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Antiviral Res. Author manuscript; available in PMC 2016 August 01.

Published in final edited form as:

Antiviral Res. 2015 August ; 120: 1–6. doi:10.1016/j.antiviral.2015.04.012.

Development of a novel cell-based assay to monitor the transactivation activity of the HSV-1 protein ICP0

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Abstract

The herpes simplex virus type 1 (HSV-1) immediate-early phosphoprotein infected cell protein 0 (ICP0) is a potent transcriptional activator of viral genes and is required for efficient viral replication and reactivation from latency. However, it is largely unknown what role specific cellular factors play in the transactivator function of ICP0. With the long-term goal of identifying these factors, we developed a cell-based assay in a 96-well format to measure this activity of ICP0. We designed a system using a set of HSV-1 GFP reporter viruses in which the expression of GFP is potently induced by ICP0 in cell culture. The initial feasibility of this system was confirmed over a 24-hour period by fluorescence microscopy. We adapted this assay to a 96-well plate format, quantifying GFP expression with a fluorescence scanner. Our results indicate that the cell-based assay we developed is a valid and effective method for examining the transactivating activity of ICP0. This assay can be used to identify cellular factors that regulate the transactivating activity of ICP0.

Keywords

HSV; ICP0; GFP; viral transcription; cell-based assay

1. Introduction

Herpes simplex virus type 1 (HSV-1) infects 70–80% of the population and commonly causes cold sores, although many infections are asymptomatic (Roizman, 2007). HSV-1 can also cause ocular infections and is the major cause of infectious blindness in western industrialized countries (Roizman, 2007). Initially, HSV-1 infects epithelial cells at a primary site, resulting in a lytic infection. The virus can then infect the sensory neurons that innervate the infected epithelial cells, where it establishes a life-long latent infection. Reactivation of latent virus can occur by different stresses, which initiates lytic infection in neurons and ultimately epithelial cells at the primary site of infection. This cycle of latent

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and lytic infections leads to recurrent disease. One viral protein that is important for efficient for viral replication and reactivation from latency is the immediate-early (IE) protein, infected cell protein 0 (ICP0) (Cai and Schaffer, 1989, Harris *et al.*, 1989, Stow and Stow, 1986).

ICP0 is one of the five HSV-1 IE proteins and has RING finger motif (Everett, 1989, Meredith *et al.*, 1995) that allows ICP0 to function as an E3 ubiquitin ligase (Boutell *et al.*, 2002). E3 ubiquitin ligases are components of an E1-E2-E3 biochemical pathway that conjugates and polymerizes chains of ubiquitin onto proteins (Kleiger and Mayor, 2014). This activity of ICP0 directs the degradation of specific cellular proteins (Chelbi-Alix and de The, 1999, Everett *et al.*, 1998a, Lees-Miller *et al.*, 1996, Muller and Dejean, 1999, Parkinson and Everett, 2000, Perusina Lanfranca *et al.*, 2013), which in turn impairs cellular antiviral defenses. The E3 ubiquitin ligase activity of ICP0 is also linked to the ability of ICP0 to act as a potent global transcriptional activator of viral genes (Everett *et al.*, 1998b, O'Rourke *et al.*, 1998). Previous studies have shown that the transactivation activity of ICP0 is important for efficient HSV-1 replication and reactivation from latency (Cai *et al.*, 1993, Cai and Schaffer, 1992, Everett *et al.*, 2009). Although the transactivating activity of ICP0 has been studied in the absence of other IE genes in transient transfection assays (Davido *et al.*, 2005, Everett, 1987, Jordan and Schaffer, 1997, Mostafa *et al.*, 2013), it has not been extensively studied independent of other IE proteins in the context of a viral infection. A limited number of cellular factors have been linked to ICP0's transactivation activity (Davido *et al.*, 2002, Li *et al.*, 2009); however, the development of a cell-based assay to monitor this activity would allow for the systematic identification of key cellular factors or activities (e.g., via siRNA knockdown) that contribute to this function.

