (1995) The interaction between human papillomavirus type 16 E1 and E2 proteins is blocked by an antibody to the N-terminal region of E2. Eur.J.Biochem., 229, 517-525.

Hirt,B. (1967) Selective extraction of polyoma DNA from infected mouse cell cultures. J.Mol.Biol., 26, 365-369.

Lee,C.W., Sorensen,T.S., Shikama,N. and La Thangue,N.B. (1998) Functional interplay between p53 and E2F through co-activator p300. Oncogene, 16, 2695-2710.

Lin,B.Y., Ma,T., Liu,J.S., Kuo,S.R., Jin,G., Broker,T.R., Harper,J.W. and Chow,L.T. (2000) HeLa cells are phenotypically limiting in cyclin E/CDK2 for efficient human papillomavirus DNA replication. J.Biol.Chem., 275, 6167-6174.

Penrose,K.J. and McBride,A.A. (2000) Proteasome-mediated degradation of the papillomavirus E2-TA protein is regulated by phosphorylation and can modulate viral genome copy number. The Journal of Virology, 74, 6031-6038.

Sakai,H., Yasugi,T., Benson,J.D., Dowhanick,J.J. and Howley,P.M. (1996) Targeted mutagenesis of the human papillomavirus type 16 E2 transactivation domain reveals separable transcriptional activation and DNA replication functions. The Journal of Virology, 70, 1602-1611.

Shah,K. and Howley,P. (1995) Papillomaviruses. Virology (ed. by B. Fields, D. Knipe and P. Howley), pp. 2077-2109. Raven Press Ltd., New York.

Sun,X.Y., Frazer,I., Muller,M., Gissmann,L. and Zhou,J. (1995) Sequences required for the nuclear targeting and accumulation of human papillomavirus type 6B L2 protein. Virology, 213, 321-327.

Swindle,C.S., Zou,N., Van Tine,B.A., Shaw,G.M., Engler,J.A. and Chow,L.T. (1999) Human papillomavirus DNA replication compartments in a transient DNA replication system. The Journal of Virology, 73, 1001-1009.

Taichman,L.B. and LaPorta,R.F. (1987) The expression of papillomaviruses in human epithelial cells. The Papovaviridae (ed. by N. P. Salzman and P. M. Howley) Plenum Press, New York, N.Y.

Thierry,F. and Howley,P.M. (1991) Functional analysis of E2-mediated repression of the HPV18 P105 promoter. New Biol., 3, 90-100.

Vance,K.W., Campo,M.S. and Morgan,I.M. (1999) An enhanced epithelial response of a papillomavirus promoter to transcriptional activators. J.Biol.Chem., 274, 27839-27844.

Yang,L., Li,R., Mohr,I.J., Clark,R. and Botchan,M.R. (1991) Activation of BPV-1 replication in vitro by the transcription factor E2. Nature, 353, 628-632.

Zhou,J., Sun,X.Y., Louis,K. and Frazer,I.H. (1994) Interaction of human papillomavirus (HPV) type 16 capsid proteins with HPV DNA requires an intact L2 N-terminal sequence. The Journal of Virology, 68, 619-625.

Legends to figures.

Figure 1. L2 inhibits the transcription transactivation function of E2. **A**, Schematic representation of luciferase reporter plasmids. The first three plasmids contain the wild type LCR of HPV-18 (pHPV18-LCR), the LCR with four point mutations in the E2 binding site 1 (pHPV18-LCR-BS1) or four point mutations in each of sites 1 to 3 (pHPV18-LCR-BS1-3). The other two plasmids are p18-6E2 containing six E2 binding sites upstream of the HPV-18 promoter and the E2-minimal promoter constructs ptk6E2, containing six E2 binding sites upstream of the tk promoter. **B**, Transactivation of HPV-18 LCR BS mutants and E2 minimal promoter constructs by HPV-16 E2 in HaCaT and C33a cells. Cells were transiently transfected with 0.1μg of each luciferase reporter construct and with increasing amounts (0, 0.01, 0.1, 1 and 10ng) of the E2 expression plasmid pCMV-$E2_{16}$. The total amount of DNA was kept constant by adding appropriate amounts of the parental plasmid pCMV. Each experiment was performed at least three times in duplicate. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2 (black bars), taken arbitrarily as 1. Standard deviation (SD) is shown. **C**, HPV-16 L2 down-regulates the transcription transactivation function of E2. HaCaT and C33a cells were transiently co-transfected with 0.1μg of each luciferase reporter construct and 1ng pCMV-$E2_{16}$ with increasing amounts of p16-L2, (0, 0.01, 0.05, 0.1 and 0.5μg). Each experiment was adjusted for total DNA by co-transfecting with the parental plasmid pCMV. Each experiment was performed at least three times in duplicate. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2 (black bars), taken arbitrarily as 1. SD is

shown. The activity of the promoters with no E2 and 0.5μg of L2 is shown as light grey bars.

Figure 2. HPV-16 L2 does not affect the replication function of HPV-16 E2 in C33a cells. (**A**) Phosphorimage of a Southern blot hybridised with ^{32}P-labelled HPV-16 ori plasmid. Cells were transfected with 1μg of p16ori-m, +/- 5μg pCMV-E1$_{16}$, +/- 10ng or 100ng pCMV-E2$_{16}$ and +/- 1μg or 2μg pL2. For each reaction, Xnm1 was used to linearise p16ori-m (odd-numbered lanes) and then Dpn1 was used to digest replicated DNA (even-numbered lanes). The open arrow on the left indicates input plasmid; the full arrow on the right indicates the replicated plasmid. Replication occurred in the presence of 10ng or 100ng of pCMV-E2$_{16}$ co-transfected with 1μg p16ori-m and 5μg pCMV-E1$_{16}$ (lanes 6 and 12). The absence of either E1 (lane 4) or E2 (lane 2) resulted in no detectable replication. The experiment was carried out three times with essentially the same results. (**B**) Quantitation of DNA replication. Southern blots were scanned and the extent of DNA replication was calculated by measuring the ratio of double cut/single cut bands, thus controlling for variations between experiments. The graph represents a summary of three independent experiments +/- standard deviation.

