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Failure to interact with Brd4 alters the ability of HPV16 E2 to regulate host genome expression and cellular movement

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Abstract

The E2 protein of the carcinogen human papillomavirus 16 (HPV16) regulates replication and transcription of the viral genome in association with viral and cellular proteins. Our previous work demonstrated that E2 can regulate transcription from the host genome. E2 can activate transcription from adjacent promoters when located upstream using E2 DNA binding sequences and this activation is dependent upon the cellular protein Brd4; this report demonstrates that a Brd4 binding E2 mutant alters host genome expression differently from wild type E2. Of particular note is that highly down regulated genes are mostly not affected by failure to interact with Brd4 suggesting that the E2-Brd4 interaction is more responsible for the transcriptional activation of host genes rather than repression. Therefore failure to interact efficiently with Brd4, or altered levels of Brd4, would alter the ability of E2 to regulate the host genome and could contribute to determining the outcome of infection.

Keywords

Human papillomavirus; E2; Brd4; Cancer; Exon array; Affymetrix

1. Introduction

Human papillomavirus 16 (HPV16) is a causative agent in human cancers including cervical, anal and head and neck (zur Hausen, 2009). HPV16 infects the basal epithelium and the double stranded DNA viral genome is replicated in the cell nucleus. Two viral

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.virusres.2015.09.008>.

proteins are required for this replication; E2 that acts as an origin recognition receptor and binds to 12 bp palindromic target sequences surrounding the viral origin of replication and E1 that is recruited to the viral origin by E2 via a protein-protein interaction (McBride et al, 1989). E1 is the viral helicase and forms a di-hexameric complex at the AT rich origin of replication and replicates the viral genome in association with host polymerases and replication factors (Conger et al, 1999; Masterson et al, 1998; Park et al., 1994). The E2 protein can also regulate transcription from the viral promoter adjacent to the origin of replication; this promoter regulates expression of the viral oncogenes E6 and E7. E2 can either activate or repress transcription from this promoter depending upon the levels of E2 protein and the cell type that assays are carried out in, primarily E2 is thought to act as a repressor. (Bernard et al., 1989; Bouvard et al., 1994; Cripe et al, 1987; Romanczuk et al., 1990; Stenlund and Botchan, 1990). In many cancers, though not all, the viral genome is integrated into that of the host and the E2 gene is lost resulting in elevated levels of E6 and E7 that are proposed to contribute to cell transformation (zur Hausen, 2009). A final direct role for E2 in regulating the viral life cycle is as a mitotic chromatin receptor for the virus (McBride et al., 2012). In this role E2 is proposed to interact with the host chromatin during mitosis via the amino terminal domain while the E2 carboxyl terminal DNA binding domain is bound to the viral genome. Using this mechanism the viral genome maintains nuclear localization during cell division, it is essential that the virus is in the nucleus for the life cycle.

There have been many cellular binding partners for the amino terminal domain of E2 identified (McBride, 2013). The most widely studied is Brd4, first implicated in E2 function due to binding to BPV1 E2 (You et al., 2004). Brd4 was first identified as a mitotic chromatin binder that marks actively transcribed genes, with a role in regulating the cell cycle and subsequent studies demonstrated a direct role in regulating transcriptional regulation (Dey et al, 2000). Brd4 is a BET family protein (it has two bromodomains, BD1 and BD2) and the bromodomains bind to acetylated proteins including histones; the interaction with histones retains Brd4 on the chromatin (Vollmuth et al, 2009). Brd4 is an essential gene (Houzelstein et al., 2002) and NUT4-Brd4 fusion proteins are causative in aggressive mid-line carcinomas (French et al., 2003). As well as interacting with mitotic chromatin Brd4 also acts as a transcription factor. During transcriptional elongation serine 2 of the CTD of RNA pol II is phosphorylated by pTEFb, Brd4 is required for pTEFb nuclear localization and association with the transcriptional machinery as well as activation of the associated CDK9 (Jang et al, 2005). More recently it has been shown that Brd4 has a kinase activity that directly targets serine 2 of the CTD and it has been proposed that this activity is required for initiation and transition to elongation while pTEFb regulates the elongation (Devaiah et al., 2012).

