

A Targeted RNA Interference Screen Reveals Novel Epigenetic Factors That Regulate Herpesviral Gene Expression

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ABSTRACT Herpes simplex virus (HSV) utilizes and subverts host chromatin mechanisms to express its lytic gene products in mammalian cells. The host cell attempts to silence the incoming viral genome by epigenetic mechanisms, but the viral VP16 and ICP0 proteins promote active chromatin on the viral genome by recruiting other host epigenetic factors. However, the dependence on VP16 and ICP0 differs in different cell lines, implying cell type-dependent functional contributions of epigenetic factors for HSV gene expression. In this study, we performed a targeted RNA interference (RNAi) screen for cellular chromatin factors that are involved in regulation of herpes simplex virus (HSV) gene expression in U2OS osteosarcoma cells, a cell line that complements *ICP0* mutant and *VP16* mutant virus replication. In this screen, we found the same general classes of chromatin factors that regulate HSV gene expression in U2OS cells as in other cell types, including histone demethylases (HDMs), histone deacetylases (HDACs), histone acetyltransferases (HATs), and chromatin-remodeling factors, but the specific factors within these classes are different from those identified previously for other cell types. For example, KDM3A and KDM1A (LSD1) both demethylate mono- and dimethylated H3K9, but KDM3A emerged in our screen of U2OS cells. Further, small interfering RNA (siRNA) and inhibitor studies support the idea that KDM1A is more critical in HeLa cells, as observed previously, while KDM3A is more critical in U2OS cells. These results argue that different cellular chromatin factors are critical in different cell lines to carry out the positive and negative epigenetic effects exerted on the HSV genome.

IMPORTANCE Upon entry into the host cell nucleus, the herpes simplex virus genome is subjected to host epigenetic silencing mechanisms. Viral proteins recruit cellular epigenetic activator proteins to reverse and counter the cellular silencing mechanisms. Some of the host silencing and activator functions involved in HSV gene expression have been identified, but there have been indications that the host cell factors may vary in different cell types. In this study, we performed a screen of chromatin factors involved in HSV gene regulation in osteosarcoma cells, and we found that the chromatin factors that are critical for HSV gene expression in these cells are different from those for previously studied cell types. These results argue that the specific chromatin factors operative in different cell lines and cell types may differ. This has implications for epigenetic drugs that are under development.

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Nuclear DNA viruses encode gene products that inhibit host epigenetic silencing factors and recruit host activating epigenetic factors to provide active euchromatin for transcription of their genes (1). Herpes simplex virus (HSV) virion DNA is not associated with histones but is rapidly chromatinized upon entry into the nuclei of cells (2, 3). Viral gene products are involved in the blocking and/or removal of repressive chromatin (3–5). Expression of viral immediate-early (IE) genes is dependent on the virion protein VP16, which forms an activator complex containing the cellular host cell factor 1 (HCF-1) and octamer-binding protein 1 (Oct-1). Oct-1 binds to sites in IE gene promoters, and HCF-1 recruits transcription factors and chromatin-modifying factors that promote IE gene transcription (5–7). The HCF-1 subunit recruits several chromatin-modifying enzymes, including (i)

the KMT2F (SETD1A) histone H3 lysine 4 (H3K4) methyltransferase (8), which introduces the histone H3K4 trimethylation euchromatin mark, (ii) the KDM1A (LSD1) demethylase (9), which removes the H3K9me1/2 heterochromatin mark, and (iii) the KDM4 (JMJD2) demethylase (10), which removes the H3K9me3 heterochromatin mark. Efficient expression of the IE genes, especially ICP0, is also dependent on the SNF2H chromatin remodeling subunit (11), but the mechanism of its recruitment to IE genes is not known. These and other chromatin modifications allow the transcription of the viral IE genes. The IE protein ICP0 then inhibits chromatin-silencing mechanisms on the entire genome, at least in part by inhibition of CoREST-HDAC1 complexes (12) and by recruitment of the CLOCK histone acetyltransferase (HAT) enzyme (13).

HSV strains that fail to encode VP16 or ICP0 proteins or that encode defective forms of them show defects in gene expression and replication, but the levels of the defects vary in different cell lines (14–16). Also, depletion of activator complex-interacting proteins, such as the histone acetyltransferases p300, CBP, PCAF, and GCN5 or the chromatin remodeling factors BRM and Brg-1, does not result in significant defects in viral gene expression (6). Epigenetic inhibitors have also shown cell type-dependent effects on the HSV life cycle. Treatment with HDAC inhibitors reactivates latently infected HSV in certain cell types (17–19). Interestingly, KDM1A (LSD1) preferentially demethylates the euchromatic marks H3K4me1/2 when associated with RCOR1/CoREST but demethylates the heterochromatic marks H3K9me1/2 when associated with the androgen receptor, which functions as a corepressor or coactivator (9, 20–22). Another type of HDM, KDM3A, is known to target H3K9me1/2 but not H3K4me1/2 (23). However, the mechanisms by which these redundant KDMs select a given substrate are not understood. It is possible that certain subsets of histone-modifying enzymes are functionally more active in different complexes, which could be cell type dependent and/or signal transduction pathway dependent.

We hypothesized that different subsets of chromatin-modifying factors are functionally more active for HSV infection in specific cell lines. In this study, we used U2OS osteosarcoma cells because they are known to complement the growth defect of HSV-1 *ICP0*-negative mutant viruses (14) and in particular to complement the epigenetic phenotypes of *ICP0*-negative mutant viruses observed in other cell types (3, 24). U2OS cells are also known to partially complement the growth defect of HSV-1 *VP16* mutant viruses (15). Therefore, we speculated that U2OS cells might contain a set of epigenetic factors required for regulation of HSV gene expression different from those in cells that require *ICP0* and/or *VP16* for HSV infection. To investigate this question, we performed a screen to identify chromatin-modifying genes that contribute to HSV gene expression in U2OS cells. RNA interference (RNAi) was used to suppress each candidate gene, and an imaging readout was used to assess viral gene expression from an HSV recombinant virus, HSV8GFP, in which an essential viral protein, ICP8, is fused to green fluorescent protein (GFP). We found that the previously documented general classes of chromatin factors are also required in U2OS cells but that the specific members of these classes are different.

RESULTS

Establishment of an HSV infection screening system. Different cell lines show differential restriction of HSV *ICP0*-negative recombinant strains (14). For example, U2OS cells are known to complement the growth defect of HSV-1 *ICP0*-negative mutant viruses (14) and *VP16* mutant viruses (15) and in particular to complement the altered viral chromatin phenotype of *ICP0*-negative mutant viruses observed in other cell types (3, 24). Potential altered epigenetic regulatory mechanisms are that U2OS cells are known to not express ATRX (25), and the IFI16 nuclear DNA sensor does not respond to HSV infection (26, 27). Therefore, we speculated that the set of epigenetic factors involved in regulating HSV gene expression in U2OS cells might differ from those in other cell types previously studied (1). We therefore conducted a targeted small interfering RNA (siRNA) screen of host chromatin factors functioning in U2OS cells to regulate HSV gene expression.

