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Article

The virus-induced upregulation of the miR-183/96/182 cluster and the FoxO family protein members are not required for the efficient replication of HSV-1

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Abstract: Herpes simplex virus 1 (HSV-1) expresses a large number of miRNAs, and their function is still not completely understood. In addition, HSV-1 has been found to deregulate host miRNAs, which adds to the complexity of regulation of efficient virus replication. In this study, we comprehensively addressed the deregulation of host miRNAs by massive-parallel sequencing. We found that only miRNAs expressed from a single cluster, miR-183/96/182 are reproducibly deregulated during productive infection. These miRNAs are predicted to regulate a great number of potential targets involved in different cellular processes and have only 33 shared targets. Among these, members of the FoxO family of proteins were identified as potential targets for all three miRNAs. However, our study shows that the upregulated miRNAs do not affect the expression of FoxO proteins, moreover these proteins were upregulated in HSV-1 infection. Furthermore, we show that the individual FoxO proteins are not required for efficient HSV-1 replication. Taken together, our results indicate a complex and redundant response of infected cells to the virus infection that is efficiently inhibited by the virus.

Keywords: herpes simplex virus 1; HSV-1; virus-host interaction; miRNA; FoxO;

1. Introduction

Viruses induce extensive deregulation of cellular biological processes and exploit their metabolism for efficient replication and spread. Such deregulation can include various strategies to optimize energy consumption (reviewed in [1]), subversion of defense mechanisms (reviewed in [2]), or signaling pathways (reviewed in [3]). It has also been observed that virus infection inevitably leads to perturbation of host microRNAs (miRNAs), which might affect any of the mentioned processes. miRNAs are small non-coding RNA molecules that posttranscriptionally regulate the expression of genes by pairing with the complementary nucleotides, usually within the three prime untranslated regions (3'UTR), of mRNAs [4]. The nucleotides from 2 to 8 of the miRNAs have an essential role in binding specificity to the target sites [5]. Multiple studies unveiled that viruses utilize host miRNAs to control a host, as well as their own, gene expression. For instance, the hepatitis C virus (HCV) shows a unique connection with miR-122 abundantly expressed in the liver which primes the environment for efficient virus proliferation and persistence within the host [6]. In particular, large DNA viruses such as herpes simplex virus 1 (HSV-

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1), have been found to encode their miRNAs, in addition to exploiting those of the host [7], [8], [9]. HSV-1 is a widespread human pathogen known as the causative agent of cold sores. In rare cases, HSV-1 can also cause life-threatening infections and diseases, such as encephalitis [10]. The primary site of infection is usually mucosal epithelium, where the virus enters cells and abundantly expresses its genes in a controlled cascade of gene expression, first immediate-early (IE), early (E), and late proteins (L), and finally leading to generation of new virions. Also, HSV-1 can infect nearby innervating sensory neurons and travel retrogradely from the peripheral axons to the neuronal body to establish lifelong latent infection. In contrast to the productive infection, during latency virus does not replicate and the only abundantly expressed transcripts are those arising from the latency-associated transcript (LAT) locus which gives rise to stable long non-coding RNAs (lncRNA) called LAT intron, and a number of microRNAs [11].

Occasionally, HSV-1 can reactivate and travel anterogradely to the periphery to re-enter the productive phase of infection and spread [10]. HSV-1 has been shown to express 29 mature miRNAs (vmiRNAs), all of which can be detected, at various levels, during the productive phase of infection. However, only a small subset of vmiRNAs has been detected in latently infected human neurons [12], [13] or latency models [14]. This includes vmiRNAs arising from the LAT region (miR-H2, -H3, -H4, -H5, -H7, and -H8) and miRNAs located just upstream of the LAT transcription start site (miR-H1 and miR-H6). The exact roles of vmiRNAs in HSV-1 infection are yet to be revealed, nonetheless, for some of them a role in the control of latency had been demonstrated [15], [16], [17], [11], [18], [19]. On the other hand, several studies have reported deregulation of host miRNAs after HSV-1 infection [20], and for some, the functional importance has been proposed. For example, miR-23a, miRNA upregulated after HSV-1 infection is decreasing the levels of interferon regulatory transcription factor 1 (IRF1), which in turn results in reduced levels of viperin, an important anti-viral gene, and thus facilitates virus infection [21], [22]. Likewise, it has been shown that miR-132 is induced in KSHV infection of lymphatic endothelial cells (LECs), as well as HSV-1 or HCMV infection of monocytes and it targets p300, a protein that associates with CREB and is an important mediator of antiviral immunity. By decreasing the levels of p300, the expression of IFN-β, ISG15, IL-1β, and IL6 is impaired, resulting in the suppression of antiviral immunity, and thus, facilitating viral replication [23]. Interestingly, miR-138, which is abundantly present in neurons, is limiting the expression of ICP0, an important IE viral protein crucial for efficient reactivation, thereby repressing the productive cycle and promoting latency in mice [24]. Additionally, miR-138 has been found to decrease the expression of host genes important for the virus replication, transcription factors Oct-1 and FoxC1, to create a suitable environment for latent infection. [25].