Figure 3. HPV-16 L2 induces the degradation of E2 in HaCaT cells but not in C33a cells. (**A**), HaCaT cells were transfected with 4μg pCMV-E2$_{16}$ (lane 1), or 4μg pCMV-E2$_{16}$ plus 4μg p16-HAL2 (lane 2), or with 4μg p16-HAL2 alone (lane 3). (**B**), C33a cells were transfected with 4μg pCMV-E2$_{16}$ (lane 1), or 4μg pCMV-E2$_{16}$ plus 4μg p16-HAL2 (lane 2). Cell extracts were analysed in independent Western blots with mouse anti-HA monoclonal antibody HA.11, anti-E2 mouse monoclonal antibody TVG261 and mouse anti-actin antibodies.

Figure 4. HPV-16 L2 decreases the level of E2 in HaCaT cells in a proteasome independent manner without down-regulation of E2 transcription. **(A)** HaCaT cells or

C33a cells were transiently transfected with 4μg pCMV-$E2_{16}$ alone ("minus" lanes) or with 4μg pCMV-$E2_{16}$ and 4μg pL2 ("plus" lanes). Twenty four hours later the transfected cells were either treated for 8hr with the indicated proteasome inhibitors or kept untreated. Cells were lysed and the extracts were analysed by Western blot with mouse anti-HPV-16 E2 and mouse anti-actin antibodies. **(B)** Total RNA from HaCaT cells transfected with HPV-16 E2 alone or with HPV-16 E2 and HPV-16 L2 was digested with DNAse and reverse transcribed with random primers. Real time PCR was performed in triplicate with primers and probes for E2 and actin. There was no difference in E2 transcription in the presence or absence of L2 and all the amplification curves are coincident.

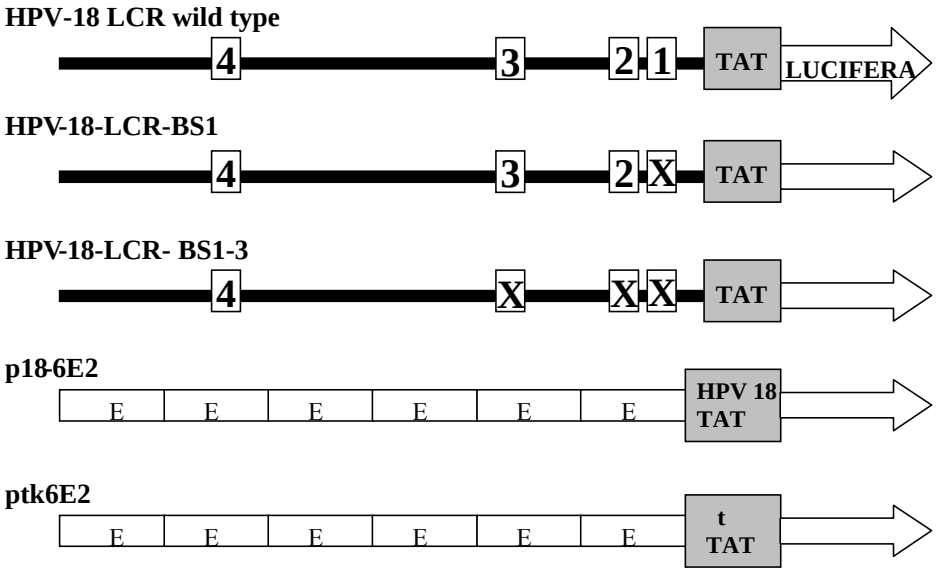
Figure 5. The N-terminus of L2 interacts physically with E2. (**A**), Diagrammatic representation of C-terminus and N-terminus deletion mutants of E2. (**B**), GST-mutant L2 fusion proteins were loaded onto glutathione beads and 10μl of immobilised beads were incubated with ^{35}S-labelled E2. Bound proteins were separated by SDS-PAGE and visualised by autoradiography.

Figure 6. L2 C-terminus, but not N-terminus, deletion mutants localise to the nucleus. 100ng each of plasmids encoding GFP, GFP-wt L2 or GFP-L2 mutant proteins were transfected into HaCaT keratinocytes. Twenty four hr after transfection cells were fixed and stained for DNA using DAPI. Cells were analysed in a Leica TCS SP2confocal microscope.

Figure 7. Expression of GFP-L2 C-terminus deletion mutants. Plasmids encoding GFP, GFP-L2 and GFP-L2 C-terminus deletion mutants (4μg) were transfected into HaCaT keratinocytes. Cells were lysed twenty four hours later and protein lysates were analysed by western blots with anti HPV-16 L2 polyclonal rabbit serum (Gornemann et al., 2002) (lanes 1-4) or anti GFP polyclonal rabbit serum SC-8334

(Santa Cruz Biotechnology, UK) (lanes 1-7). The arrowheads on the left point to the fusion proteins and the arrowheads on the right represent MW markers.