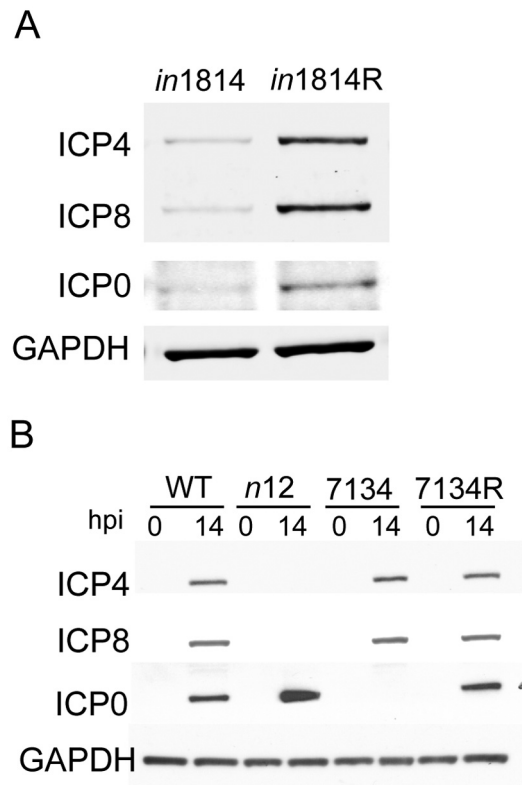


FIG 1 Viral factors required for IE and E gene expression in U2OS cells. (A) U2OS cells were infected with *in1814* *VP16* mutant virus or *in1814R* rescued virus at an MOI of 0.1 PFU/cell for 8 h, and ICP4, ICP8, and ICP0 expression levels were detected by immunoblotting using specific antibodies. (B) U2OS cells were either mock infected (0) or infected with wild-type (WT) KOS, *n12* *ICP4*-null, 7134 *ICP0*-null, or 7134R rescued virus at an MOI of 0.1 for 14 h, and ICP4, ICP8, and ICP0 expression levels were determined by immunoblotting.

We first defined the viral gene products that regulate HSV gene expression in U2OS cells used for the screen by measuring viral *ICP8* (*U_L29*) gene expression in cells infected with different viral mutant strains. Infected cell protein 8 (ICP8) is an early gene product, and therefore this screen would identify factors that are important for regulating stages of the productive HSV life cycle, including immediate-early and early gene expression, and the transition to late gene expression. To test the role of *VP16*, which is required for optimal IE gene expression (16), we infected U2OS cells with a *VP16* mutant virus, *in1814*, or the rescued virus strain *in1814R*. We observed that at 8 hours postinfection (hpi) *in1814*-infected cells showed reduced expression of ICP4, ICP8, and ICP0 compared with *in1814R*-infected cells (Fig. 1A), indicating that *VP16* is needed for optimal HSV immediate-early and early gene expression in U2OS cells. When the *n12* *ICP4*-null mutant was compared with wild-type (WT) HSV-1, *n12* virus-infected cells showed virtually no ICP8 expression and enhanced ICP0 expression (Fig. 1B), indicating that *ICP4* is essential for early HSV gene expression in U2OS cells. Finally, the 7134 *ICP0* mutant virus showed normal levels of ICP8 expression in these cells (Fig. 1B), consistent with previous reports that U2OS cells complement HSV gene expression by other *ICP0* mutant viruses (14). These experiments suggested that cellular factors normally functioning

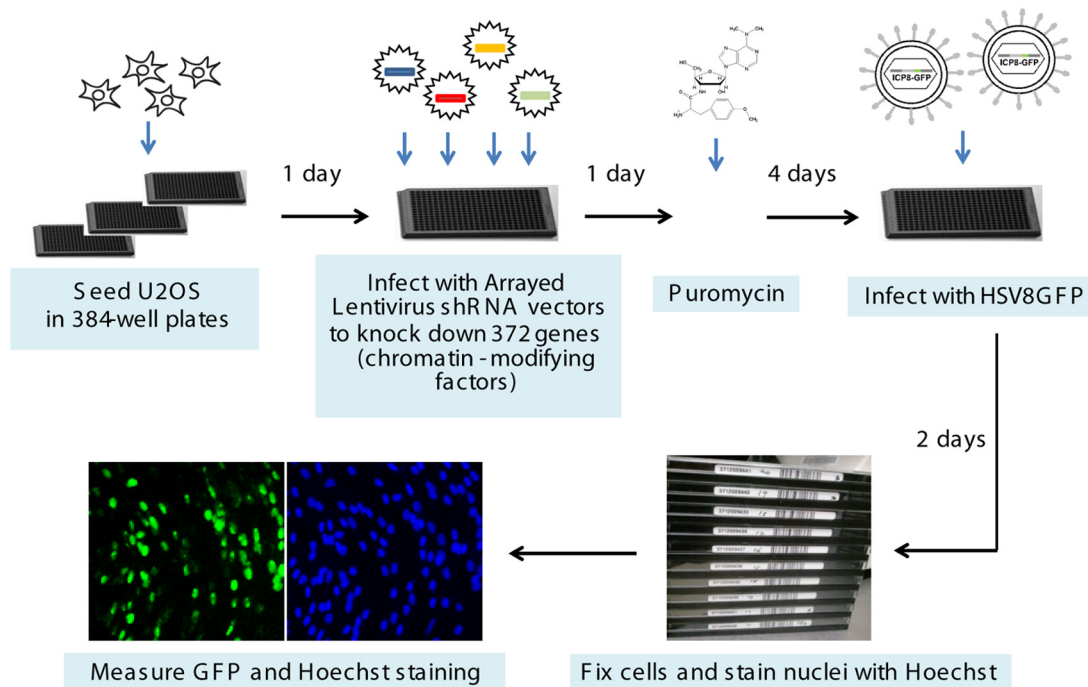


FIG 2 Schematic diagram of the shRNA and high-content imaging screen. U2OS cells were seeded in 384-well plates and incubated for 1 day at 37°C. Lentiviruses expressing shRNAs were added to the U2OS cells, and puromycin was added the next day. Four days later, the cells were infected with HSV8GFP and incubated for 2 days. The cells were fixed, nuclei were stained with Hoechst, and GFP fluorescence was measured with an automated Cellomics high-content screening and analysis system.

with VP16 and ICP4 in other cells are likely involved in regulation of HSV gene expression in U2OS cells.