The initial role for Brd4 in the viral life cycle was proposed to be as the host mitotic chromatin receptor (You et al, 2004). For some E2 types there is a co-localization of E2 and Brd4 on the host chromatin during mitosis and the presence of E2 enhances the affinity of Brd4 for the mitotic chromatin (Cardenas-Mora et al., 2008; McBride et al, 2004; McPhillips et al., 2005; Silla et al, 2010). However, for other E2 types, including HPV16, it is less clear whether Brd4 is the mitotic receptor protein for E2 (McPhillips et al., 2006). Other candidate proteins for E2 in this regard are TopBP1 and ChLR1 (Donaldson et al.,

2007; Parish et al., 2006). However, for the transcriptional activation properties of E2 the interaction with Brd4 is essential; all E2 types tested for mutations in the amino terminal domain that abrogate E2 interaction with Brd4 also abrogate E2 transcriptional activation function (Lee and Chiang, 2009; McPhillips et al., 2006; Schweiger et al., 2006). A role for Brd4 in repression of the papillomavirus transcriptional control region has also been suggested (Smith et al., 2010). There is of course the possibility that this mutant fails to interact with another cellular protein that is responsible for the mutant phenotypes. However Brd4 is a strong candidate to explain the mutant phenotype for several reasons: the crystal structure between Brd4 and E2 indicates that arginine 37 is a contact point on E2 for Brd4; the mutation abrogates E2 transcriptional activation function as would be predicted from Brd4 function; the mutant fails to interact with Brd4. However, we cannot definitively rule out that other cellular partners for E2 could be involved and known interactors are described in McBride, (2013).

There has also been a proposed role for the interaction between E2 and Brd4 regulating the DNA replication properties of E2 (Baxter and McBride, 2005; Gauson et al., 2015; Sakai et al., 1996; Wang et al., 2013). A recent report demonstrates co-localization of E2 with Brd4 on fragile sites of chromatin in C33a cells and proposed that this co-localization was involved in regulating E1-E2 mediated DNA replication in areas where viral genome breakage would promote viral integration, very often found in HPV related cancers (Jang et al., 2014). Other reports of E2 binding sites on the human genome do not indicate association with actively transcribed genes (Vosa et al., 2012).

Recently we reported that HPV16 E2 can regulate transcription from a host genome when expressed stably (Gauson et al., 2014). We hypothesized that an interaction between E2 and Brd4 would be involved in transcriptional regulation of the host chromatin. To investigate this we carried out exon array studies on U2OS cells stably expressing HPV16 wild type E2 (E2^{wt} from now on) and HPV16 E2 with arginine 37 mutated to an alanine, a mutation previously shown to be compromised in Brd4 interaction (E2^{-Brd4} from now on) (Schweiger et al., 2006). We confirmed that E2^{-Brd4} is compromised in transcriptional activation in U2OS cells and that cells expressing both E2^{-Brd4} grow significantly slower than cells expressing E2^{wt} or vector control cells. The exon array results demonstrated that E2^{wt} and E2^{-Brd4} regulate overlapping and distinct sets of genes. There was no reduction in the number of genes that E2^{-Brd4} regulated, but there were E2^{wt} activated genes that depend upon the Brd4 interaction. E2^{-Brd4} was also not as compromised as E2^{wt} in wound healing (Gauson et al., 2014). Overall the results suggest that interaction with Brd4 assists E2^{wt} in regulating the host genome and in the absence of Brd4 interaction E2 may locate to and regulate other parts of the genome. More work is required to determine how and why E2 regulates the host genome, this is an important topic as this could facilitate viral infection.

2. Material and methods

2.1. Generation of stable clones in U2OS cells

To generate E2 expressing U2OS clones, 4×10^5 U2OS cells were plated onto 100 mm² plates and fed with Dulbecco's modified eagle medium (DMEM) supplemented with 10% Fetal Calf Serum and 1% (v/v) penicillin/streptomycin mixture (Invitrogen). The following

day cells were transfected using the calcium phosphate method with 1 μ g of E2^{wt}, E2^{-Brd4} expressing plasmid or empty vector plasmid DNA as a control. Plasmid vectors encoded neomycin resistance (G418) for selection. Cells were treated with 0.5% trypsin EDTA (Invitrogen) 48 h post-transfection, and re-plated at the following dilutions; 1:5, 1:20 and 1:50. E2 expressing cells were selected for using DMEM media containing G418 (Geneticin) at a concentration of 0.75 mg/ml. Cells were re-fed with fresh DMEM media containing G418 every 3–4 days. Once colonies had started to form they were transferred using cloning rings to 6-well plates. Cells continued to be maintained in G418 DMEM medium, allowing for the potential E2 clones to expand. Potential clones were then screened for E2 expression using western blot.