Figure 8. Residues 301-400 of L2 inhibit E2 transactivation function but the full length protein is needed to induce E2 down-regulation. (**A),** 0.1μg of ptk6E2, 1ng of pCMV-$E2_{16}$ and increasing amounts (0.1-1μg) of each plasmid encoding C-terminus deletion mutants of L2 were co-transfected into HaCaT cells and luciferase activity measured as above. The experiment was repeated three times in duplicate and SD is shown. (**B**), 4μg pCMV-$E2_{16}$ were transfected into HaCaT keratinocytes with or without 4μg p16-L2 or 4μg of each plasmid encoding L2 C-terminus deletion mutants. One day later the transfected cells were lysed and the cell extracts were analysed by Western blot with mouse anti-HPV-16 E2 and mouse anti-actin antibodies.


HPV-18 LCR wild type
4
3
2
1
TAT
LUCIFERA
HPV-18-LCR-BS1
4
3
2
X
TAT
HPV-18-LCR- BS1-3
4
X
X
X
TAT
p18-6E2
E
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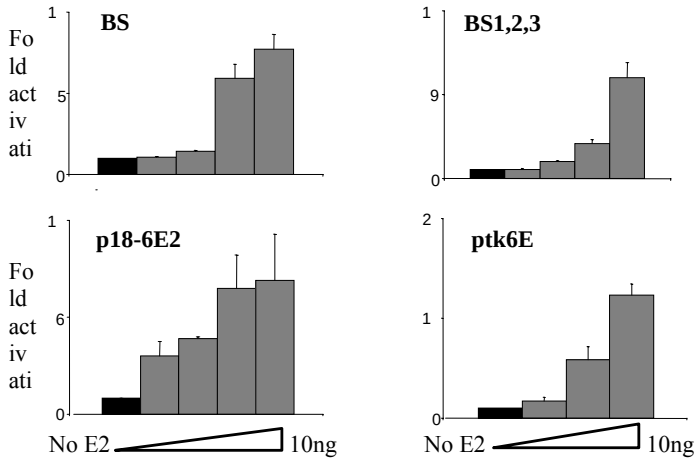
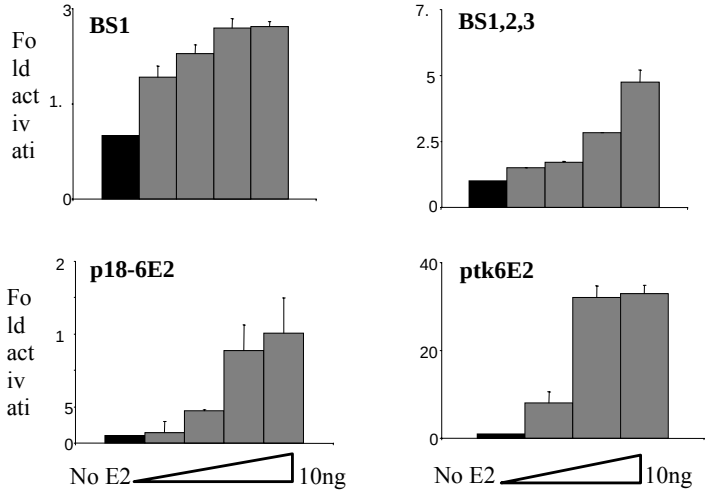
Okoye et al., Figure 1B