To begin to elucidate cellular factors that affect ICP0's transactivating activity, we have developed an assay that measures this activity of ICP0 in a 96-well plate format. The assay was developed using two HSV-1 reporter viruses that express only ICP0 of the 5 IE genes (*d*106) or none of the IE genes (*d*109). Both viruses have a human cytomegalovirus major immediate early promoter (HCMV MIEp) driven green fluorescent protein (GFP) in the HSV-1 *ICP27* locus (Samaniego *et al.*, 1998). Similar to other published reports (Eidson *et al.*, 2002, Everett *et al.*, 2009, Minaker *et al.*, 2005, Preston and Nicholl, 1997), we show here that ICP0 is capable of transactivating the HCMV MIEp; in the case of *d*106, ICP0 transactivation activity is quantified by measuring GFP fluorescence levels using a Typhoon Imager. To ultimately adapt this screen to use siRNAs against cell targets, we demonstrate that ICP0's transactivating activity was minimally affected when cells were transfected with an siRNA against the cellular house keeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Using a 96-well plate format, we established that *d*106 (+ICP0) has higher levels of GFP than *d*109 (-ICP0), which increase from 12 to 24 h post infection (hpi). Lastly, we use this assay in an siRNA library screen and identify that an siRNA against ubiquitin activating enzyme E1-like (UBEL1) impairs the transactivation activity of ICP0, validating the development of our assay.

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2. Materials and methods

2.1. Cell culture and viruses

HeLa S3 (human cervical carcinoma) cells (kindly provided by Ira Blader) were utilized in the assay and grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 U/mL penicillin, 10 U/mL streptomycin, and 50 μg/mL gentamycin at a temperature of 37°C in 5% CO_2.

HSV-1 recombinant viruses, *d*106 and *d*109 (Fig 1, modified (Samaniego *et al.*, 1998), kindly provided by Neal DeLuca) were used in our cell-based assay. Of the 5 IE genes, *d*106 only expresses ICP0 (+ICP0), whereas *d*109 does not express any IE proteins (-ICP0). Both viruses express the GFP gene from the human cytomegalovirus major IE promoter (HCMV MIEp). Both *d*106 and *d*109 viruses were grown on M17 cells, which are a derivative of Vero cells stably transformed with the HSV-1 ICP4 and ICP27 genes (kindly provided by David Leib). Adenoviral vectors, Ad-crtTA and Ad-T-ICP0, were used to infect M17 and transiently express ICP0 to increase titers of *d*109. Ad-crtTA and Ad-TICP0 were grown and titered as previously described (Halford *et al.*, 2001). *d*106 was titered on Vero cells and *d*109 was titered on L7 cells (Vero cells that contain the ICP0 gene) (Samaniego *et al.*, 1997), and fluorescent forming units were counted 24 hpi. M17, Vero, and L7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 10 U/mL penicillin, 10 U/mL streptomycin, and 50 μg/mL gentamycin at a temperature of 37°C in 5% CO_2.

2.2. Reverse transfection

HeLa S3 cells were reverse tranfected in BD Falcon black well clear bottom optilux 96-well plates (San Jose, California, catalog # 08-772-104). Ambion siRNAs (Grand Island, NY) were used at a volume of 10 μL per well and final concentration of 10 μM per well. The ubiquitin pathway siRNA library (Ambion, Grand Island, NY, Catalog number #AM80991V3) was used at the same volume and concentration. siRNA was pre-plated in wells of the 96-well plate. 0.2 μL of Lipofectamine 2000 per well was mixed with 9.8 μL Opti-MEM per well for 10–25 min. The Lipofectamine 2000/Opti-MEM mix is added to the pre-plated siRNA and allowed to incubate for 20 minutes. After the 20 min incubation, HeLa S3 cells were added to the wells at 8,000 cells per well in 80 μL per well in penicillin/ streptomycin free 10% FBS DMEM. Transfected and mock-transfected wells were washed twice with complete medium 24 h post transfection (hpt) and then 100 μL of HeLa S3 cell medium was added to each well. 48 hpt, medium was removed from the cells and the well was either infected with previously described reporter viruses or harvested for RNA isolation.

2.3. Infection

HeLa S3 cells were infected 48 hpt with *d*106 or *d*109 reporter viruses at a multiplicity of infection (MOI) of 1. Viruses were diluted in HeLa S3 medium that did not contain phenol red. Infections were allowed to proceed for 12, 18, and 24 hpi.