The miRNA cluster miR-183/96/182, which is transcribed as a single pri-miRNA transcript and further processed into three separate pre-miRNAs, has been shown deregulated in cells infected with different herpesviruses, including HSV-1, human cytomegalovirus (HCMV), and Epstein Barr Virus (EBV) [26], [27], [28]. In HSV-1 infected cells this cluster, which is suppressed by host protein ZEB, is regulated by ICP0 that directs host protein ZEB for ubiquitin-dependent proteasomal degradation, which in turn leads to derepression of miR-183/96/182 cluster [29]. However, the biological relevance of this deregulation is not known.

In this study, we comprehensively investigate host miRNAs expression during productive HSV-1 infection using massive parallel sequencing of miRNAs coupled with transcriptome analysis. We report that only miRNAs transcribed from the single cluster, miR-183/96/182 are reproducibly and significantly upregulated in HSV-1 infected HFFs. These miRNAs target the Forkhead box O (FoxO) family of transcription factors tightly regulated by the insulin/PI3K/Akt signaling pathway. We show that individual FoxO family protein members are not required for the efficient HSV-1 infection and that miR-183, -96, and -182 have a minor role in the regulation of these proteins.

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2. Materials and Methods

Cells and viruses

Human embryonic kidney cells (HEK293, CRL-1573), epithelial cells of the African green monkey kidney (Vero, CCL-81), primary human foreskin fibroblast (HFF), primary mouse embryonic fibroblast (MEF, generous gift of S. Jonjić, University of Rijeka) and human lung fibroblast (WI-38, CCL-75) were grown in Dulbecco's Modified Eagle Medium (DMEM) (PAN-biotech) supplemented with 10% fetal bovine serum (FBS; PAN-biotech), Penicillin / Streptomycin 100 µg / µl, 2 mM L-glutamine (Capricorn), and 1mM Sodium Pyruvate (Capricorn) at 37°C in the presence of 5% CO2. Mouse Bone Marrow-Derived Macrophages (BMDM; a generous gift from I. Munitić, University of Rijeka) were maintained in RPMI (Lonza) medium containing 10% of FBS, Penicillin / Streptomycin 100 µg / µl, and 2 mM L-glutamine. Human Neuroblastoma cells (SH-SY5Y, CRL-2266) were maintained in MEM (Minimum Essential Medium) with Earle's Salts (Capricorn), supplemented with 15% fetal bovine serum (FBS; PAN-biotech), Penicillin / Streptomycin 100 µg / µl, 2 mM L-glutamine (Capricorn) at 37°C in 5% CO2. Human retinal pigment epithelial cells immortalized with hTERT (hTERT RPE-1, CRL-4000) were maintained in MEM (Minimum Essential Medium) with Earle's Salts (Capricorn), supplemented with 10% fetal bovine serum (FBS; PAN-biotech), Penicillin / Streptomycin 100 µg / µl, 2 mM L-glutamine (Capricorn), 0.5 mM Sodium Pyruvate, 15 mM HEPES, 10uG/ml Hygromycin at 37°C in 5% CO2. HSV-1 strain KOS (kindly provided by DM. Coen, Harvard Medical School, Boston, USA) was prepared and titrated in Vero cells as previously described [29] and stored at -80°C. Mouse cytomegalovirus (MCMV) strain C3X (MCMV BAC pSM3fr cloned from MCMV Smith strain, ATCC VR-1399; kindly provided by Stipan Jonjić, Medical Faculty, University of Rijeka, Croatia) was prepared in MEF cells and infections were performed using centrifugal enhancement as previously described [30].