HaCa

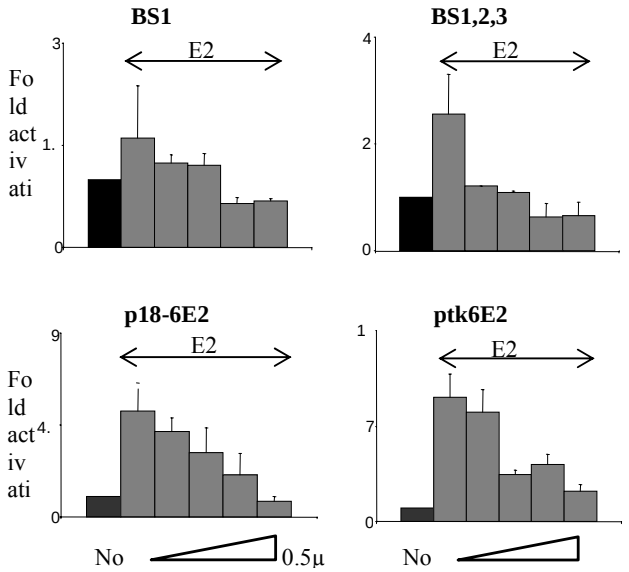


C33

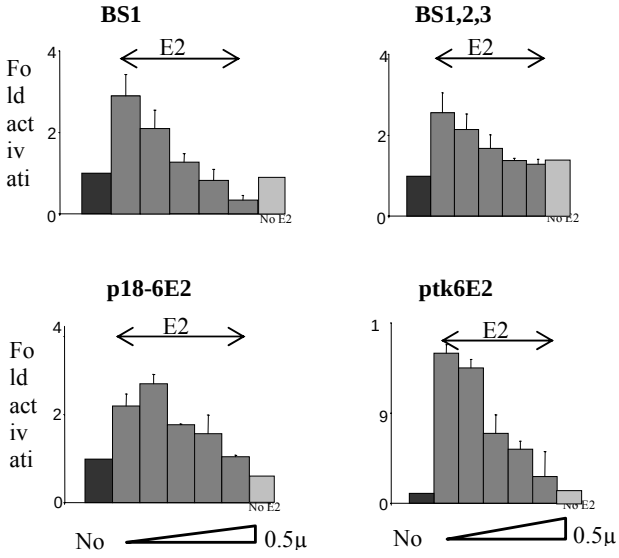


Okoye et al., Figure 1C

HaCa

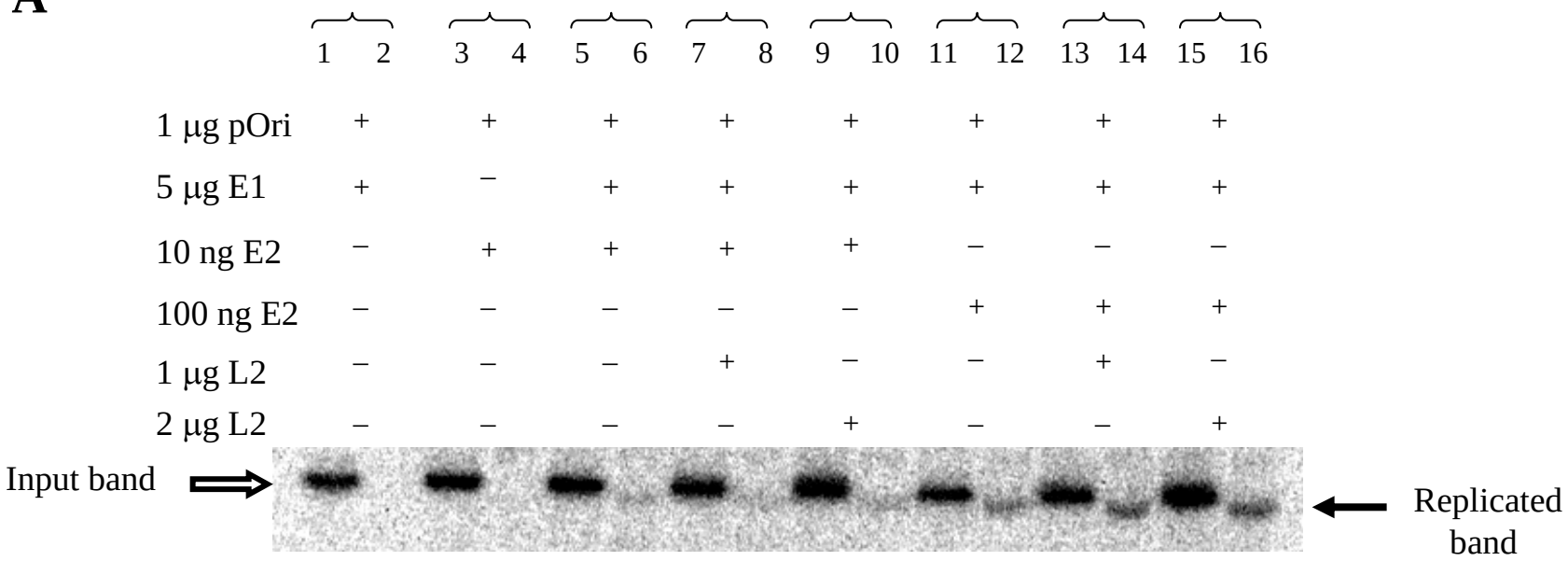


C33



Okoye et al., Figure 2