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2.4. Detection and analysis of fluorescent signals

Plates were scanned using a Typhoon image scanner (General Electric Healthcare) at standard settings according the manufacturer's recommendations. Settings were chosen to scan green fluorescence at an excitation of 488, emission of 520, and photomultiplier tube (PMT) of 475 and excitation of 633, emission of 670, and photomultiplier tube (PMT) of 475 for the deep red. Scans were done at 12, 18, and 24 hpi. At the 24 h time point for the siRNA screen, cells were stained with deep red mitotracker in FBS-free DMEM at a final concentration of 250 nM as per protocol (Invitrogen, Grand Island, NY, catalog # M22426) to account for cell density. The fluorescence intensity of each scanned well was analyzed in ImageJ by using a circle the size of the well & measuring the intensity of each well. Data was copied into Excel (Microsoft) software for analysis. Wells that contained only cells and medium were used as background controls, and this value was subtracted from values of infected wells. For the siRNA screen, the GFP intensity was normalized to the mitotracker (deep red) intensity. *d*106 values were set at 100% and mock transfected, *GAPDH* siRNA transfected *d*106, *d*109 values were compared to the 100% value. For the siRNA screen, similar calculations were done, but normalized values were used instead of GFP only values. Lastly, the percent average variation for all *d*106 signals using the data in presented in Fig. 5 was calculated to be 3% of the mean *d*106 signal..

2.5. RNA isolation and reverse transcription real time PCR

Cells were lysed and harvested using Trizol (Invitrogen, Grand Island, NY) according to manufacturer's recommendations. RNA was isolated using phenol-chlorform extraction. 500 ng of RNA was reverse transcribed to make cDNA using the iScript BioRad cDNA synthesis kit (BioRad, Hercules, CA, catalog # 170-8890) according to manufacturer's conditions. Real time PCR was done by using SYBR green master mix (Roche) and standard conditions were used for the cellular target, *GAPDH* (5′ CGGATTTGGTCGTATTGGGCGC 3′ and 5′ TCCCGTTCTCAGCCTTGACGGT 3′), and the normalization control, *hTBP* (5′-TGCACAGGAGCCAAGAGTGAA-3′ and 5′-CACATCACAGCTCCCCACCA-3′)(Smith *et al.*, 2013). Standard curves were obtained for the target and normalization control. Comparisons were made using the ^{2}CT method, setting the untransfected control at 100%.

3. Results

3.1. ICP0 transactivates the HCMV MIEp-GFP

With the long-term goal of identifying cellular factors that play a role in the transactivating activity of ICP0, we sought to develop a 96-well plate cell-based assay. We initially wanted to establish if the replication-defective reporter viruses, *d*106 (+ICP0) and *d*109 (-ICP0) (Samaniego *et al.*, 1998), could be used in this format. Specifically, we wanted to use fluorescence as an output of ICP0's transactivation activity. Both *d*106 and *d*109 viruses contain an HCMV MIEp-GFP reporter, and previous studies indicate that ICP0 can transactivate the HCMV MIEp (Fig. 1). We first tested whether the expression of GFP is dependent on ICP0 in HeLa cells, a cell type commonly used to study HSV-1 replication that is highly transfectable for nucleic acids (e.g., siRNA and DNA plasmids) (Everett, 1987, Sarma *et al.*, 2008). HeLa S3 cells were infected with *d*106 (+ICP0) or *d*109 (-ICP0),

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and GFP expression was examined by microscopy at 2, 6, 12, and 24 h post infection (hpi). Activation of the HCMV MIEp-GFP was first detected by 6 hpi for *d*106 (+ICP0) and steadily increased until 24 hpi (Fig. 2). In contrast, activation of HCMV MIEp-GFP in *d*109 (-ICP0) became apparent by 12 hpi in a subset of cells (Fig 2), with increased GFP expression by 24 hpi (Fig. 2). Although the GFP is induced in *d*109 (-ICP0)-infected cells, it does not reach the level, both in relative fluorescence intensity and cell number, as *d*106 (+ICP0)-infected cells. This experiment indicated that ICP0 potently induced the HCMV MIEp-GFP reporter construct in *d*106; thus, we established that *d*106 can be used as a reporter virus for monitoring ICP0 transactivating activity.