Infections and reagents

Cells were seeded a day before the experiment and infected with HSV-1 or MCMV at indicated MOI (multiplicity of infection) or MOCK infected (uninfected). One hour after infection the infectious medium was replaced with a fresh growth medium. Samples for protein or nucleic acids analyses were collected at indicated hours post-infection (h.p.i.). As indicated, specific inhibitors or reagents were added 30 minutes before infection and maintained during the time course of the infection. High molecular weight polyinosinic:polycytidylic acid (poly [I:C]), 100 µg/mL (Invivogen), and recombinant IFN-β (rIFN-β), 1 U/µL (PBL Biomedical Laboratories), 20 µM MG132 (Sigma) and 100 µM acyclovir (ACV) (Sigma), and Puromycin (ROTH) were used according to the manufactures instructions.

Plaque Assay

Virus titers were determined as previously described [29]. Briefly, confluent monolayers of Vero cells in 6 well plates were infected with serial 10-fold dilutions of the supernatant sampled from the HSV-1-infected cells and incubated with the infectious media for 1 h at 37°C. The infectious medium was then removed and the cells were overlaid with a methylcellulose solution (3% FBS, DMEM, 100 µg / µl Penicillin / Streptomycin, 1mM Na-Pyruvate in 1.2% methylcellulose) and incubated in 5% CO2 at 37 °C for 3 days. Cells were fixed using 5% (v/v) methanol/10% (v/v) acetic acid and stained with 5% Giemsa in PBS. The viral titers were determined as the number of plaque-forming units (PFU) / mL.

RNA extraction and quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from MOCK and HSV-1 infected cells using TRI Reagent solution (Ambion), according to the manufacturer's instructions. Total RNA samples were transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and levels of the transcripts were determined by RT-qPCR using FastStart Essential DNA Green Master (Roche) according to the manufacturer's instructions. The results were

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normalized to the expression of the 18S ribosomal RNA or mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and presented as the relative mRNA expression compared to 18S rRNA. The primers used were: FoxO1 - forward, 5′ –GCTTCCCACACAGTGTCAAGAC-3′; reverse, 5′ –CCTGCTGTCAGACAATCTGAAGTAC-3′; FoxO3 – forward, 5′ -GGGGAACTTCACTGGTGCTA-3′; reverse; 5′- TGTCCACTTGCTGAGAGCAG-3′; 18S rRNA – forward, 5′ –GTAACCCGTTGAACCCCATT-3′; reverse, 5′ –CCATCCAATCGGTAGTAGCG-3′; GAPDH-mouse – forward, 5′ –GGTGCTGAGTATGTCGTGGA-3′; reverse, 5′ –GTGGTTCACACCCATCACAA-3′.

For the analysis of miRNAs, total RNA was transcribed using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions and used as a template in following RT-qPCR reactions using TagMan Universal PCR Master Mix (Applied Biosystems). Specific TaqMan miRNA assays were used for the detection of human miR-23a-3p, miR-101-3p, miR-132-3p, miR-138-5p, miR-183-5p, miR-96-5p, miR-182-5p, let-7a-5p, and HSV-1 vmiR-H6-3p (Applied Biosystems). All reactions were performed in biological triplicates using LightCycler 96 (Roche).