Targeted chromatin factor RNAi and high-content imaging screen. To identify U2OS cell-specific epigenetic factors, we performed a targeted chromatin factor RNAi and high-content imaging screen using U2OS cells infected with a recombinant HSV, HSV8GFP, which expresses the viral ICP8 protein fused to green fluorescent protein (GFP). We measured GFP fluorescence in HSV8GFP-infected cells to monitor viral gene expression, similar to a previous small-molecule screen (28). To deplete host epigenetic factors, we used an arrayed lentivirus-expressing short hairpin RNA (shRNA) library (The RNAi Consortium, Broad Institute) targeting 372 host chromatin-modifying factors for this screen (see Table S1 in the supplemental material). This library consists of 1,846 individual shRNAs targeting human genes (shChromatin), with 4 or 5 unique shRNA sequences designed to target the coding or 3' untranslated region sequence of each gene. We also included shRNAs targeting GFP as a positive control (shGFP) and shRNAs targeting RFP, luciferase, and *lacZ* as negative controls (shNegControl). For the screen, which is summarized in Fig. 2, we seeded U2OS cells in 384-well plates and infected them the next day with the lentivirus vectors expressing shRNAs. Lentivirus-infected cells were selected in puromycin-containing medium for 4 days and then infected with the reporter virus HSV8GFP. At 2 days postinfection (dpi), the cells were fixed and their DNA was stained with Hoechst dye to allow visualization of all nuclei. We used a Cellomics automated high-content imaging system to identify individual cells in each well by Hoechst staining and then to measure the GFP intensity per nucleus. The Cellomics software then calculated the average GFP intensity per cell and the percentage of GFP-positive cells in each well.

To validate the screen, we first examined the distributions of the average GFP intensity values and percent GFP-positive cells in the shNegControl wells, the shGFP positive-control wells, and shChromatin wells (see Fig. S1A in the supplemental material). As expected, shGFP lentivirus-infected wells showed decreased average GFP intensity and percent GFP-positive cells relative to the shChromatin-treated wells. In contrast, cells expressing shNegControl showed no change in GFP expression (see Fig. S1A). These results validated the ability of the screen to detect changes in GFP expression due to shRNA activity.

We sought to identify genes with direct effects on viral gene expression but not genes that produced indirect effects on the GFP signals due to influence on host cell viability, proliferation, and/or density. To this end, we calculated *Z* scores for the average GFP intensity and percent GFP-positive cells. The *Z* scores were determined as $(x - m)/SD$, where *x* is the average GFP intensity or percent GFP-positive cells for an individual shRNA, *m* is the average GFP intensity or percent GFP-positive cells for the complete shChromatin data set, and SD is the standard deviation of its respective *m* value (see Table S1 and Fig. S1B in the supplemental material). We examined whether the per-cell average GFP intensity or the percent GFP-positive cells was correlated to cell number and found that neither of these metrics exhibited a strong dependence on cell number (see Fig. S1B in the supplemental material). We also evaluated the correlation of *Z* scores between average GFP intensity and percent GFP-positive cells (Fig. 3A). Linear regression analysis showed that the slope was 0.8705 ± 0.01176 (Pearson $r = 0.8632$; $P < 0.0001$), indicating a strong correlation between the average GFP intensity and percent GFP-positive cells.

We then used the two *Z* scores, the number of shRNAs for the specific gene that showed the effect, and the cell number per well

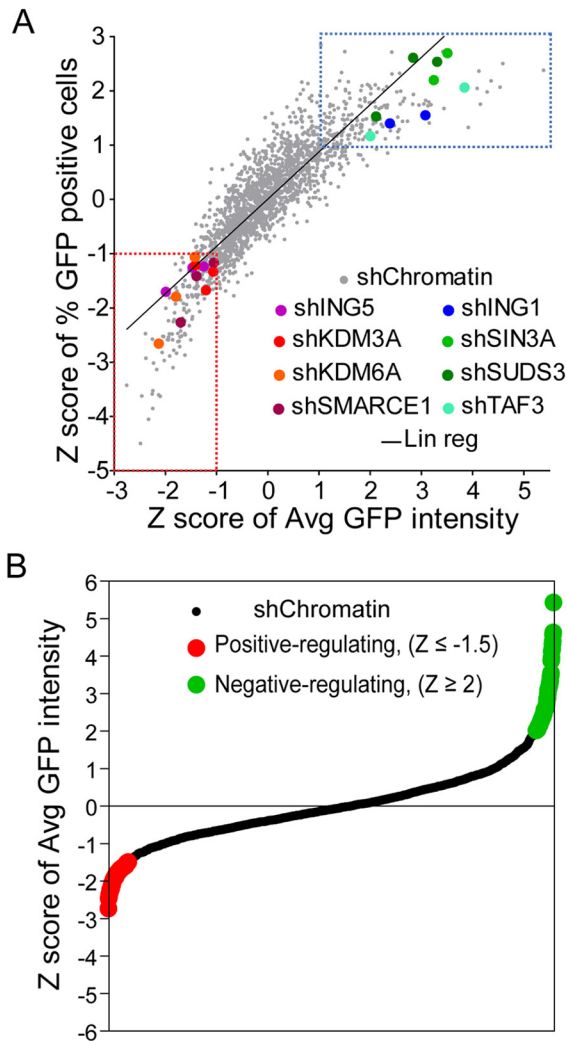


FIG 3 Results from a targeted screen of chromatin factors. The average GFP intensity and percent GFP-positive cells for each sample were analyzed to determine the positively and negatively regulating hits. (A) Relationship between Z scores for average GFP intensity and percent GFP-positive cells. The values for average GFP intensity and percent GFP-positive cells for the shChromatin wells (gray dots) are shown. Selected positively regulating (including weak hits, $Z \leq -1$) and negatively regulating (including weak hits, $Z \geq 1$) hits from the shChromatin shRNAs are shown in red and blue dotted boxes, respectively. (B) Distribution of Z scores. Red dots represent strong positively regulating hits ($Z \leq -1.5$), and green dots represent strong negatively regulating hits ($Z \geq 2$).