3.2. siRNA depletion of *GAPDH* does not appear to affect ICP0's transactivation activity by fluorescence microscopy

We next wanted to know if transfecting an siRNA against a cellular housekeeping gene would affect the transactivating activity of ICP0. For this experiment, HeLa S3 cells were untransfected, mock transfected, or transfected with an siRNA targeted against *GAPDH*. Mock-transfected cells were included in these experiments to determine if the transfection reagent alone modulated ICP0's transactivating activity. As indicated in Figure 3, we observed no notable differences in GFP levels of *d*106 (+ICP0)- or *d*109 (-ICP0)-infected cells that were untransfected, mock transfected, and *GAPDH* siRNA-transfected via microscopy.

3.3. *GAPDH* transcript levels are depleted in a 96-well plate format

Once we showed that transfection of siRNAs into cells did not appear to affect GFP levels of *d*106 (+ICP0) or *d*109 (-ICP0)-infected cells as imaged by microscopy, we next wanted to confirm that we could deplete the transcript levels of a cellular target using an siRNA in a 96-well plate format. HeLa S3 cells were untransfected, mock transfected, or transfected with a siRNA targeting *GAPDH* in triplicate wells. Forty-eight h post transfection (hpt), RNA from each well was isolated, reverse transcribed into cDNA, and analyzed by Real Time PCR. Transcript levels of *GAPDH* were significantly reduced to 3.4% of untransfected levels in the siRNA-transfected samples and to 75% in mock-transfected samples compared to untransfected samples, which were given the value of 100% (Fig. 4). This experiment shows that we are able to reduce mRNA levels of a specific cellular target in a 96-well plate substantially.

3.4. An siRNA against *GAPDH* has a minimal affect on ICP0's transactivation activity in a 96-well plate

After we established that an siRNA against *GAPDH* depleted its transcript levels using a 96-well plate, we adapted this assay using the *d*106 and *d*109 reporter viruses to a 96-well plate format. HeLa S3 cells were untransfected, mock transfected, or transfected with a siRNA against *GAPDH*. At 48 hpt, HeLa S3 cells were mock infected in triplicate or infected with *d*106 or *d*109 at an MOI of 1. The 96-well plate was scanned for fluorescence using a Typhoon Imager at 12 hpi, 18 hpi, and 24 hpi. GFP expression was detected at 12 hpi for *d*106 (+ICP0) (Fig. 5A), which gradually increased at 18 and 24 hpi (Fig. 5A). For each time point, *d*106 (+ICP0) GFP levels are visibly higher than *d*109 (-ICP0), with *d*109 GFP

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intensities were reduced 5–6 fold at 12 hpi, 9.5–12 fold at 18 hpi, and 10.4–14 fold at 24 hpi (Fig. 5B) relative to *d*106, whether in the absence or presence of the *GADPH* siRNA. Lastly, there results confirmed our microscopy studies in which an siRNA against *GAPDH* did not appreciably affect GFP intensities for *d*106- or *d*109-infected wells compared their respective untransfected controls (Fig. 5B). These experiments demonstrate that we can examine the transactivation activity of ICP0 using *d*106 in a cell-based assay in a 96-well plate format.

3.5. An siRNA agajnst *UBE1L* impairs the transactivation activity of ICP0

Utilizing our 96-well plate cell-based assay, we screened a small siRNA library that targeted components of the ubitiquitin pathway. Wells were reverse-transfected and infected with *d*106. From this screen, an siRNA against *UBE1L*, an E1 enzyme of the ubiquitin-proteasome pathway, impaired the transactivation activity of ICP0 5.5-fold at 24 hpi (Fig. 6), which began to approach *d*109 (-ICP0) GFP levels (Fig. 6). Thus, we demonstrate that this assay can be used in an siRNA screen having identified a target in the ubiquitin-proteasome pathway.

4. Discussion

In this paper, we have demonstrated, in line with other studies (Everett *et al.*, 2009, Minaker *et al.*, 2005, Preston and Nicholl, 1997), that ICP0 can potently transactivate the HCMV MIEp (Fig. 2). We also found that an siRNA against the housekeeping gene, *GAPDH*, does not appear to affect the transactivation activity of ICP0 as measured by microscopy (Fig. 3) and that *GAPDH* transcripts could be depleted to significant levels in a 96-well plate format (Fig. 4). These results indicate that siRNA technology can be used in this cell-based assay in a 96-well plate format. We also established that the Typhoon Imager is a quick and valid method to examine ICP0's transactivating activity in a 96-well plate format (Fig. 5), and we verified that the assay can be used to screen an siRNA library by identifying an ubiquitin-proteasome pathway target, *UBE1L* (Fig. 6). Overall, we have concluded that the reporter viruses, *d*106 and *d*109, can be utilized in a cell-based assay by using a 96-well plate format and Typhoon image scanner.