RNA sequencing and data analysis

For the miRNA analysis, HFF cells were infected with HSV-1 and total RNA was extracted at 8 h.p.i. and 18 h.p.i. Small RNA libraries were generated using TrueSeq small RNA sample preparation kit (Illumina) according to the manufacturer's protocol and sequenced by single read sequencing (50 nt) using Illumina HiSeq 2500 sequencer (Institute Ruđere Bošković, Laboratory for Advanced Genomics). The total number of aligned sequence reads (read count) for each host and viral miRNA was normalized using read counts for human let-7a, the expression of which does not change during HSV-1 infection, or to the size of the library (RPM value) as previously described [14]. The sequence reads were analyzed with sRNAtoolbox, a versatile compilation of software for miRNA analysis [31], [32] as previously described [20]. Briefly, microRNAs were mapped using Bowtie and miRbase sequence database (release 22) annotations [33]. Of note, newly reported HSV-1 miRNAs, miR-H28 and miR-H29, are not represented within the miRbase but were included in the analysis. We were looking for reads that did not contain mismatches in the first 19 nucleotides but accepted all sequences that start at most 3 nt upstream and end at most 5 downstream of the reference sequence, and the minimum read length of input reads was 15. All sequence reads detected at least once in any condition were used for differential expression.

The transcriptome sequencing was performed using the TruSeq Stranded Total RNA Sample Preparation Guide (Illumina) as previously described [34]. Briefly, samples were rRNA depleted and fragmented, primed for first and second-strand cDNA synthesis, adenylated 3′ ends, adapters were ligated and DNA fragments were enriched. The libraries were validated, using the Agilent Bioanalyzer and sequenced on NextSeq 500 Illumina platform at Rudjer Boskovic Institute, Zagreb, Croatia. The sequencing quality was assessed using FastQC, and reads were mapped to the reference HSV-1 genome, strain KOS (GenBank accession number JQ673480.1), and Homo sapiens (human) genome assembly GRCh38 (hg38) from Genome Reference Consortium, using TopHat. Mapped reads were then visualized using Integrative Genomics Viewer (IGV). Finally, Cufflinks was used for differential expression and normalized expression levels were reported in FPKM (Fragments Per Kilobase Of Exon Per Million Fragments Mapped).

Protein extraction and Western Blot

To extract proteins, cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris (ph 8.0) and protease inhibitors (cOmplete, Roche)), mixed with 2x Laemmli buffer with β-mercaptoethanol – (Santa Cruz Biotech), and denatured for 6 minutes at 95 °C. Proteins were separated in 10% SDS–PAGE gels. and transferred onto a nitrocellulose membrane (Santa Cruz Biotech). Membranes were blocked in 5% w/v nonfat dry milk in 1× Tris-buffered saline (TBS) for 30 minutes at room

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temperature and incubated with the specified primary antibodies at 4 °C with gentle rotation overnight. Primary antibodies and dilutions used in experiments: α -actin (Millipore) - 1:10000, α -gC (Abcam) - 1:2000, α -ICP0 (Abcam) – 1:2000, α -ICP4 (Abcam) – 1:2000, α/β tubulin (Cell Signaling) - 1:1000, α -FoxO1 (Cell Signaling) - 1:1000, α -FoxO3 (Cell Signaling) - 1:1000, α -FoxO4/AFX-1 (Santa Cruz) - 1:100, α -P-FoxO1/3 (Cell Signaling) - 1:1000, α -P-FoxO3 (Cell Signaling) - 1:1000. Blots were washed for 30 minutes with TBS-0.05% Tween 20 (TBS-T) and primary antibodies were detected using horseradish peroxidase-conjugated goat α -rabbit or α -mouse secondary antibodies both diluted 1:2000 (Cell Signaling) and incubated at room temperature for 1h. Blots were again washed for 30 min and visualized using Amersham ECL reagent or SuperSignal West Femto Maximum Sensitivity Substrate (TermoFisher) and ChemiDoc MP (Bio-Rad).

CRISPR-Cas9 constructs and validation of knockout (-/-) cells

Three guide RNAs for each gene of interest were designed using the GPP sgRNA Designer tool (Broad Institute) [35], [36] based on the human reference GRCh38. The List of the guide RNAs (gRNA) sequences and constructs used in the study can be found in Table S1. Briefly, synthetic dsDNA oligonucleotides were directly cloned using BsmBI (New England Biolabs) into pLentiCRISPRv2 (Addgene) as previously described [37] and the cloned sequence was confirmed by sequencing (Eurofins). HEK293 cells were transfected with the generated plasmid, together with the lentiviral packaging system based on psPAX2 and pMD2.G [37] using Lipofectamine 2000 (ThermoFisher, Germany) according to the manufacturer's instructions. Lentiviral vectors carrying individual guide RNAs were collected from the supernatant, and vectors targeting the same gene were pooled, filtered, and added with polybrene at the final concentration of 8 μg/ml (Sigma) to the SH-SY5Y cells. Twenty-four hours after transduction, cells were washed in PBS and the selection media was added (DMEM 10% FSC supplemented with puromycin at 1 μg/mL). After establishing the resistant culture, the depletion of the targeted protein was confirmed by Western Blot analysis.