2.2. Western Blotting

Cells were trypsinized and washed twice with phosphate buffered saline, pelleted then re-suspended in 100 μ l of lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.8, 150mM NaCl with a protease inhibitor mixture (Roche Molecular Biochemicals) dissolved in the lysis buffer). The cell and lysis buffer mixture was incubated on ice for 30 min, and then centrifuged in a refrigerated microfuge for 10 min at 20,800 rcf at 4°C. Protein levels were determined using a BCA assay (Sigma). Equal amounts of protein were boiled in 2 μ l of 10 $\times$ Sample Reducing Agent (Invitrogen) and 5 μ l 4 $\times$ LDS Buffer (Invitrogen). Samples were then loaded onto a 4–12% gradient gel (Invitrogen), ran at 200V for 1 h and transferred onto nitrocellulose membranes using the wet blot method. The membrane was then blocked in Odyssey blocking buffer (diluted 1:1 with PBS), for one hour at room temperature. After blocking the membrane was probed with TVG261 anti-HPV16 E2 mouse antibody (ab17185) diluted 1:500 in blocking buffer, and rabbit β -actin (sc-130656) diluted 1:5000 in blocking buffer, and incubated O/N at 4 °C. Following incubation with primary antibody, the membrane was washed with 0.1% PBS-Tween wash buffer before probing with Odyssey secondary antibody diluted 1:20,000 (Goat anti-mouse IRdye 800 cw) for one hour at room temperature. The membrane was then washed in 0.01% PBS-tween before infrared scanning using the Odyssey Li-Cor imaging system.

2.3. Transcription assay

5×10^5 U2OS cells were plated out on a 60 mm² plate and transfected 24 h later with either 10 ng, 100 ng or 1000 ng of E2-WT or E2-Brd4 plasmid DNA, using the calcium phosphate method. The day following transfection, cells were washed twice with PBS and re-fed with complete DMEM. 24 h later the cells were harvested for transcription assay. Cells were washed twice with PBS and then lysed with 300 μ l of reporter lysis buffer (Promega) at room temperature for 10 min. The cell/lysis buffer mixture was then scraped and transferred into a 1.5 ml Eppendorf tube and spun in a refrigerated microfuge for 10 min at 4°C with 3293 rcf to clear debris. 80 μ l of the supernatant was assayed for luciferase activity using the luciferase assay system (Promega). The protein concentrations of the lysates were also determined by BCA assay (Sigma), and the results are expressed as the luciferase activity relative to the protein concentration. The assay shown is representative of three independent experiments carried out in duplicate.

2.4. RNA extraction

U2OS cells expressing E2^{wt}, E2^{-Brd4} and a vector control line (non-E2 expressing) were plated out at 1×10^6 on 100 mm² plates. The following day cells were harvested for the preparation of RNA. Cells were washed twice with PBS before adding 600 µl of buffer RLT from the Qiagen RNeasy kit directly onto the monolayer of cells, and left to incubate for 5 min at room temperature. Post-incubation with buffer RLT, cells were scraped using a scraper and the cell/buffer RLT mix was then added to a Qias shredder column (Qiagen) and centrifuged following the manufacturer's instructions to homogenize the sample. The Qiagen RNeasy protocol was then followed to extract RNA from the U2OS cells stably expressing E2. The DNA was removed using DNase treatment (Qiagen) on column.

2.5. cDNA synthesis

cDNA was made using the Finnzymes (Thermo Scientific) DyNAmo SYBR Green 2-Step qRT-PCR kit (F-430S, F-430L), following the protocol provided.