(the latter to compensate for toxic effects of shRNAs) to prioritize the results from the screen. Strong hits were defined as those having Z scores for average GFP intensity of ≥ 2 or ≤ -1.5 and Z scores for percent GFP-positive cells of ≥ 1 or ≤ -1 for wells containing more than 2,000 cells (Fig. 3B). Weak hits were also defined as those having Z scores for both average GFP intensity and percent GFP-positive cells of ≥ 1 (Fig. 3A, blue dotted box) or ≤ -1 (Fig. 3A, red dotted box) for wells containing more than 1,430 cells, which is approximately twice the SD of the complete shChromatin set for negatively regulating or for positively regulating hits, respectively. We applied a stricter cutoff to select strong negatively regulating hits, because U2OS cells are known to be

more permissive than many other cell lines for HSV replication. Table 1 shows the top hits with 2 or more shRNAs from strong hits with positive or negative regulation effects and 3 or more shRNAs from weak hits (Fig. 3A and B). These criteria resulted in 4 strong and 7 weak positively regulating hits and 6 strong negatively regulating hits (Table 1).

Proteomic network and interactome analysis. We next performed an interactome analysis to determine whether the epigenetic factors identified in our screen were part of known functional complexes. We first generated protein-protein interaction maps by analyzing the selected hits in Table 1 in the GeneMANIA human interactome database in Cytoscape (Fig. 4). We also investigated individual hits that did not generate protein-protein interactions from the interactome analysis using UniProt, GeneCards, and BioGRID protein and genetic databases to identify their binding partners and their functional properties. Several different types of chromatin modifying complexes were identified (Fig. 4).

Chromatin remodeling complexes. Interestingly, 4 of the 11 positive regulators, PHF10, ARID1A, BAZ1B, and SMARCE1, are members of SWI/SNF-type complexes (Fig. 4, top), which are nucleosomal or chromatin-remodeling complexes. Notably, the hSNF2H subunit of the ISWI chromatin remodeling complex that was previously shown to promote HSV-1 replication in Hep-2 cells (11) was not one of the hits.

Histone demethylases. Two of the positive regulators identified were lysine demethylases, KDM3A and KDM6A (Table 1). KDM3A removes dimethyl and monomethyl modifications from histone H3 lysine 9 (H3K9me1/2) (23). Another lysine demethylase, KDM1A (LSD1), which has the same substrate specificity as KDM3A and was previously shown to regulate HSV-1 gene expression (7), was not observed as a hit in our study, either because it is not critical in U2OS cells or because the shRNAs did not sufficiently reduce its expression levels to block HSV gene expression. KDM6A removes the trimethyl modification of histone H3 lysine 27 (H3K27me3) (29), which raises the possibility that H3K27me3 is associated with lytic viral chromatin as well as latent viral chromatin (30, 31).

Histone acetyltransferases. ING5 is a subunit of the H4-specific acetyltransferase (HAT) HBO1 and the H3-specific MOZ/MORF HAT complex (32). ING5 interacts with p300 and acetylates p53, implying a tumor-related function (33). The CLOCK HAT, which was shown to complement *ICP0* mutant viruses in Hep-2 cells (13), was not one of the hits in our screen.

Other positive regulators. METTL17 is a member of the methyltransferase-like family. HDAC7 is a class II histone deacetylase, and HOXD3 is a member of homeobox family of highly conserved site-specific transcription factors. The prior knowledge about these factors does not provide a specific mechanism by which they might positively regulate HSV gene expression.

Histone deacetylase (HDAC) complexes. Of the genes with negatively regulatory effects, three hits are part of HDAC complexes as determined by proteomic network analysis (Fig. 4, bottom). Sin3A and SUDS3 are core subunits in a histone deacetylase (HDAC1/2) complex, and ING1 interacts with Sin3A (34). The ING1-lamin A interaction is critical for cellular gene regulation (35), and interestingly, depletion of lamin A/C increases viral heterochromatin and reduces HSV replication (36).

TBP-associated factors. Surprisingly, two of the down-regulating factors, TAF1 and TAF3, were TBP-associated factors

TABLE 1 Top hits of epigenetic factors regulating HSV-1 in U2OS cells

Z score	No. of shRNAs	Gene	Gene product description
Positively regulating genes			
≤ -1.5 (strong)	2	HDAC7	Histone deacetylase 7A
		HOXD3	Homeobox protein HoxD3
		KDM6A	Lysine (K)-specific demethylase 6A
≤ -1 (weak)	4	PHF10	PHD finger protein 10
	3	METTL17	Methyltransferase-like 17
		ARID1A	AT-rich interactive domain 1A (SWI like)
		BAZ1B	Bromodomain adjacent to zinc finger domain, 1B
		JMJD8	Jumonji domain containing 8
		ING5	Inhibitor of growth family, member 5
		KDM3A	Lysine (K)-specific demethylase 3A
		MARCE1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin e1
Negatively regulating genes			
≥ 2 (strong)	3	SUDS3	Sin3 histone deacetylase corepressor complex component SDS3
	2	ING1	Growth inhibitor ING1/tumor suppressor ING1
		PDS5B	PDS5, regulator of cohesion maintenance, homolog B
		SIN3A	SIN3 homolog A, transcriptional regulator/transcriptional corepressor Sin3A
		TAF1	TBP-associated factor 1, 250 kDa
		TAF3	TAF3 RNA polymerase II, TATA box-binding protein (TBP)-associated factor, 140 kDa

(TAFs) (Table 1; Fig. 4, bottom), usually thought to be transcriptional activating factors. TAF1 and TAF3, previously shown to bind to HSV-1 ICP4 *in vitro* and in mass spectrometry analysis (37–39), may therefore play a role in ICP4’s negatively regulatory properties.

Other negative regulators. ING1 was a negatively regulating hit in our screen. Human ING1 proteins are known to differentially regulate histone acetylation (40). Another negative regulator, PDS5B, is a chromatin cohesion protein (41).

Validation of shRNA hits using siRNA-mediated knock-down. To validate our primary screen hits, we depleted U2OS cells of selected chromatin factors using double-stranded siRNA treatment, and we then infected the cells with WT HSV-1 and measured viral gene expression using Western blot detection of the viral ICP8 protein. We focused on 4 upregulating chromatin factor hits, KDM3A, KDM6A, ING5, and SMARCE1, and 4 down-regulating hits, ING1, TAF3, SIN3A, and SUDS3, from our primary screen. When we transfected U2OS cells with ON-TARGETplus SMARTpool siRNAs (Thermo-Fisher Scientific) prior to HSV-1 infection, we observed that the siRNAs specific for KDM3A, ING5, SMARCE1, TAF3, SIN3A, and SUDS3 reduced the target protein levels efficiently (see Fig. S2A in the supplemental material). KDM6A and ING1 protein levels could not be evaluated by immunoblotting due to the lack of specific antibodies, so we measured the transcript levels of these genes using quantitative real-time PCR (Fig. 5A). The *ING5* RNA level was reduced by 40 to 60%, and transcripts of the other genes were reduced by more than 70% without any significant toxicity. To evaluate their effects on viral gene expression, siRNA-transfected U2OS cells were infected with wild-type HSV-1 strain KOS at a multiplicity of infection (MOI) of 0.1 PFU/cell and harvested at 0, 8, and 14 hpi. siRNA knockdown of KDM3A, KDM6A, ING5, or SMARCE1 reduced ICP8 expression levels by 50% at 14 hpi (Fig. 5B; also, see Fig. S2A in the supplemental material), which was consistent with the positively regulatory effects observed in our primary screen. Knockdown of SUDS3 increased ICP8 expression levels (1.5-fold; $P = 0.0231$), consistent with the negatively regulatory effects seen