A



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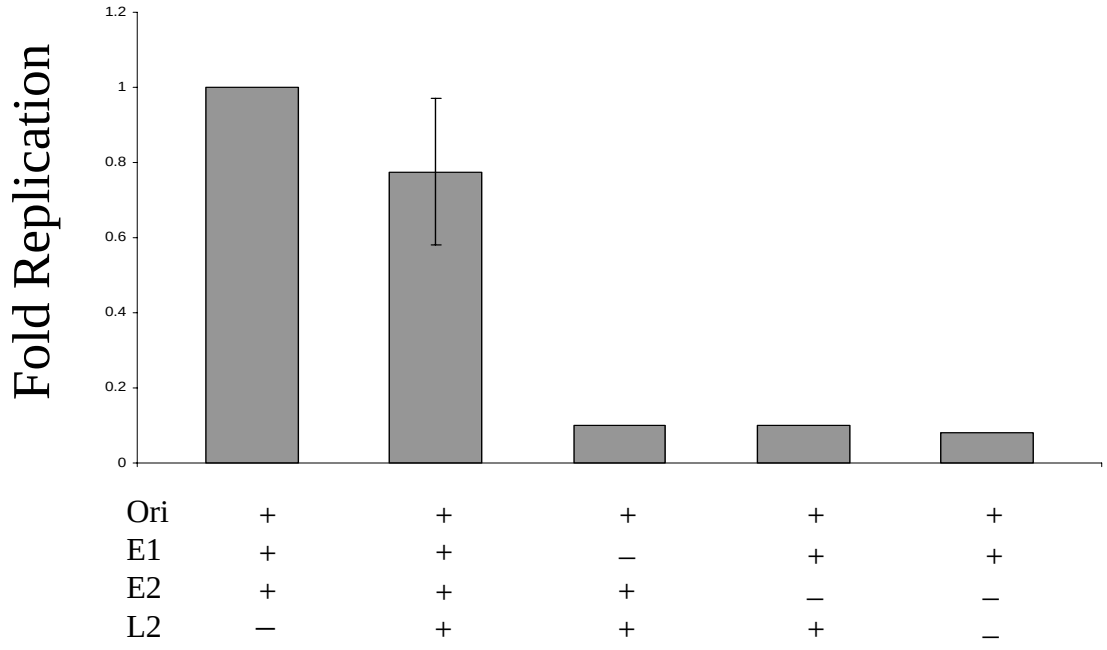
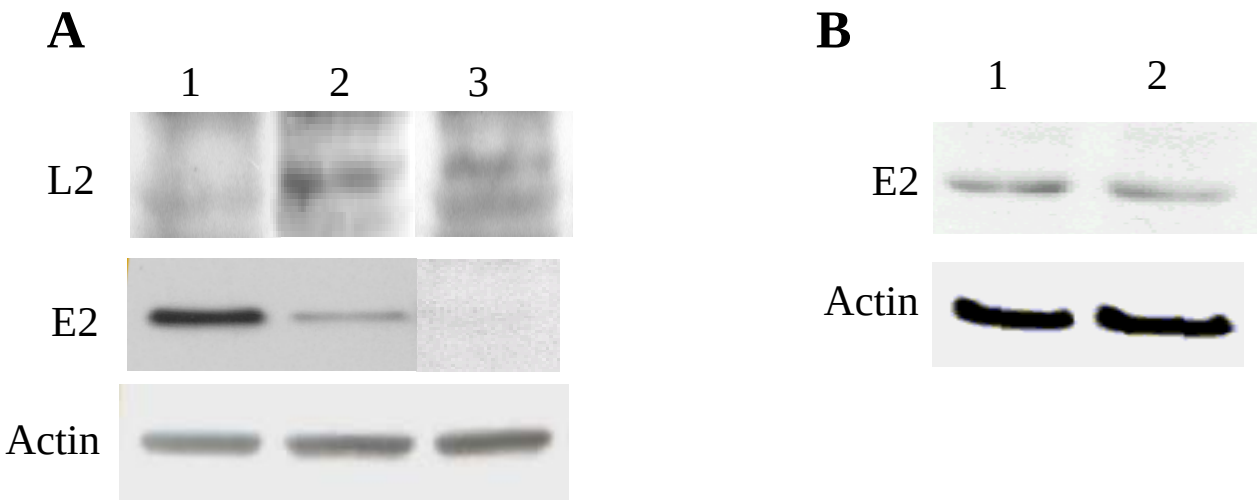
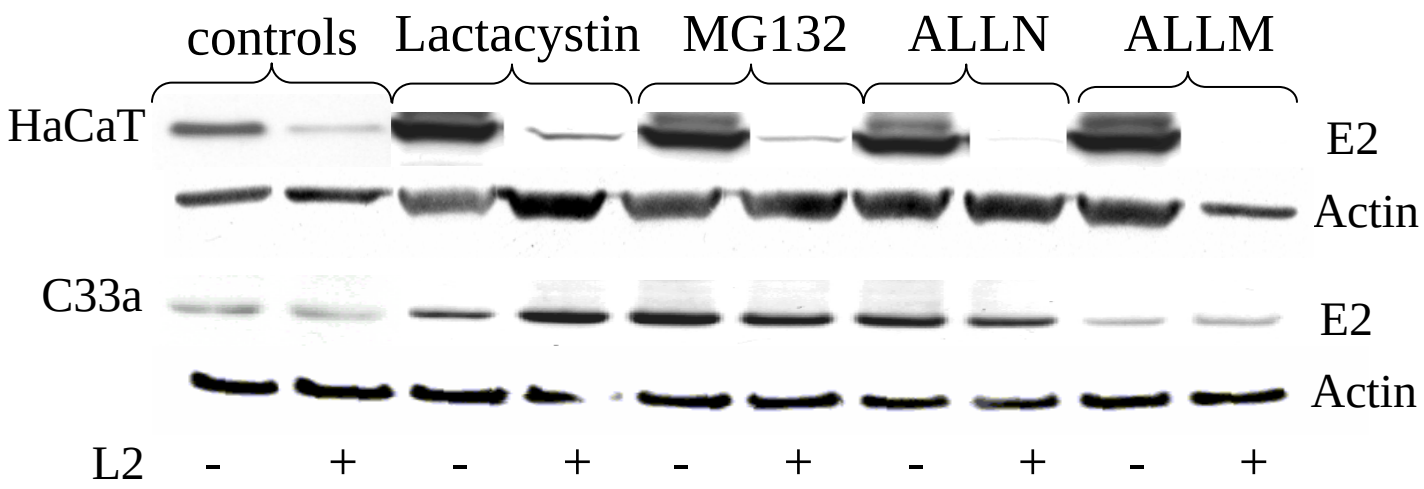