2.6. Sybr Green qPCR

To validate the genes regulated by E2-WT and E2-Brd4 expressing U2OS clones, a Sybr green protocol was followed (DyNAmo SYBR Green qPCR Kit with ROX, Cat no: F-400RL), using primers designed by Qiagen, (Qiagen QuantiTech primer assay). Results were all normalized to a GAPDH endogenous control, and then further normalized to VEC 1:1 (non-E2 control), which was made 1. The $\Delta\Delta C_t$ method was used for this validation.

2.7. Exon-array

Exon array analysis was carried out as described (Gauson et al., 2014). The data was analyzed using Affymetrix Exon array software and core data analysis was performed using Partek genomics suite as described previously (Gauson et al., 2014).

2.8. Growth curve

2×10^5 cells were plated out into 100 mm² plates in triplicate and maintained in complete DMEM. 3 days after seeding the cells, they were trypsinized and counted using a haemocytometer. This was repeated 3 times in 3 day intervals. Growth curves were generated from the cell counts of E2^{wt}, E2^{-Brd4} and Vector control clones, calculated from the cell number counts at 0, 3, 6 and 9 days. The data was analyzed using a 2 tail t-test to investigate whether there was a significant difference in growth rates between the different cell line populations.

2.9. Wound assay

In order to have consistent "wounds" for the cells to heal, cell culture inserts were used (Ibidi, cat #80209). The width of the "wound" is approximately 500 µm (± 50 µm). The cell inserts were attached to six well plates and seeded with 5×10^4 cells either side of the artificial wound. This was done in duplicate for E2^{wt}, E2^{-Brd4} and Vector clones (two sets of clones were monitored). Cells were left to grow for a 24 h time period until each side of the chamber was confluent. Cell inserts were removed and images taken at 0, 12 and 24 h time intervals (Zeiss, Axiovert 200 M microscope and AxioCam). This experiment was repeated

three times and the same result was observed on all occasions. Measurements were made using Axiovision software at multiple points of the wound for each time point and used to determine the differences in wound healing rates in Fig. 4b.

3. Results and discussion

3.1. E2^{-Brd4} expressing cell lines grow slower than E2^{wt} cells

The goal of this project was to investigate the role of the HPV16 E2 (E2 from now on) interaction with Brd4 in regulation of the host genome. Our previous studies demonstrated that, in U2OS cells, expression of E2^{wt} had no effect on cellular growth. This is an important point as it allowed us to conclude that gene expression changes were actually due to expression of the E2 proteins. U2OS cell clones expressing a mutant of E2 that does not bind to Brd4 (mutated at arginine 37 to an alanine, E2^{-Brd4}) were generated as for wild type E2 (Gauson et al., 2014). Fig. 1a shows expression of E2^{wt} and E2^{-Brd4} in two independent clones with two VEC control lines (these were selected with G418 following transfection with a G418 resistance plasmid but do not express E2). Both of the E2^{wt} (lanes 2 and 5) and E2^{-Brd4} (lanes 3 and 6) clones express E2 protein. Actin levels are shown to control for protein loading and transfer. In Fig. 1b growth curves carried out with the with VEC 1.1, E2^{wt}-Clone1 and E2^{-Brd4}-Clone 1 are shown and the representative cell numbers at each time point shown below. This experiment was carried out with the other VEC, E2^{wt} and E2^{-Brd4} clones with essentially similar results, only a representative clone is shown for clarity on the figure. In these experiments the E2^{wt} clones grow similarly to VEC with perhaps a little growth increase after 9 days, this was observed in both clones. For E2^{-Brd4} the cells grow significantly slower than the E2^{wt} cells and this is detected at days 6 and 9; a 2 tail t-test gave p-values less than 0.01 at these time points. The conclusion from these experiments is that both the E2^{wt} and E2^{-Brd4} proteins are expressed well in the selected clones and that all clones can grow healthily, albeit at slightly different rates.