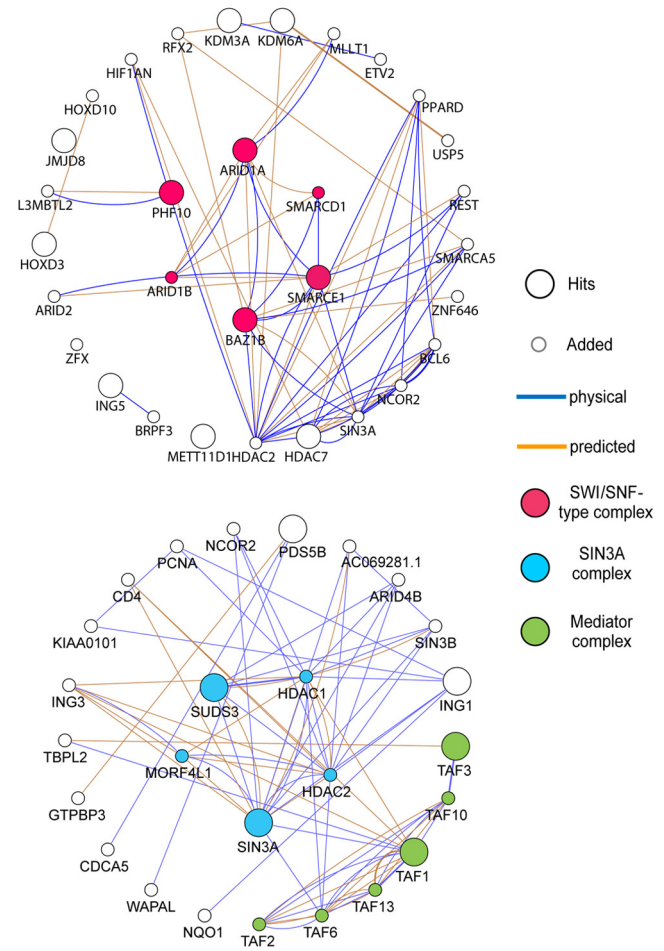


FIG 4 Protein-protein interaction network maps for the major hits. Protein-protein interaction networks of positively regulating (top) or negatively regulating (bottom) hits were generated using GeneMANIA in Cytoscape. Extra 20 proteins (small circles and dots) that have known physical (blue lines) or predicted (yellow lines) interactions with the input hits are shown to generate the network maps.

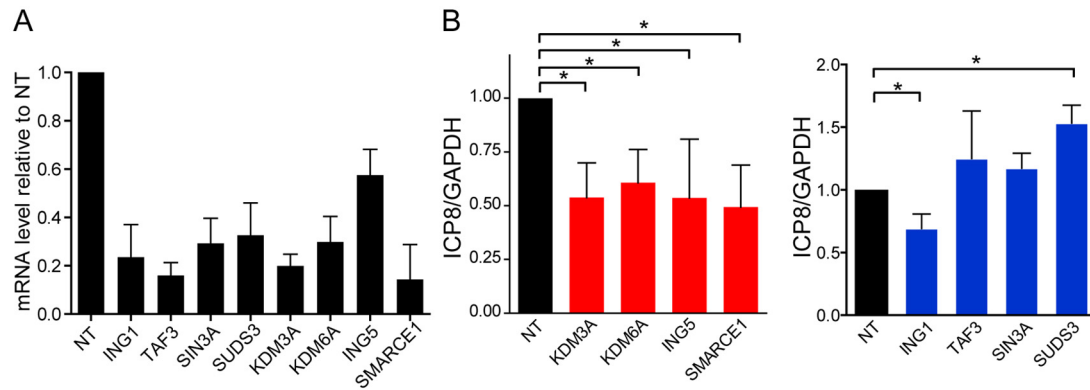


FIG 5 Validation of selected hits using siRNA-mediated knockdown followed by HSV-1 infection. (A) Depletion of chromatin factors using siRNAs. Nontarget control (NT) and KDM3A-, ING5-, SMARCE1-, TAF3-, SIN3A-, and SUDS3-targeting siRNAs were transfected into U2OS cells, and transcript levels of the indicated genes in siRNA-transfected cells were determined by qRT-PCR. (B) Effects of chromatin factor depletion on viral gene expression. U2OS cells were infected at an MOI of 0.1 with WT HSV-1, and ICP8 expression levels were determined in cell extracts prepared at 14 hpi using immunoblotting. ICP8 levels were quantified using ImageJ or Image Studio Lite (LI-COR). The ICP8 levels were normalized to GAPDH and the NT siRNA values. Values are from more than three independent experiments, and values that are statistically significantly different from the NT value are indicated (*, $P \leq 0.05$ [two-tailed paired *t* test]).

in the screen. Knockdown of TAF3 or SIN3A showed a trend toward increased ICP8 expression, but the effects were not statistically significant (Fig. 5B; also, see Fig. S2 in the supplemental material). Interestingly, knockdown of ING1 decreased the accumulation of ICP8, the opposite of the primary screen phenotype. Because we have not validated the knockdown of ING1 in our primary screen, it was possible that this discrepancy could result

from the knockdown efficiency or shRNA-mediated effects in the primary screen. In general, the siRNA knockdowns confirmed the results of the primary shRNA-containing lentivirus-mediated screen (Table 2).