Okoye et al., Figure 3



A



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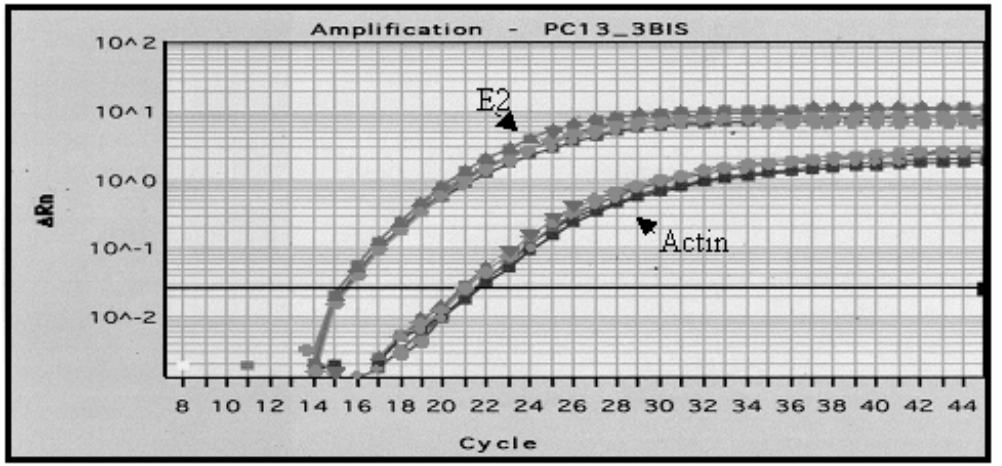
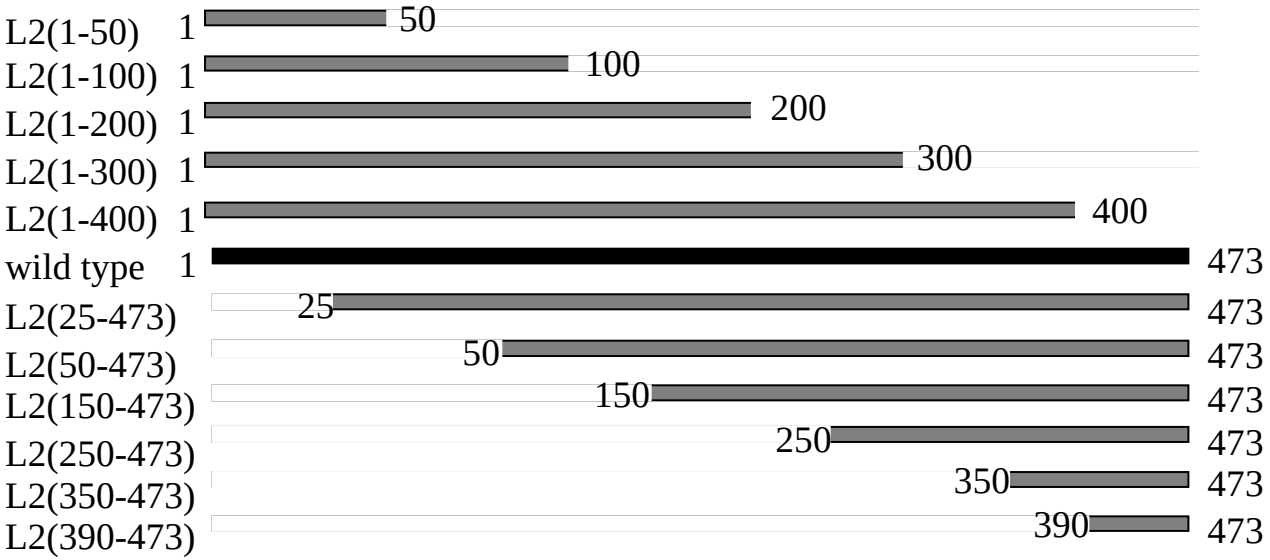


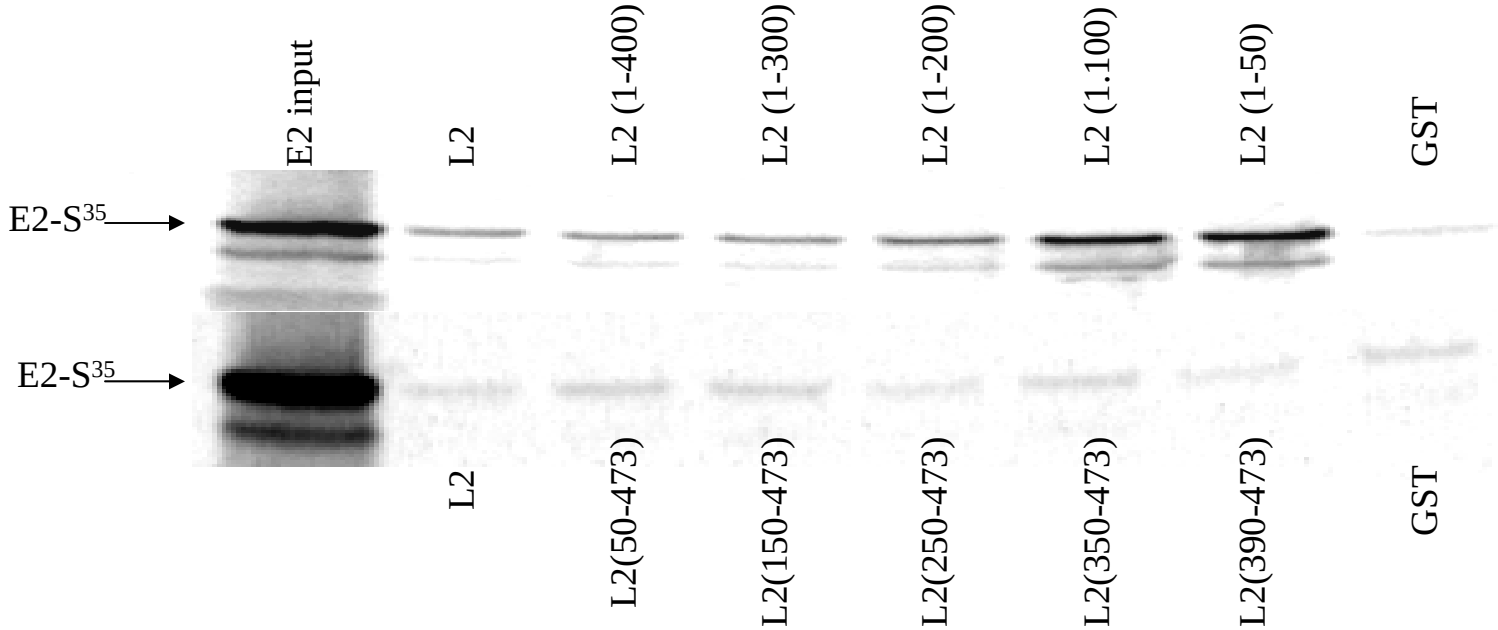
▼, ▲, ● : HaCaT cells transfected with E2

■, ▪, ◆ : HaCaT cells transfected with E2+L2

A


L2(1-50) 1 50
L2(1-100) 1 100
L2(1-200) 1 200
L2(1-300) 1 300
L2(1-400) 1 400
wild type 1 473
L2(25-473) 25 473
L2(50-473) 50 473
L2(150-473) 150 473
L2(250-473) 250 473
L2(350-473) 350 473
L2(390-473) 390 473