Statistical analysis

To analyze statistical significance, Student's t-test was used (GraphPad Prism software). A level of $p \leq 0.05$ was considered statistically significant. Level of statistical significance is marked with asterisks: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$. Non-significant samples were not highlighted on the graphs.

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3. Results

3.1. miR-183/96/182 cluster is upregulated in HSV-1 infected primary HFFs

To comprehensively address the deregulation of host miRNAs during productive HSV-1 infection and its potential biologically functions, we infected primary human foreskin fibroblasts (HFFs) at high MOI, extracted RNAs from mock-infected cells, and cells infected with HSV-1 strain KOS at 8 and 18 hours post-infection (h. p. i.), and analyzed the expression of miRNA by massive parallel sequencing. The obtained sequences were analyzed using sRNAtoolbox. In each experiment, we detected about 1500 human miRNA expressed at a wide abundance range, i.e. from 0.02 – 279598 reads per million (RPM) (Table S2). On the other hand, HSV-1 encoded miRNAs represented only a minor fraction of the total miRNA in the sample, i.e. 0.24% and 0.68% at 8 and 18 h.p.i., respectively (Figure S1), which is in accordance with the similar previous studies. Our differential expression analysis showed significant deregulation (fold change ≥2) of 134 human miRNAs at 18 h.p.i. (Figure 1A), however, only a small fraction of miRNAs were deregulated with a fold change ≥3. As expected, at 8 h.p.i. we did not observe significant deregulation of miRNAs compared to the mock, nonetheless a slight trend of deregulation of the same miRNAs that were significantly deregulated at 18 h.p.i. can be observed. To further validate the reproducibility of the sequencing approach, we compared this data set with an unrelated sequencing performed previously (Jurak et al. not published), using the identical virus strain (KOS) infecting the matching cell type (HFFs), under similar conditions (10 and 24 h.p.i.) and the same sequencing platform. Of note, the overall sequencing quality was comparable (i.e. total number of reads, the HSV-1 miRNA pattern, and the relative quantity) (Table S3). Surprisingly, the overlap between these two experiments was rather limited, i.e. only four miRNAs (miR-182, -183, -96, and -375) were found reproducibly upregulated and two miRNAs downregulated (miR-29 and -27a) in all experimental conditions (Figure S2). The fold change of the reproducibly deregulated miRNAs is shown in Figure 1B. These results suggest strong dependence on the cells used in the experiments and can explain observed discrepancies between different studies. Nonetheless, miRNAs expressed from a single transcriptional cluster, miR-183, -96, and -182 [38] were reproducibly upregulated and showed the highest level of deregulation, indicating its importance. The same cluster has been found upregulated in a limited study on two in vitro HSV-1 latency models (rat superior cervical ganglia derived neurons quiescently infected with HSV-1 GFP-US11 (Patton strain) and HFF quiescently infected with HSV-1 KOS strain) [29], but also in cells infected with other herpesviruses [27], [28]. Important to note, we detected only minor fluctuations (<1.5x) in the expression levels for several miRNAs previously shown significantly deregulated in HSV-1 infected cells [39], [23], [40], [41], [21], [42], [43], [44], [26], [29], [45], [46], [47]. As mentioned above, the use of different viruses, different cells, and conditions might explain this discrepancy.