While this system is adaptable to siRNA or small molecule inhibitor screens, this method also has several advantages over other established methods used to examine ICP0's transactivation activity (Davido *et al.*, 2002, Everett, 1987, Jordan and Schaffer, 1997, Mostafa *et al.*, 2013). Specifically, ICP0's transactivation activity can be monitored in the context of a viral infection, in the absence of other IE proteins, and at multiple time points in one experiment or infection by the assaying of live cells. In contrast, other methods monitor this activity in the context of a viral infection but in the presence of other IE proteins (Davido *et al.*, 2002), which may complicate the interpretation of certain studies. Transient transfection reporter assays have been used to examine ICP0's transactivation activity independent of other IE proteins, but these assays are not done in the setting of a viral infection (Everett, 1987, Jordan and Schaffer, 1997, Mostafa *et al.*, 2013). Finally, most viral reporter assays and transient transfection assays can only monitor one time point per sample because the cells will either have to be lysed or fixed to analyze the sample, in contrast to our system. Thus, the *d*106/*d*109 reporter assay is advantageous because it can be

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used to monitor multiple time points for one experiment and monitor ICP0's transactivation activity in the absence of IE proteins while in the context of a viral infection. Furthermore, the inclusion of *d*109 can also be used in counter-screens to verify that targets or inhibitors are specific for ICP0.

In summary, we have developed a cell-based assay for ICP0 that can use siRNA technology or small molecule inhibitors in a 96-well plate format. This assay can also be modified for use in a high throughput screen with the inclusion of viability indicators such as alamarBlue (Osaka and Hefty, 2013). Notably, this assay will likely increase our knowledge related to the regulation of ICP0's transactivating activity, which may eventually lead to potential treatments against HSV-1 and its recurrent diseases.

Acknowledgments

This work was supported in part by Public Health Service grant RO1AI72357 from the National Institute of Allergy and Infectious Diseases. The content of the manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We thank members of the Davido lab for assistance and comments related to this study, and Miles Smith and Steve Benedict for critical review of this manuscript.

Glossary

Ad	adenovirus
CHX	cyclohemiximde
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
FBS	fetal bovine serum
FFU	fluorescent forming units
HCMV	human cytomegalovirus
hpi	hours post infection
hpt	hours post transfection
HSV-1	herpes simplex virus type 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
IE	immediate early
ICP0	infected cell protein 0
ICP27	infected cell protein 27
MIEp	major immediate early promoter
MOI	multiplicity of infection
PFU	plaque forming unit

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SEM	standard error of the mean
siRNA	small interfering RNA

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Highlights

- HSV-1 ICP0 potently transactivates an HCMV MIEp-GFP reporter construct in the HSV-1 mutant, *d*106.
- Using *d*106, ICP0 transactivation activity increases over time as determined by fluorescence microscopy and scanning.
- This reporter assay can be adapted to a 96-well plate format and is compatible with siRNA and inhibitor testing.

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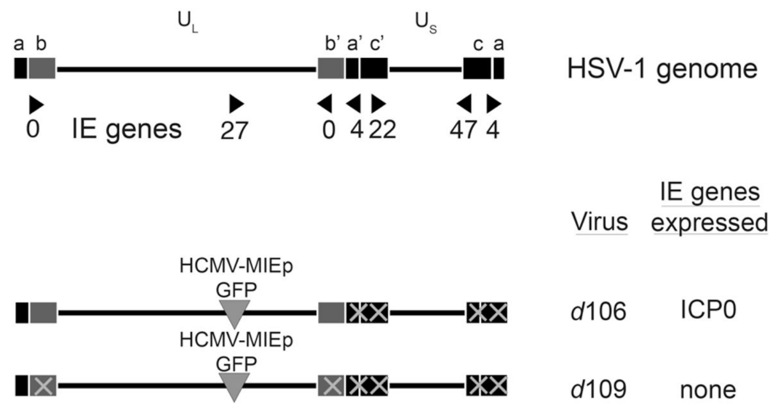

Fig. 1. HSV-1 recombinant viruses, *d*106 and *d*109, utilized in our cell-based assay

Of the 5 IE genes, *d*106 only expresses ICP0 (ICP0+), whereas *d*109 does not express any IE proteins (ICP0-). Both viruses express the *GFP* gene from the human cytomegalovirus major IE promoter (HCMV MIEp). This figure is modified from Samaniego, *et. al.,* 1998.