B


E2 input
L2
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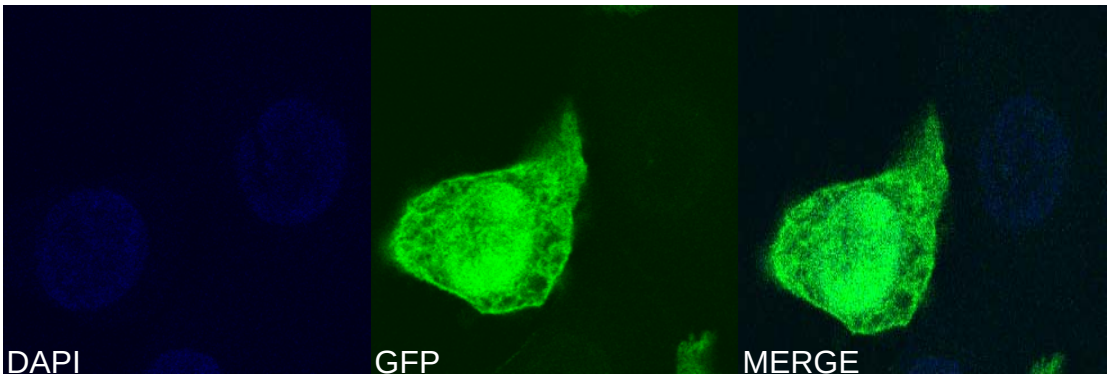
L2 (1-300)
L2 (1-200)
L2 (1.100)
L2 (1-50)
GST
E2-S35
E2-S35
L2
L2(50-473)
L2(150-473)
L2(250-473)
L2(350-473)
L2(390-473)
GST

Okoye et al., Figure 6

A



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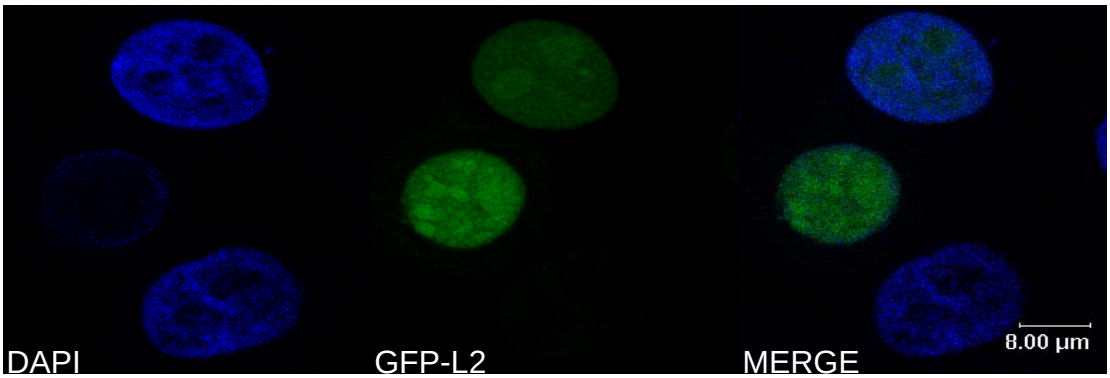
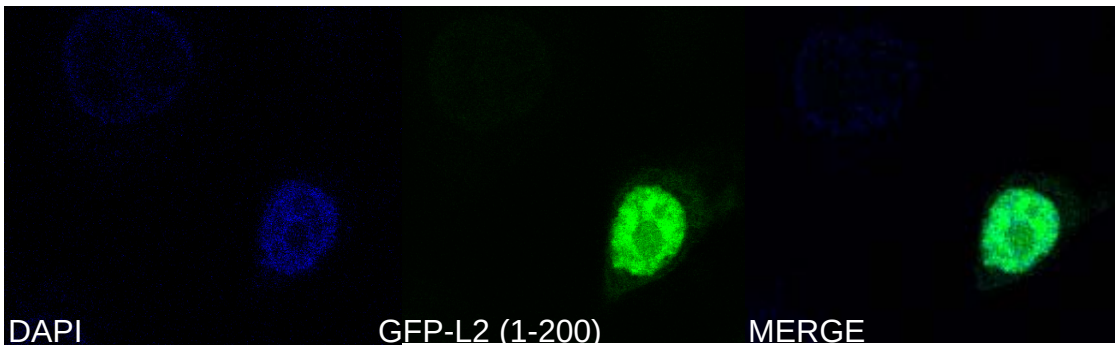
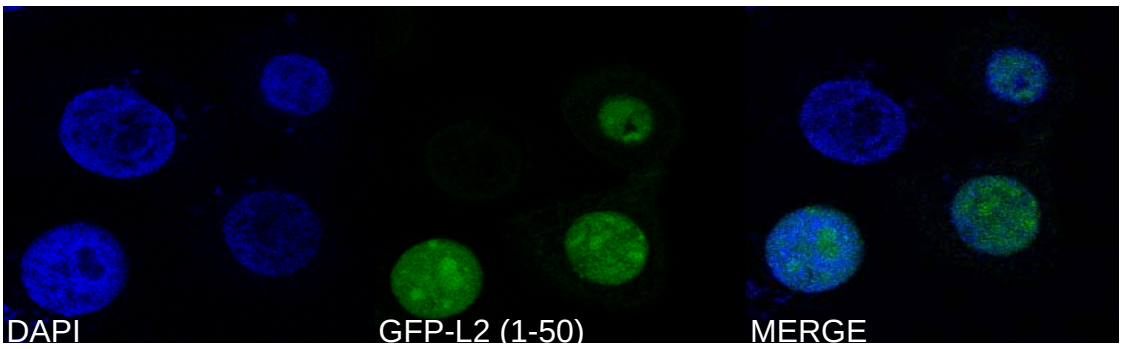