3.2. E2^{-Brd4} is severely compromised in transcriptional activation in U2OS cells

Interaction between E2 and Brd4 is required for transcriptional activation by E2 (Lee and Chiang, 2009; Schweiger et al., 2006) and is also implicated in regulating transcriptional repression by E2 (Schweiger et al., 2007). To confirm that the E2^{-Brd4} protein is behaving as predicted in U2OS cells the ability of both wt and Brd4 mutant E2 in activation of transcription from a tk promoter with 6 E2 binding sites located upstream (Vance et al., 1999) was tested in luciferase assays and the results are shown in Fig. 2. The results are expressed as fold increase in activation over levels of the reporter plasmid in the absence of E2 equaling 1. Lanes 1 and 2 demonstrates that at 10ng and 1000ng of E2^{wt} expression plasmid respectively there is a 9 and 25 fold increase in transcription. The response to E2^{wt} expression plasmids is never linear, i.e., 100 times more plasmid never gives 100 times more activation, so these results are in line with those from other cell lines (Donaldson et al., 2012; Gauson et al., 2015). In lanes 3 and 4 it is clear that E2^{-Brd4} is severely compromised in the ability to activate transcription, 10ng and 1000 ng give reporter levels of 0.75 and 1.56 respectively relative to no E2. Therefore in U2OS cells the E2^{-Brd4} mutant is behaving as predicted from previous studies in other cell lines.

3.3. Identification of host genes regulated by E2^{wt} and E2^{-Brd4} expression

In Fig. 2 we demonstrated that E2^{-Brd4} is compromised in transcriptional activation. Previously we have demonstrated that E2^{wt} regulates transcription of the host genome, results in Fig. 2 suggest that E2-Brd4 may be compromised in this E2 property. This was investigated by carrying out exon array analysis of E2^{wt}-Clone1 and E2^{-Brd4}-Clone 1 and generating altered gene lists from this data. The results were generated from the average results from three independent RNA/cDNA preparations from the clones and compared with the gene levels in VEC 1.1. E2^{wt} generated changes in 889 cellular genes equal to or greater than 1.5 fold when compared with the VEC control line; 417 genes were up-regulated and 472 genes down regulated. The full list of genes is given in supplementary Table S1. E2^{-Brd4} generated changes in 1884 cellular genes equal to or greater than 1.5 fold when compared with the VEC control line; 957 genes were up-regulated and 827 genes were down regulated. The full list of genes is given in supplementary Table S2. Validation of the array was carried out with 8 genes for E2^{wt}, 7 of which validated in E2^{wt}-Clone 1 and E2^{wt}-Clone2, see Table S3 for details. Validation of the array was carried out with 7 genes for E2^{-Brd4}, 7 of which validated in E2^{Brd4}-Clone 1 and E2^{Brd4}-Clone2, see Table S4 for details. Considering that E2^{-Brd4} cannot activate transcription from adjacent promoters via E2 DNA binding sites, as shown in Fig. 2, this suggests that E2 can regulate the cellular genome via mechanisms that are independent of Brd4 interaction. This is in agreement with recent studies that demonstrated that E2 and Brd4 have overlapping interaction domains on host chromatin, but that these domains do not necessarily contain E2 DNA binding sites (Jang et al., 2014).

3.4. E2^{wt} regulates some host genes in a Brd4 dependent manner

Table 1 lists the top 50 up and down regulated genes by E2^{wt} and Supplementary Table S5 lists the top 50 up and down regulated genes by E2^{-Brd4}. In Table 1 the E2^{wt} regulated genes also regulated by E2^{-Brd4} in the same direction by at least two fold are highlighted in grey. 32% of the up regulated E2^{wt} genes are also increased by E2^{-Brd4} but strikingly 96% of the E2^{wt} down regulated genes are also down regulated by E2^{-Brd4}. This suggests that, at least in these highly regulated genes, E2 uses Brd4 to up regulate a significant percentage of the genes with enhanced expression. To test this hypothesis we selected three genes that E2^{wt} up regulates and that E2^{-Brd4} does not; *Lrrc15*, *Serpina1* and *SLN*. We then checked for the expression level of these three genes in all of the cell lines shown in Fig. 1a and the results of this are shown in Fig. 3; the results shown are summaries of three independent experiments. Below each lane the fold changes with standard errors are shown, all results are expressed relative to levels in VEC 1.1 equaling 1, lanes 1–3. In VEC1.2 none of the genes are significantly changed, lanes 4–6, whereas in E2^{wt}-Clone1 (lanes 7–9) and E2^{wt}-Clone2 (lanes 10–12) all three genes are significantly increased as predicted. In E2^{-Brd4}-Clone1 (lanes 13–15) and E2^{-Brd4}-Clone2 (lanes 16–18) all three genes are substantially down compared with the two wild type clones. Only *Lrrc15* in E2^{-Brd4}-Clone2 showed a small significant increase. Overall the results suggest that some of the E2^{wt} highly up regulated genes are increased due to interaction with Brd4.