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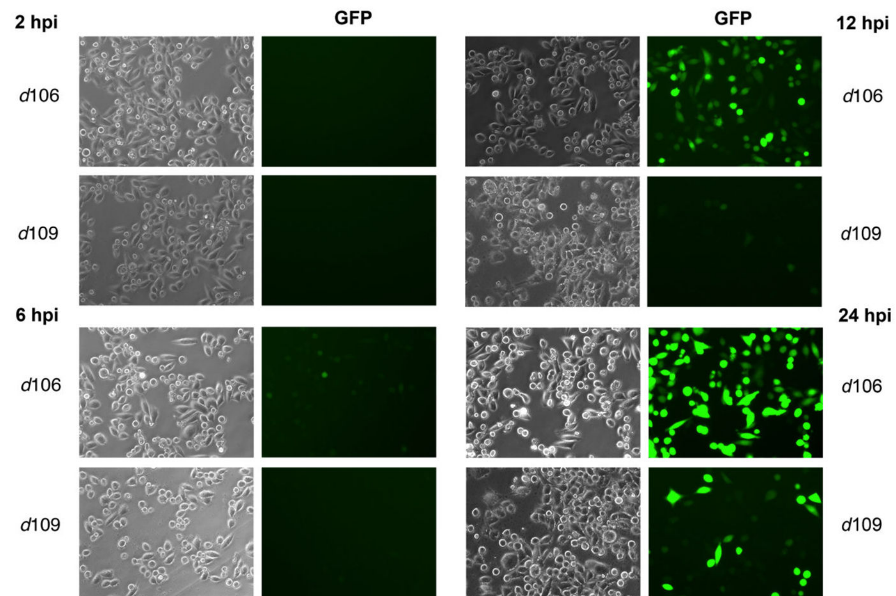

Fig. 2. ICP0 transactivates the HCMV MIEp-GFP

HeLa S3 cells were seeded in a 12-well plate, and mock-infected or infected at an MOI of 1 FFU per cell of *d*106 or *d*109 per cell. Cells were examined by fluorescence microscopy at 2, 6, 18, and 24 h post infection (hpi), and images were captured with a digital camera. Light microscopy images are located on the left and fluorescent images are located on the right for each virus

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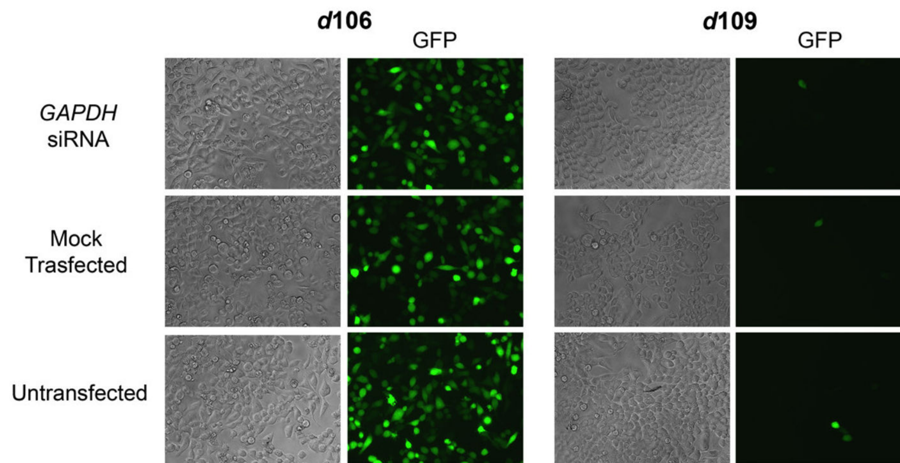

Fig. 3. siRNA depletion of *GAPDH* does not affect ICP0's transactivation activity

HeLa S3 cells were untransfected, mock transfected or transfected with an siRNA directed against *GAPDH*. Mock transfected cells were included in these experiments to determine if the transfection reagent alone modulates ICP0's transactivating activity. All samples were compared to the untransfected control. Forty-eight hpt, HeLa S3 cells were infected with an MOI of 1 with *d*106 or *d*109 for 24 h and viewed by fluorescence microscopy. Light microscopy images are located on the left and fluorescent images are located on the right per virus. Images were captured with a digital camera.