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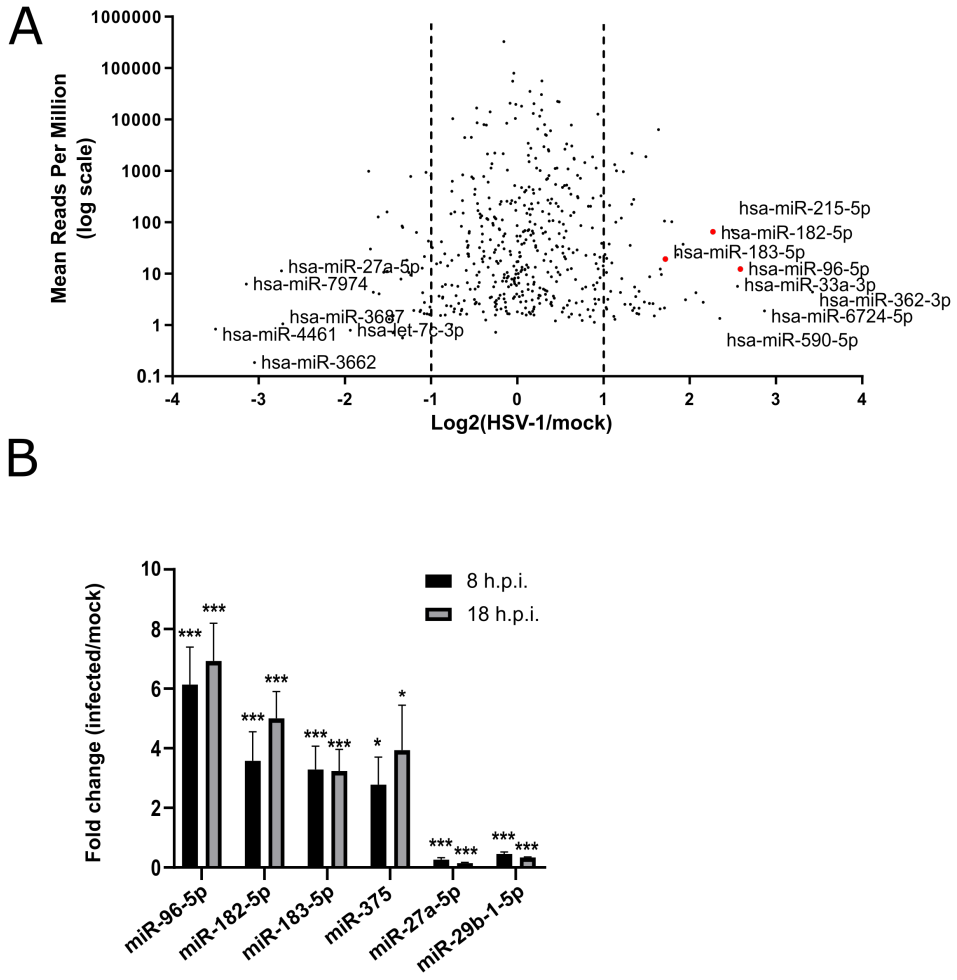

Figure 1. Host miRNAs are deregulated in HSV-1 infection. (A) Log2 value of 18 hours after infection over uninfected (mock-infected) samples shown as mean of reads per million. The most deregulated miRNAs are indicated, and the position of miRNAs of the miR-183/96/182 cluster is indicated with red dots. (B) Fold change of miRNA expression at 8 (black bars) and 18 (gray bars) hours after infection over mock-infected cells. Statistical significance is indicated with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

To additionally challenge the reproducibility of our sequencing results and to address upregulation of the miR-183/96/182 cluster in different cells, we infected two primary cells (i.e. human foreskin fibroblasts HFFs and mouse bone marrow-derived macrophages BMDM), WI38 cells (human lung fibroblasts), and two widely used transformed cell lines (i.e. human embryonic kidney cells HEK293 and human neuroblastoma cells SH-SY5Y) and analyzed the miRNA expression by the stem-loop-RT-qPCR at 8 and 18 or 24 h.p.i. In the analysis, we included additional four host miRNAs, miR-23a-3p, miR-101-3p, miR-132-3p, and miR-138-5p, previously reported deregulated and/or important for HSV-1 infection [21], [40], [43], [24]. Of note, the expression of HSV-1 late gene product, miR-H6 served as the indicator of the virus replication. Surprisingly, only miR-182, miR-96, and miR-183 were upregulated more than >2x, in both fibroblasts cells (HFFs and WI38; ~10x) (Figure 2A and 2E), but not in other cells (Figure 2B, C, D). Moreover, we observed only minor deregulation for all other miRNAs tested, previously reported deregulated. Thus, we selected the miR-183/96/182 as the main target for further functional analysis.