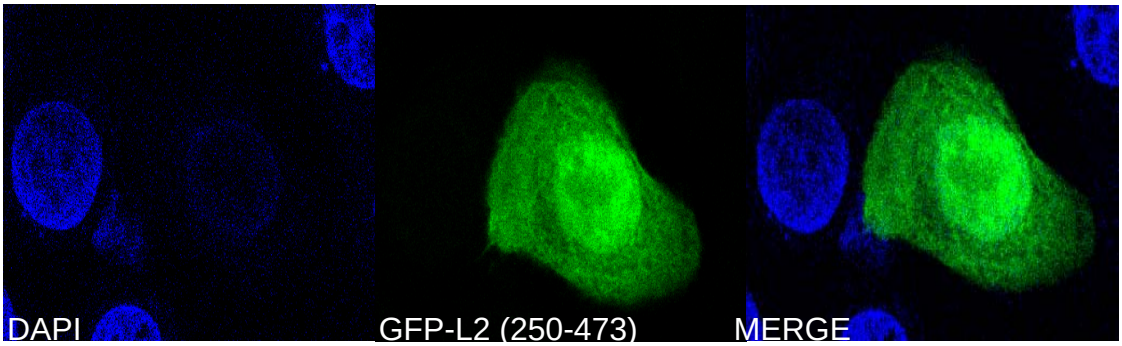
C



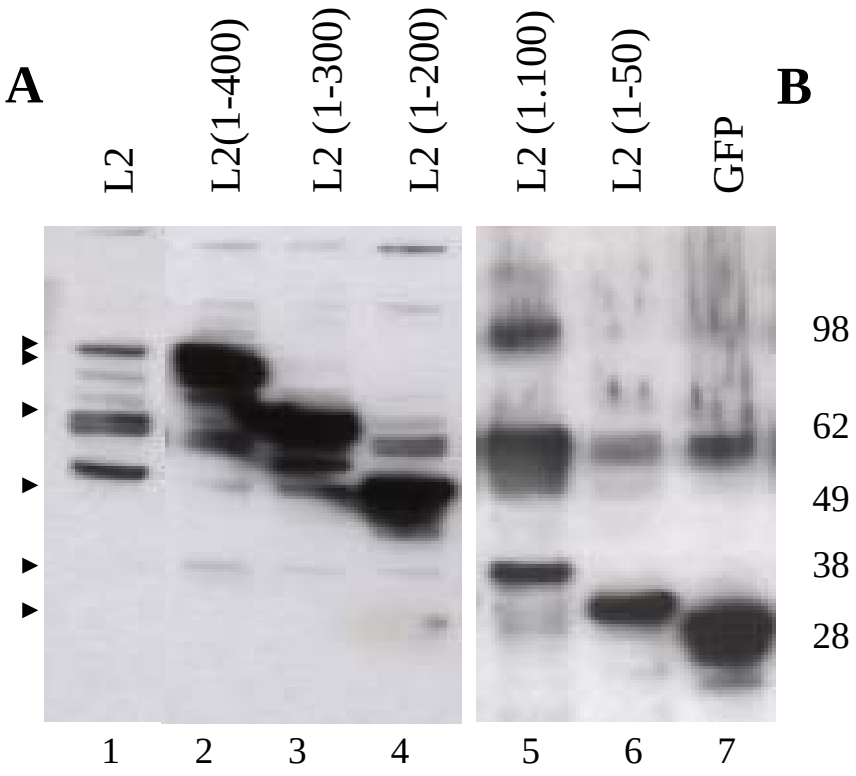
D



E

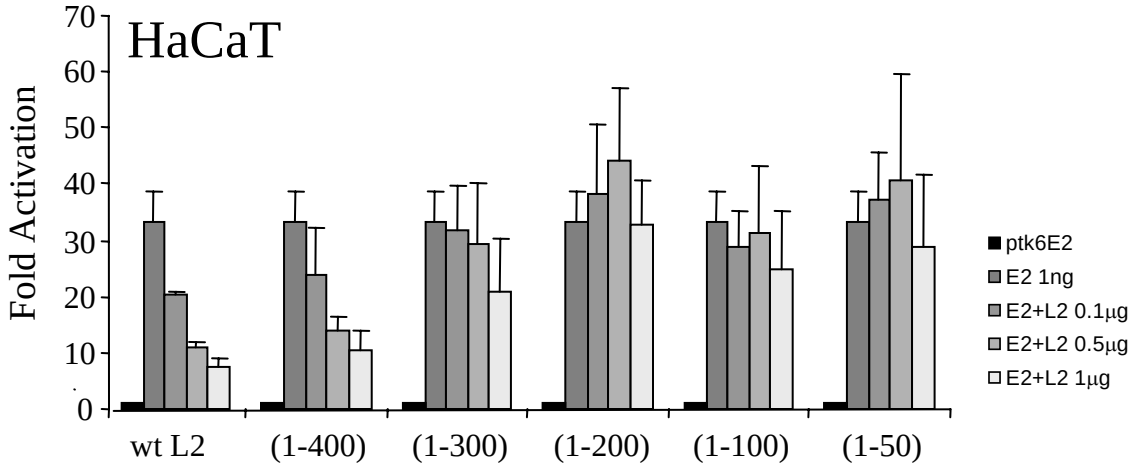


Okoye et al., Figure 7



Okoye et al., Figure 8

A



B