3.5. Ingenuity Pathway Analysis (IPA) of E2^{wt} and E2^{Brd4} regulated genes

To investigate the potential functional consequences of the genes regulated by E2^{wt} and E2^{Brd4}, genes regulated up or down by 2.5 fold or more were subjected to IPA analysis. A summary PDF document of this analysis is given as PDF S1 for E2^{wt} and PDF S2 for E2^{Brd4}. Not surprisingly there are many differences between the gene sets indicating that failure to interact with Brd4 could influence the affect that E2 expression has on the cell. For both the wild type and Brd4 mutant E2 proteins the number one disease and disorder associated with the gene sets was cancer and the genes in these groups are given in Table S6 (E2^{wt}) and Table S7 (E2^{Brd4}). Previously we demonstrated that cellular movement was disrupted due to E2 expression (Gauson et al, 2014) and for both wt and Brd4 mutant E2 Cellular Movement was the number one molecular and cellular function identified in the gene data set. The genes associated with Cellular Movement are given in Table S8 (E2^{wt}) and Table S9 (E2^{Brd4}).

Overall the IPA analysis demonstrated both similarities but also differences between the altered gene data set from E2^{wt} and E2^{Brd4}. Previously we have demonstrated that the E2^{wt} expressing U2OS clones have an altered cellular movement and a delayed closure in wound healing (Gauson et al., 2014). We next investigated whether this phenotype was retained by E2^{Brd4}.

3.6. E2^{wt} expressing U2OS clones are more compromised in wound healing than E2^{Brd4} clones

U2OS VEC 1.1, E2^{wt}-Clone1, E2^{Brd4}-Clone2 cells were plated out and investigated for wound healing as described in materials and methods and previously (Gauson et al, 2014). Following removal of the strip to create a wound cells were photographed at 0, 12 and 24 h and a typical result from these experiments is shown in Fig. 4a (no E2 = VEC 1.1; E2-WT = E2^{wt}-Clone1, E2-Brd4 = E2^{Brd4}-Clone1). Fig. 4b gives a graphic representation summarizing three independent experiments carried out with two clones from each sample set. Lanes 1–6 are time 0, lanes 7–12 are 12 h after wound induction, lanes 13–18 are 24 h after induction. It is clear that at 12 h, in the absence of E2, the wound is almost closed (top panel Fig. 4a, middle, lanes 7 and 8 in Fig. 4b) and at 24 h is closed (top panel Fig. 4a, right, lanes 13 and 14 in Fig. 4b) while the E2^{wt} clone has delayed closing at 12 h (middle panel Fig. 4a, middle, lanes 9 and 10 in Fig. 4b) and is still not completely closed at 24 h (middle panel Fig. 4a, right, lanes 15 and 16 in Fig. 4b). The E2^{Brd4} mutant has a phenotype in between these two; at 12 h the wound is healed more than wild type but not quite to VEC control levels (bottom panel Fig. 4a, middle, lanes 11 and 12 Fig. 4b) and at 24 h the wound is healed although the cell density is not as high as with VEC (bottom panel Fig. 4a, right, lanes 17 and 18 Fig. 4b). This difference was seen reproducibly three times with two independent clones as summarized in Fig. 4b. This altered wound healing is not due to altered speed of growth of the cells as, if anything, the E2^{Brd4} cells are the slowest growing cells while E2^{wt} are the fastest growing (Fig. 1b). Therefore we conclude that the interaction between E2 and Brd4 is involved in the regulation of cellular movement by E2 in U2OS cells.