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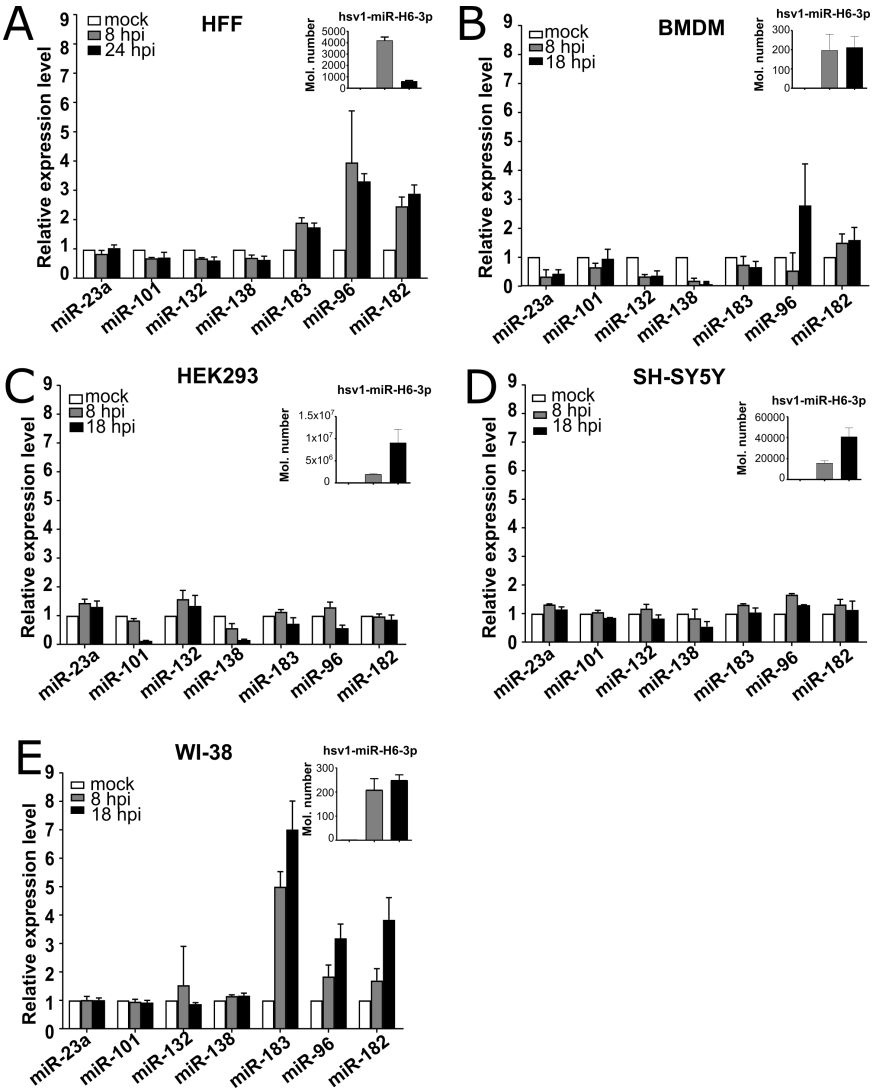

Figure 2. A host miRNA cluster miR-183/96/182 is upregulated in human fibroblasts. (A-E) Indicated cells (upper part of panels) were infected at an MOI of 10 and total RNA was extracted at indicated time points. The expression levels of miRNAs were determined by RT-qPCR using stem-loop specific assays and normalized to let-7a expression. The expression of vmiR-H6-3p is shown it the upper right corner of the panels.