Comparison of functional activity of KDM3A and KDM1A (LSD1) in HeLa and U2OS cells. To study in more depth the role of two apparently redundant enzymes in different cell lines, we

TABLE 2 Epigenetic factors regulating HSV-1 in different cell types^a

Cell type	Chromatin factor					Histone modification	Method	Reference
	Remodeling factor	HAT	HDAC	HMT	HDM			
HeLa	HIRA						RNAi	55
	Asf1a						RNAi	56
				KMT2F (SETD1A)		H3K4me3	RNAi	52
				KMT7 (SETD7/9)		H3K4me1	RNAi	52
					KDM1A (LSD1)	H3K9me1/2	RNAi, inhibitor	7
HepaRG	ATRX-Daxx ^b		ATRX-Daxx ^b				RNAi	25
	Asf1b						RNAi	57
Sensory neuron			REST			H3Kac	dnREST	45
U251-MG			CoREST-REST			H3Kac	RNAi	53
MRC5					KDM4A-D	H3K9me3	RNAi, inhibitor	10
HFF					KDM1A (LSD1)	H3K9me1/2	Inhibitor	10
					KDM4A-D	H3K9me3	Inhibitor	10
							RNAi	11
HEp-2	SMARCA5 (SNF2H)						RNAi	11
U2OS		CLOCK				H3, H4Kac	RNAi	58
	SMARCE1						RNAi	This study
	TAF3						RNAi	This study
		ING5				H3, H4Kac	RNAi	This study
		ING1 ^b		ING1 ^b		H3, H4Kac	RNAi	This study
				SIN3A-SUDS3		H3, H4Kac	RNAi	This study
				KDM6A	H3K27me2/3	RNAi	This study	
				KDM3A	H3K9me1/2	RNAi	This study	

^a Abbreviations: HAT, histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; HDM, histone demethylase.

^b Not classified.

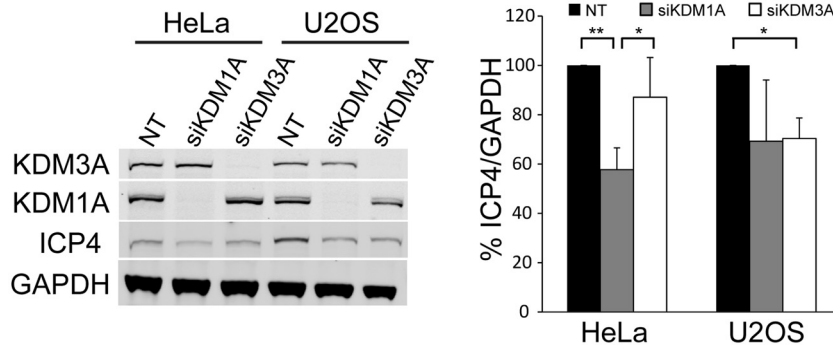


FIG 6 Comparison of HSV gene expression following depletion of KDM1A or KDM3A in HeLa and U2OS cells. (Left) KDM1A- or KDM3A-specific siRNA-transfected HeLa and U2OS cells were infected with WT HSV-1 at an MOI of 0.1, and ICP4 expression levels were determined by immunoblotting. (Right) ICP4 expression level was quantified using Image Studio (LI-COR) and normalized to the GAPDH value. Normal and depleted levels of KDM1A and KDM3A in each cell type were determined by immunoblotting using specific antibodies. Values are from more than three independent experiments, and comparisons that are statistically significant are indicated (*, $P < 0.05$; **, $P \leq 0.01$ [two-tailed paired t test]).

then focused on the utilization of KDM1A (LSD1) and KDM3A in two different cell lines, HeLa and U2OS cells. KDM3A and KDM1A (LSD1) both target H3K9me1/2 for demethylation (42); however, it is not clear how the activity of these functionally redundant methylases is regulated in different cell types. KDM1A (LSD1) promotes euchromatin on and expression of IE genes in HeLa and HFF cell lines (7, 10) but did not show a significant effect on ICP8 expression in our primary screen. However, depletion of KDM3A resulted in decreased HSV-1 gene expression in U2OS cells (Fig. 5B).

To compare the functionality of KDM1A (LSD1) and KDM3A in the different cell lines, we depleted KDM1A or KDM3A in HeLa or U2OS cells using siRNAs (Fig. 6, left), infected the cells with WT HSV-1, and measured IE ICP4 expression by Western blotting (Fig. 6, right). First, these blots showed approximately equal levels of KDM1A (LSD1) in HeLa and U2OS cells and approximately equal levels of KDM3A in HeLa and U2OS cells (Fig. 6, left). Second, depletion of KDM1A (LSD1) in HeLa cells reduced the ICP4 level by about 45% ($P < 0.01$) compared to that in cells transfected with nontargeting siRNAs (Fig. 6, right). In contrast, depletion of KDM3A in HeLa cells showed only a 10 to 15% reduction. Depletion of KDM1A (LSD1) in U2OS cells did not cause a statistically significant decrease in ICP4 expression, but depletion of KDM3A did cause a statistically significant decrease in ICP4 expression in U2OS cells (Fig. 6, right). These results are consistent with the idea that KDM1A (LSD1) is more essential in HeLa cells while KDM3A is equally or more critical in U2OS cells.

To confirm the apparent cell type-specific functionality of KDM1A (LSD1) using a different approach, we tested the effect of the monoamine oxidase inhibitors (MAOIs), tranylcypromine (TCP) and pargyline, which are known KDM1A (LSD1)-specific inhibitors (7), on HSV gene expression in HeLa and U2OS cells. Treatment of HeLa cells with TCP reduced ICP4 expression in HeLa cells (Fig. 7A; also, see Fig. S3 in the supplemental material), which was consistent with previous reports (7, 10), but TCP treatment had no effect on ICP4 expression in U2OS cells. Pargyline treatment of HeLa cells similarly reduced ICP4 expression in HeLa cells (Fig. 7B; also, see Fig. S3 in the supplemental material) but led to an increase in ICP4 expression in U2OS cells. Similar to the protein levels, TCP treatment reduced *ICP4* transcripts in HeLa cells (Fig. 7C; also, see Fig. S3) but led to increased levels of *ICP4*

transcripts in U2OS cells. Similar effects were observed with *ICP8* RNA levels in HeLa and U2OS cells (Fig. 7D). These results confirmed that KDM1A (LSD1) is more critical for HSV gene expression in HeLa cells than in U2OS cells.

DISCUSSION

HSV-1, like other nuclear DNA viruses (43), is subject to and manipulates the host cell epigenetic pathways to promote its own gene expression during lytic infection. The viral VP16 and ICP0 proteins have been shown to combat host-cell silencing mechanisms in normal human cells (3, 5, 12). The dependence on VP16 and ICP0 differs in different cell types (14, 15, 24), suggesting that the host epigenetic factors that are functional in these different cell types may differ. We tested this hypothesis by conducting a screen to identify the epigenetic factors that regulate HSV gene expression in U2OS osteosarcoma cells, which complement *ICP0* mutant virus replication. We found that the specific epigenetic factors that regulate HSV-1 gene expression in U2OS cells are different from those previously shown to be critical in other cell types. Furthermore, HSV-1 gene expression was differentially sensitive to a drug that inhibits a specific histone demethylase in U2OS cells compared with HeLa cells. There appeared to be equivalent levels of KDM1A (LSD1) and KDM3A proteins expressed in U2OS and HeLa cells, indicating that there are different activities of these enzymes in the cells. These results demonstrate the need to probe the functionality of epigenetic factors rather than just their levels of expression. Furthermore, these results argue that although HSV can replicate in many cell types, different epigenetic mechanisms may be important for facilitating viral gene expression and replication in these cells.