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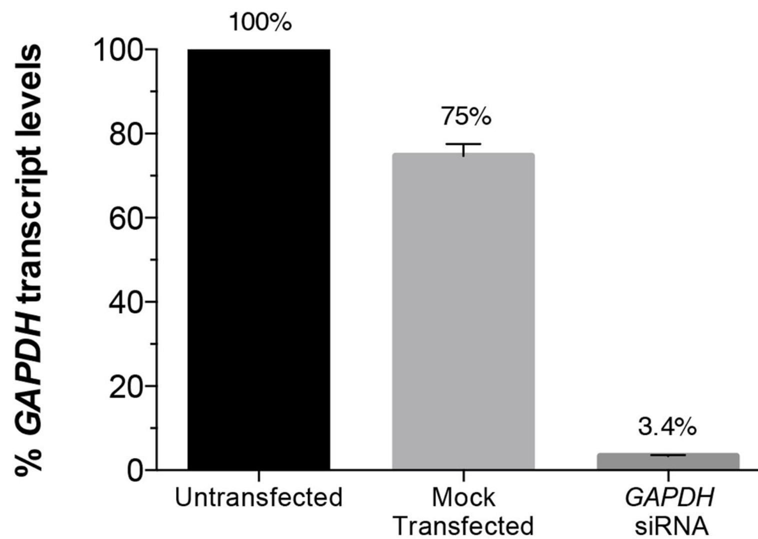

Fig. 4. *GAPDH* transcript levels are depleted in a 96-well plate

HeLa S3 cells were untransfected, mock transfected, or transfected with a siRNA targeting *GAPDH* in triplicate wells in a 96-well plate. Twenty-four hpt, well were washed as described in materials and methods. Forty-eight hpt, RNA was isolated from each well, reverse transcribed into cDNAs, and analyzed by Real Time PCR. Comparisons were made using the ^{2}CT method. *GAPDH* transcript levels were compared between mock- and *GAPDH*-transfected cells relative to the untransfected control, which was set at 100%. Error bars represent SEMs. This experiment was repeated in triplicate 3 times.

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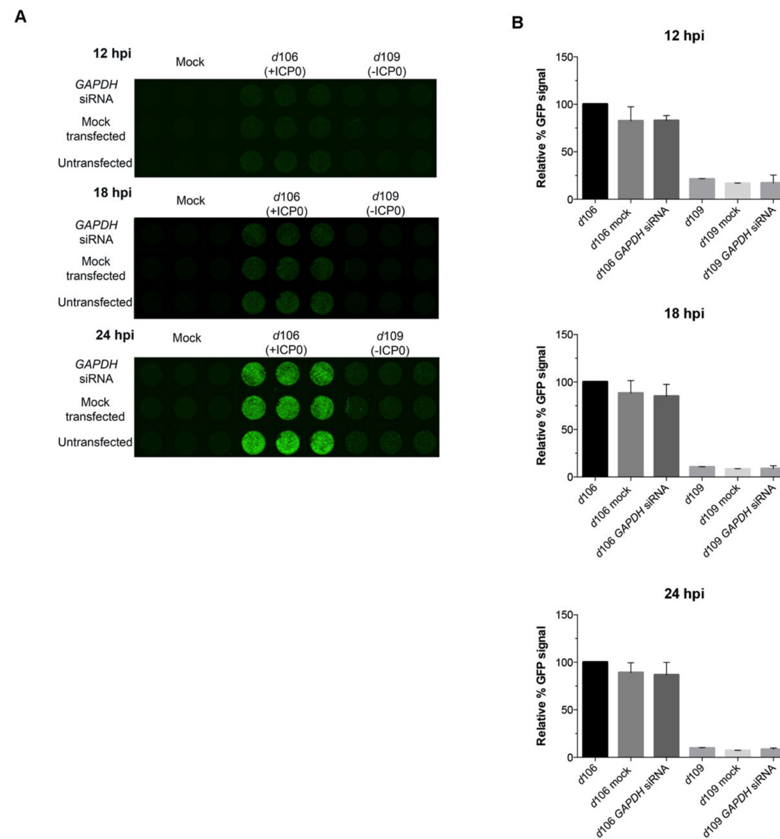