4. Conclusions

Previously we demonstrated that HPV16 E2 regulates transcription from the host genome (Gauson et al., 2014). This was the first report investigating the ability of E2 to carry out this function where the E2 levels were not induced or putting stress on the cell. Here we extend our studies to investigate the role of Brd4 in host genome regulation by E2. The results allow us to conclude several things. Firstly, failure to bind Brd4 results in an altered regulation of the host genome. Secondly, in highly regulated genes the failure to bind Brd4 is more aligned with failure to activate E2^{wt} up regulated genes. Thirdly, there are some host genes regulated by E2^{wt} that are Brd4 dependent as shown in Fig. 3 and some that are Brd4 independent as evidenced by the validated genes in Table S3 and Table S4. Fourthly, failure to bind Brd4 results in an altered ability to would heal as shown in Fig. 4. Previous studies demonstrate that expression of Brd4 can reduce invasiveness without altering cellular growth rate (Crawford et al., 2008), we postulate that E2^{wt} may interact with Brd4 to promote the slowing down of wound healing.

We propose that regulation of the host genome by E2 is an important property for successful infection by HPV16. The results presented here suggest that altered levels or interaction of E2 with Brd4 could deregulate this function. Future studies will focus on determining whether endogenous Brd4 can be manipulated to alter infection outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

E2^{wt} and E2^{Brd4} expression is tolerated stably in U2OS cells. (a) G418 resistant U2OS clones were selected for and grown out with either VEC (lanes 1 and 4), E2^{wt} (lanes 2 and 5) or E2^{Brd4} (lanes 3 and 6) were generated as described in materials and methods. Expression of the E2 proteins in these clones was investigated using western blotting and the upper panel demonstrates E2 expression in the E2^{wt} and E2^{Brd4} clones. Actin is shown as a loading control in the bottom panel. (b) Growth curves were carried out with the selected clones. Cellular growth was estimated as described in materials and methods and shown on

this graph. Only a representative clone for each (VEC 1.1, E2^{wt}-Clone1 and E2^{-Brd4}-Clone1) is shown for the sake of clarity. Both of the E2^{wt} and E2^{-Brd4} clones behaved very similarly to that shown. These growth curves were carried out on triplicate samples for all clones.

Fig. 2. E2^{-Brd4} has compromised transcriptional activation properties in U2OS cells. U2OS cells were transfected as described in materials and methods with ptk6E2-luc reporter plasmid (Vance et al., 1999) along with control vector or the indicated E2 expression vectors. 48 h after transfection cells were harvested and processed for luciferase assays as described in materials and methods. The results are expressed relative to no E2 expression plasmid equaling 1. Both low and high levels of E2^{wt} (lanes 1 and 2) activate transcription while E2^{-Brd4} fails to significantly increase transcription at either level of input plasmid (lanes 3 and 4). The results shown represent the average of three independent experiments carried out in duplicate with the standard error bars shown.

Fig. 3.

Some host gene regulation by E2^{wt} is dependent upon interaction with Brd4. Table 1 demonstrates that several of the genes up regulated by E2^{wt} are not regulated by E2^{Brd4} indicating that the regulation of these genes are dependent upon an interaction between E2 and Brd4. To investigate this three of these genes, *Lrrc15*, *Serpina1* and *SLN* were chosen for further investigation. The level of expression of these genes was investigated in VEC1.1, VEC1.2, E2^{wt}-Clone1 (E2Clone 1), E2^{wt}-Clone2 (E2Clone 2), E2^{Brd4}-Clone1 (Brd4Clone 1) and E2^{Brd4}-Clone2 (Brd4Clone 2). All results are expressed relative to the levels in VEC1.1 equaling 1 (lanes 1–3). It is clear that in VEC1.2 there is no change in expression of these genes (lanes 4–6). In both E2^{wt} clones (lanes 7–9 and 10–12) there is a significant increase in expression of the three genes while in both E2^{Brd4} clones any increase is undetectable or drastically reduced (lanes 13–15 and 16–18). The graph represents the average from three independent experiments and the standard error bars are shown.