Classes of chromatin modifiers and HSV-1 replication. HSV-1 gene expression has been shown to be regulated by several classes of host chromatin modifiers, including histone demethylases (e.g., KDM1A [LSD1]) (7, 10), histone methyltransferases (e.g., KMT2F [SETD1A]) (44), histone deacetylases (e.g., CoREST complexes with HDAC1) (45), and chromatin-remodeling factors (e.g., hSNF2H) (11). In this screen we identified the same general classes of chromatin modifiers in U2OS cells, but the specific chromatin factors were different.

Histone demethylases. Previous studies had found that the KDM1A (LSD1) and KDM4 (JMJD2) demethylases are required

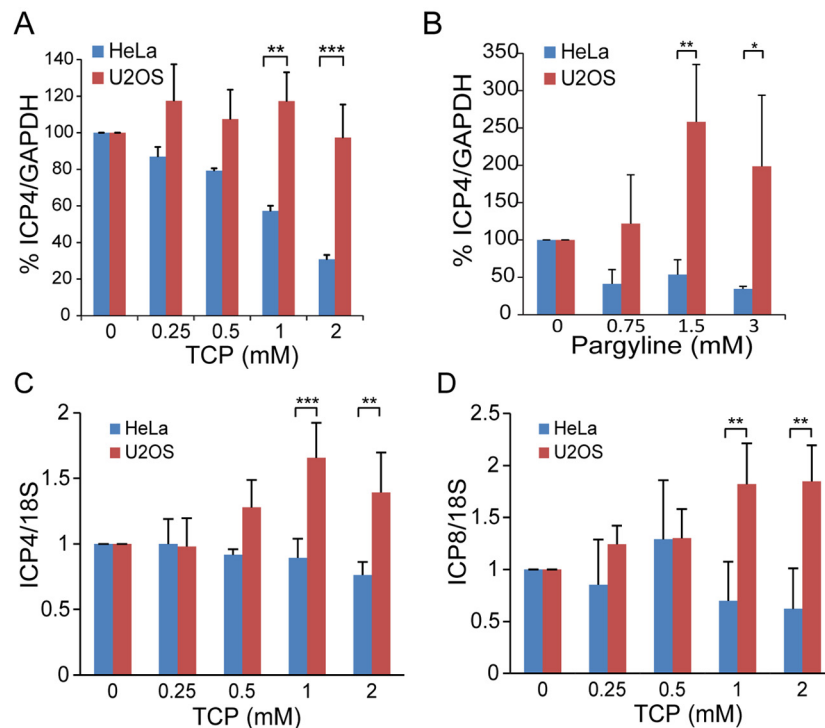


FIG 7 HSV gene expression in the presence of KDM1A (LSD1) inhibitors in U2OS and HeLa cells. (A) Effects of TCP on viral gene expression in HeLa and U2OS cells. HeLa and U2OS cells were pretreated with the indicated concentrations of TCP for 4 h, infected at an MOI of 0.1, and harvested at 4 hpi. The same concentrations of TCP were maintained until the cells were harvested. ICP4 expression was quantified relative to GAPDH. (B) Effects of pargyline on viral gene expression in HeLa and U2OS cells. HeLa and U2OS cells were pretreated with the indicated concentrations of pargyline for 4 h, infected at an MOI of 0.1 and harvested at 4 hpi. The same concentrations of pargyline were maintained until the cells were harvested. ICP4 expression was quantified relative to GAPDH. (C and D) Effects of TCP on viral RNA expression. ICP4 (C) and ICP8 (D) transcript levels were quantified using qPCR at 4 hpi under the conditions described above. Values that are statistically significantly different at the same concentration of TCP treatment are indicated (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; two-way analysis of variance [ANOVA] with Bonferroni posttest).

for HSV-1 IE gene expression in HeLa cells and human fibroblasts (7, 10), with KDM4 demethylating HeK9me3 and KDM1A LSD1 demethylating H3K9me1/2. In our screen, we found that KDM3A was required for HSV gene expression in U2OS cells. KDM3A has the same substrate specificity as KDM1A (LSD1) (42). Therefore, this result highlights the requirement for one or the other of these demethylase activities and strengthens the idea of the necessity of demethylation of H3K9 for expression of HSV-1 gene expression, although U2OS and HeLa cells likely differ in the KDMs used. KDM1A (LSD1) is recruited by HCF-1 to the HSV genome for this function, but we do not know if KDM3A is recruited to the HSV genome or if KDM3A is responsible for directly regulating HSV gene expression.

We found that KDM6A (UTX) is required for HSV-1 gene expression, presumably to remove the H3K27me2/3 heterochromatic marks from the viral chromatin. Although H3K27me3 heterochromatic marks have been found on latent HSV-1 genomes (30, 31, 46), there is no published evidence that this histone modification occurs during lytic infection. Recent studies in our laboratory have shown a transient H3K27 trimethylation of viral chromatin during HSV-1 infection of normal human fibroblasts (P. Raja, J. S. Lee, and D. M. Knipe, unpublished results). Further studies are needed to determine whether there is a direct role for KDM6A in HSV gene expression.

Histone acetyltransferases. Previous studies had shown the involvement of the CLOCK HAT (Table 2), which was shown to

complement *ICP0* mutant viruses in HEp-2 cells (13). The CLOCK HAT was not one of the hits in our screen, but in our studies we found that the ING5 and ING1 acetyltransferases are necessary for HSV-1 gene expression in U2OS cells. ING5 is a component of both the histone H4-specific acetyltransferase HBO1 and the histone H3-specific acetyltransferase MOZ/MORF complexes (32). However, we did not observe a significant effect on GFP signal following depletion of ING5-associated HATs (KAT6A, KAT7, and KAT8; KAT6B was not tested) in our screen. This might be due to inefficient knockdowns, a requirement for all or more than one to be knocked down together, or a role for ING5 in other complexes. Depletion of ING1 showed reduced ICP8 expression in our validation, which was opposite to the results from our primary screen. It should be noted that *ING1* expresses five isoforms and two of them, p33ING1b and p47ING1a, are shown to be functionally opposite. p33ING1b binds to CBP-p300 (HAT) and mSin3-HDAC1/2 and p47ING1a binds the mSin3-HDAC1/2 complex (34, 40, 47, 48). Although p33ING1b also interacts with the Sin3 repressor complex, overexpression of p33ING1b increases acetylation of histones H3 and H4, and conversely, overexpression of p47ING1a enhances deacetylation (40, 47–49). Therefore, the possibility of isoform-dependent effects needs to be further evaluated to clarify our results.