Fig. 5. An siRNA against *GAPDH* has a minimal effect on ICP0's transactivation activity in a 96-well plate format

HeLa S3 cells were untransfected, mock transfected, or transfected with an siRNA against *GAPDH*. At 48 hpt, HeLa S3 cells were mock infected or infected with *d*106 or *d*109 at an MOI of 1. (A) The 96-well plate was scanned using a Typhoon Imager at 12 hpi, 18 hpi, and 24 hpi. This experiment was repeated in triplicate 2 times. (B) Comparisons of *d*106 and *d*109 samples – untransfected *d*106 samples were set at 100% for all time points, and mock transfected and siRNA-transfected samples were compared to it. Error bars represent SEMs.

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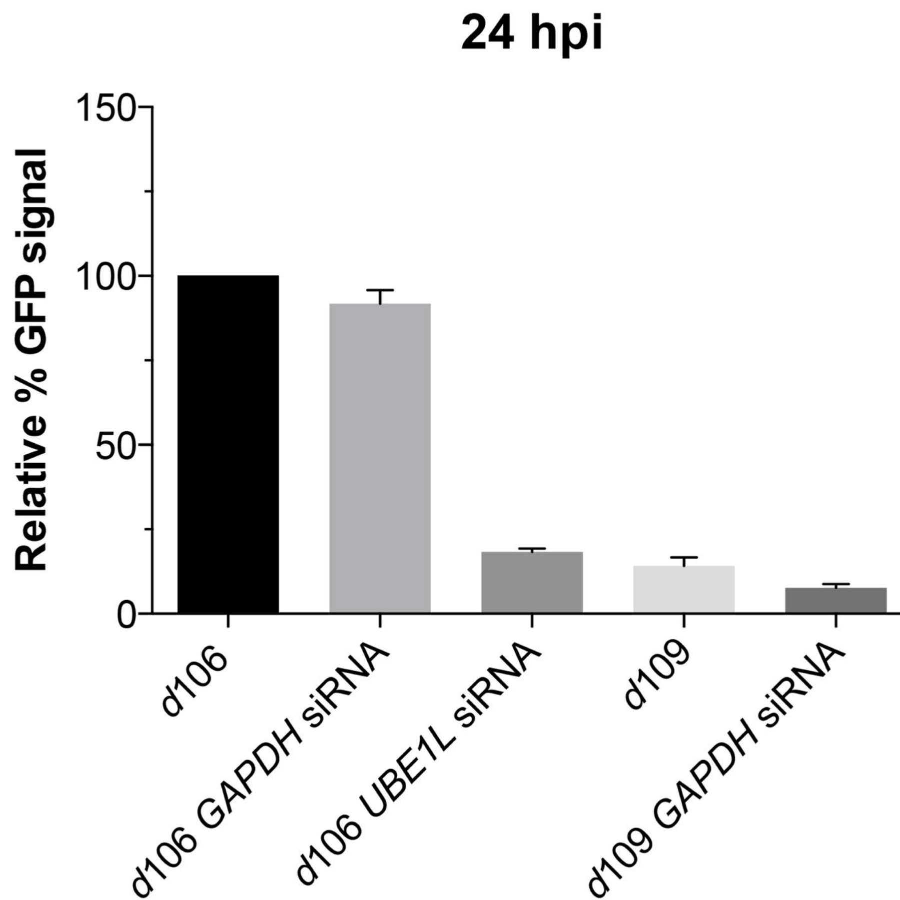

Fig. 6. An siRNA agajnst *UBE1L* impairs the transactivation activity of ICP0

HeLa S3 cells were seeded in a 96-well plate. Cells were reverse transfected as described in the protocol. Wells that contained siRNA against the ubiquitin ligase pathway were infected with *d*106 at an MOI of 1, 48 hpt. Cells were untransfected or transfected with *GAPDH* siRNA for controls. 48 hpt, control wells were infected *d*106 or *d*109 or mock infected. The 96-well plate was scanned using a Typhoon Imager at 24 hpi. Comparisons were made relative to *d*106-untransfected infection, which was set at 100% (far left bar for each graph). *UBEL1* is ubiquitin activating enzyme E1-like. Error bars represent SEMs. This experiment was repeated 3 times.