Fig. 4. E2^{-Brd4} expressing clones are less compromised in wound healing than those expressing E2^{wt}. (a) Cells were plated out and 24 h the wound was induced following removal of barrier. Shown is a typical result for VEC 1.1 (No E2), E2^{wt}-Clone1 (E2-WT) and E2^{-Brd4}-Clone 1 (E2-Brd4). This was repeated three times with 2 independent clones of each data set with essentially identical results. (b) This figure summarizes three independent experiments carried out with 2 clones from each background. The gap size was measured at 12 and 24 h and the averages shown from the three experiments with standard error bars shown. It is

clear that the $E2^{-Brd4}$ clones are less compromised than $E2^{-wt}$ in wound healing as represented in Figure 4a. Figure 4a was carried out with the number 1 clones from each pair.

Table 1

The E2^{wt} top 50 up and down regulated genes. These lists are generated from Table S1. Those genes that are highlighted in grey were regulated in the same direction by at least two fold by E2^{-Brd4} (Table S2). It is striking that the main difference between E2^{wt} and E2^{-Brd4} in these highly regulated genes is in the up regulated genes. Only 16 out of 50 of the E2^{wt} up regulated genes are also up regulated by E2^{-Brd4} whereas 46 out of 50 down regulated genes are common to both E2^{wt} and E2^{-Brd4}.

Up-regulated by E2 ^{wt}	Fold-change	Down-regulated by E2 ^{wt}	Fold-change
HIST1H3H	90.7546	GTSF1	219.713
HIST1H2BM	46.231	NFE4	-33.2709
MAGEC1	30.2533	BGN	-22.9955
SLN	20.2274	KCNIP1	-21.732
HOXB2	9.13427	MIR31	-18.8279
C6orf15	7.1056	MGP	-18.0495
FAM198B	7.06128	KRT75	-17.2035
CLDN4	6.71694	FGF5	-14.8293
LRRC15	6.61625	RIMS1	-14.4025
SERPINA1	5.93304	SEMA3A	-12.5742
LRRC17	5.67212	SULT1B1	-12.5642
CXCL14	5.30661	ESM1	-12.2765
CDK15	4.91096	IL1RL1	-9.54772
SPTLC3	4.61896	CCL2	-8.46037
IL2RB	4.54159	HHIP	-8.32544
LIPH	4.41975	PDCD1LG2	-7.53008
TRIM16	4.35329	CD274	-7.42495
PLAC8	4.3376	MC5R	-7.17821
MGST1	4.26395	DCHS2	-7.10103
UNC13D	4.13402	SEMA3E	-6.75657
CA12	4.07438	ADAMTSL1	-6.52013
SLC12A8	4.00346	KRTAP4-12	-6.49334
LUM	3.96639	ST6GALNAC3	-6.15485
LIMCH1	3.91481	TMEM154	-6.04587
LCP1	3.51652	TMPRSS15	-6.0099
CALHM2	3.42559	CLDN1	-5.66267
IGFBP5	3.42263	MTAP	-5.63307
KCNJ8	3.41959	SLITRK6	-5.55367
GFPT2	3.38769	VLDLR	-5.38956
KLK6	3.33344	RAB38	-5.2863
PAGE2	3.29191	LPAR1	-5.20042
GPR56	3.26354	KIAA1797	-5.17872
SPP1	3.22762	KITLG	-4.94164
CDO1	3.20934	KRT34	-4.93765
PTP4A3	3.20919	FBXL13	-4.75785

Up-regulated by E2 ^{wt}	Fold-change	Down-regulated by E2 ^{wt}	Fold-change
TNNC1	3.16845	FAM180A	-4.7544
SERPINF1	3.14816	KCNB2	-4.63984
GPR45	3.12373	KIAA1432	-4.60797
KRT86	3.09044	EGLN3	-4.43062
RASD2	3.0506	DENND4C	-4.40059
FAM49A	3.04373	AK3	-4.38385
AQP1	3.03761	ADAMTS5	-4.27239
PDGFRB	3.02402	TM4SF18	-4.24862
B3GNT1	3.00978	CYP24A1	-4.20666
MAOA	2.98823	DPY19L2P2	-4.20305
CERCAM	2.98504	KLHL9	-4.19991
RASSF5	2.97825	LGR5	-4.14646
TGM2	2.95568	CDC37L1	-4.14413
TTC39A	2.95362	PGM5	-4.13192
TET1	2.92418	AFAP1	-4.12776