Histone deacetylases. Previous studies have shown that HDAC1 complexes with CoREST and REST (50, 51), and these complexes are disrupted and inactivated by HSV-1 ICP0, which

promotes HSV-1 gene expression (12). In this study, we found that the SIN3A-SUDS3-HDAC complex plays a role in repression of ICP8 expression in U2OS cells.

Chromatin-remodeling enzymes. A previous study from our lab had shown a requirement for the hSNF2H chromatin-remodeling complex subunit for efficient HSV-1 IE gene expression, and in particular for *ICP0* gene transcription, in human HEp-2 cells (11). In this study, we found a requirement for the SMARCE1 protein in U2OS cells. Therefore, a different chromatin-remodeling complex appears to be functional in these cells.

In total, our results show a different set of chromatin-modifying factors that are operative in U2OS osteosarcoma cells compared with cells previously studied. This study shows the power of an HSV-1 infection screen in the identification of the critical factors in these classes of chromatin remodelers. This system could be used to determine the chromatin factors that are functional in cells from cancer and other disease states.

Support for the idea of epigenetic regulation of HSV-1 gene expression. We observed that U2OS cells have the same major classes of epigenetic factors regulating HSV-1 gene expression as other cell types but that the specific members of these classes differ. This supports the general concept of epigenetic regulation of HSV gene expression during lytic infection (3) in which cellular functions attempt to silence viral genes and viral gene products recruit other cellular epigenetic factors to reverse and/or negate the host silencing response.

The mechanistic basis for the differential activity of KDM1A and KDM3A in HeLa versus U2OS cells remains to be defined. The two enzymes appear to be expressed at similar levels in the two cell lines, so a difference in expression levels is not the explanation. The enzymes may be mutationally altered in the different cells, or their activities may be regulated differently by other gene products. Further studies are needed to explain these differences.

Epigenetic drugs and HSV-1 replication. Consistent with the essential role of host epigenetic factors in HSV-1 gene expression and replication, pharmacological inhibition of the KMT2F (SETD1A) methyltransferase (52) or KDM1A (LSD1) and KDM4 (JMJD2) demethylases reduce IE gene expression, productive infection, and reactivation (7, 10). A key observation in this field was that the KDM1A (LSD1) inhibitors TCP and pargyline (7) inhibit IE gene expression of HSV in HeLa cells and in explant reactivation of latent HSV-1 (7). In this study, we also found that MAOI treatment reduced HSV gene expression in HeLa cells. However, MAOI treatment did not reduce IE gene expression in U2OS cells, supporting the hypothesis that different epigenetic factors are required to promote HSV-1 gene expression in the different cell lines. Surprisingly, the *ICP4* and *ICP8* RNA levels increased in U2OS cells as the MAOI concentrations increased. Depending on the specific proteins associated with it, KDM1A (LSD1) can play a role as an activator or repressor (7, 53), and it is possible that KDM1A (LSD1) has higher repressive activity than activating activity for HSV gene expression in U2OS cells directly and/or indirectly. This is of interest because KDM1A (LSD1) has been shown to be overexpressed in osteosarcoma tumor cells (54), and those investigators suggested that KDM1A (LSD1) is an epigenetic drug target in these cells. It is possible that *in vivo* conditions change the requirements for these HDMs. Further studies are needed to test the role of KDM1A in different cell types. In any event, our MAOI results obtained with U2OS cells were consistent with the screen

results showing a reduced KDM1A (LSD1) activator effect in this cell type. Functional studies like HSV-1 infection should be employed to show that KDM1A (LSD1) is truly functional in these cells and participates in a rate-limiting step in epigenetic regulation to justify the use of these drugs for these cancers.

HSV infection as an epigenetic screen. Our results suggest that HSV-1 infection can be used as a screen for the chromatin-modifying factors that are critical within a specific cell line or cell type. This could have two applications. First, an HSV infection epigenetic screen could test tumor cells or cells from other disease states to define the specific chromatin factors that are functional with these cells and thereby identify potential targets for chemotherapy in various disease states, including cancer. Second, an HSV infection screen may also provide an assay for the efficacy of epigenetic drugs in specific cell types as antiviral treatments for HSV, as has been proposed by others (7).

MATERIALS AND METHODS

Cells, viruses, and drug treatments. HeLa, U2OS, and Vero cells were obtained from the American Type Culture Collection (Manassas, VA). HSV8GFP was constructed in the HSV-1 KOS genetic background (8GFP is in the HSV-1 KOS1.1 genetic background). The HSV-1 KOS wild-type strain, the 7134 *ICP0*-null virus, 7134R rescued virus, the ICP4-null *n12* virus, the *in1814* VP16 insertion mutant, and *in1814R* rescued viruses were grown and used. See the supplemental material for more details.

For drug treatment experiments, the cells were pretreated with drugs at various concentrations for 4 h and infected with virus at the concentrations described above, and the medium was changed to Dulbecco's modified Eagle medium (DMEM)–1% calf serum containing the same concentration of the appropriate drug.

shRNA and high-content imaging screen. U2OS cells (450/well) seeded in 384-well plates were infected with lentivirus vectors (1.5 μ l/well). After 1 day, lentivirus-infected cells were selected using puromycin (1 μ g/ml; catalog no. P8833; Sigma-Aldrich) for 4 days followed by HSV8GFP infection (2.5×10^5 PFU/well). After 2 days, the cells were fixed, stained with Hoechst 33342 (catalog no. H3570; Invitrogen). The average GFP intensity per cell and the percent GFP-positive cells in each well were calculated as described in the supplemental material.

Interactome analysis. We generated functionally enriched protein-protein interaction networks for the up- and down-regulating hits in Table 1 using GeneMANIA (<http://genemania.org/>) in Cytoscape (<http://www.cytoscape.org/>). See the supplemental material for more details.

siRNA depletion of specific gene products. For the validation of the primary screen hits, we used ON-TARGETplus SMARTpool siRNAs (Thermo Fisher Scientific) to knock down expression of specific gene products. See the supplemental material for more details.

SDS-PAGE and immunoblotting. For details about the procedures used for SDS-PAGE and immunoblotting, see the supplemental material.

Quantification of RNA levels using reverse transcription and real-time PCR. DNase I-treated total RNA (0.5 μ g) was reverse transcribed and quantified by real-time PCR. RNA levels were normalized to that of 18S rRNA to adjust for recovery. See the supplemental material for more details.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01086-13/-/DCSupplemental>.

Figure S1, TIF file, 2.3 MB.

Figure S2, TIF file, 2.5 MB.

Figure S3, TIF file, 2.5 MB.

Text S1, DOCX file, 0.1 MB.

Table S1, XLSX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

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