3.2. miRNAs miR-183, -96, and -182 regulate a number of common targets

To determine potential targets of miR-183, -96, and -182, we implemented TargetScan 7.1, and miRDB, two widely used algorithms that predict mRNA targets [48], [49]. The prediction revealed a large number of highly conserved potential targets for each of these miRNAs (between 300 and 778 targets found by both prediction tools for each of miRNAs; not shown). Notably, only 33 potential targets were identified as shared by all three miRNAs, and these largely represent genes encoding proteins involved in the regulation of gene expression (Figure 3A and Table S4). Among these, FoxO1 was identified as a potential target for all three miRNAs, and two other members of the Forkhead box protein family, FoxO3 and FoxO4, were found as potential targets for miR-182 and miR-96, and solely miR-96, respectively. (Figure 3A). The binding sites for each miRNA of the cluster predicted by TargetScan are shown in Figure S3. Members of the FoxO protein family are well-known transcriptional factors that are regulated by the insulin/PI3K/Akt signaling pathway and are known to have many diverse roles in cell cycle progression, apoptosis,

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metabolism, differentiation, and oxidative stress resistance [50], [51], [52], [53], including roles in viral infections [54], [55], [56], [57], [58], [59], [60], [61], [62], [63], [64], [65]. The regulation of the FoxO family by miRNAs of the mir-183/96/182 cluster is well established and has been investigated in various cellular pathways, including modulation of the innate immune response in infections or autoimmune diseases, or in various cancers [66], [67]. Thus, we did not additionally validate this regulation in this study.

3.3. HSV-1 induces the expression and posttranslational modifications of FoxO protein family members

Based on the target prediction we hypothesized that the increase of miRNAs miR-96, -182, and -183 will consequently lead to decreased levels of the FoxO family members. To address this, we infected various cells with HSV-1 at an MOI of 1 and collected samples for the western blot and RNA analysis at different time points after infection. We observed an initial increase in FoxO1 and FoxO3 levels between 1 h.p.i. and 12 h.p.i. and a slight decrease later in infection, which could be explained by the increase of miRNAs late in infection (Figure 3B). FoxO4 levels were steadily increased through the time of infection (Figure 3B). Of note, the same dynamic pattern of the FoxO proteins (first upregulation followed by downregulation) was observed in all cell lines tested (HEK293, RPE1, HFF, SH-SY5Y; WI-38, not shown). However, surprisingly, we observed FoxO1 and FoxO3 downregulation in cells in which we did not observe upregulated levels of miR-183/96/182 during the time course of infection (e.g. HEK293 (not shown)), indicating additional mechanisms, other than miRNAs, for the depletion of FoxO family members late in infection (Figure 3C). Moreover, in cells infected and treated with different inhibitors, including cycloheximide (CHX, protein synthesis inhibitor) and actinomycin D (ActD, RNA synthesis inhibitor), or in cells infected with UV inactivated virus, we did not observe downregulation of FoxO proteins (Figure 3C), regardless of the upregulation of the miR-183/96/182 cluster. These results indicated that IE /E viral gene expression is required for the depletion of FoxO proteins. Interestingly, infection in the presence of acyclovir, i.e. a condition that allows expression of IE but prevents expression of L proteins and at the same time triggers the overexpression of the cluster, the depletion was not fully achieved. On the other hand, the addition of a proteasome inhibitor (MG132) 30 min prior to infection (*), but not 2 h.p.i. (**), successfully prevented the depletion. Taking together, these results indicate a role of IE protein ICP0, a well-characterized ubiquitin ligase, but also additional mechanisms including miRNAs and late virus functions.

Furthermore, by carefully analyzing the expression of FoxO protein members by Western blot, we observed the appearance of double bands, i.e. a shift of a few kilodaltons (kDa) during the time course of infection (Figure 3B), which might represent phosphorylated forms of proteins (i.e. inactive forms). Indeed, using phospho-specific antibodies we confirmed the phosphorylation of FoxO1 and FoxO3 (Figure 3B). Interestingly, the addition of acyclovir, an inhibitor of viral DNA replication and late gene expression prevented the shift of a few kDa of FoxO proteins. The observed phosphorylation is consistent with the previous study by Chuluunbaatar et al. who have found that FoxO1 can be phosphorylated by Akt kinase and viral kinase Us3.

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Taken together, we show that the virus is equipped with multiple mechanisms to control the expression and function of FoxO proteins and that miRNAs might have only a minor contribution to this.

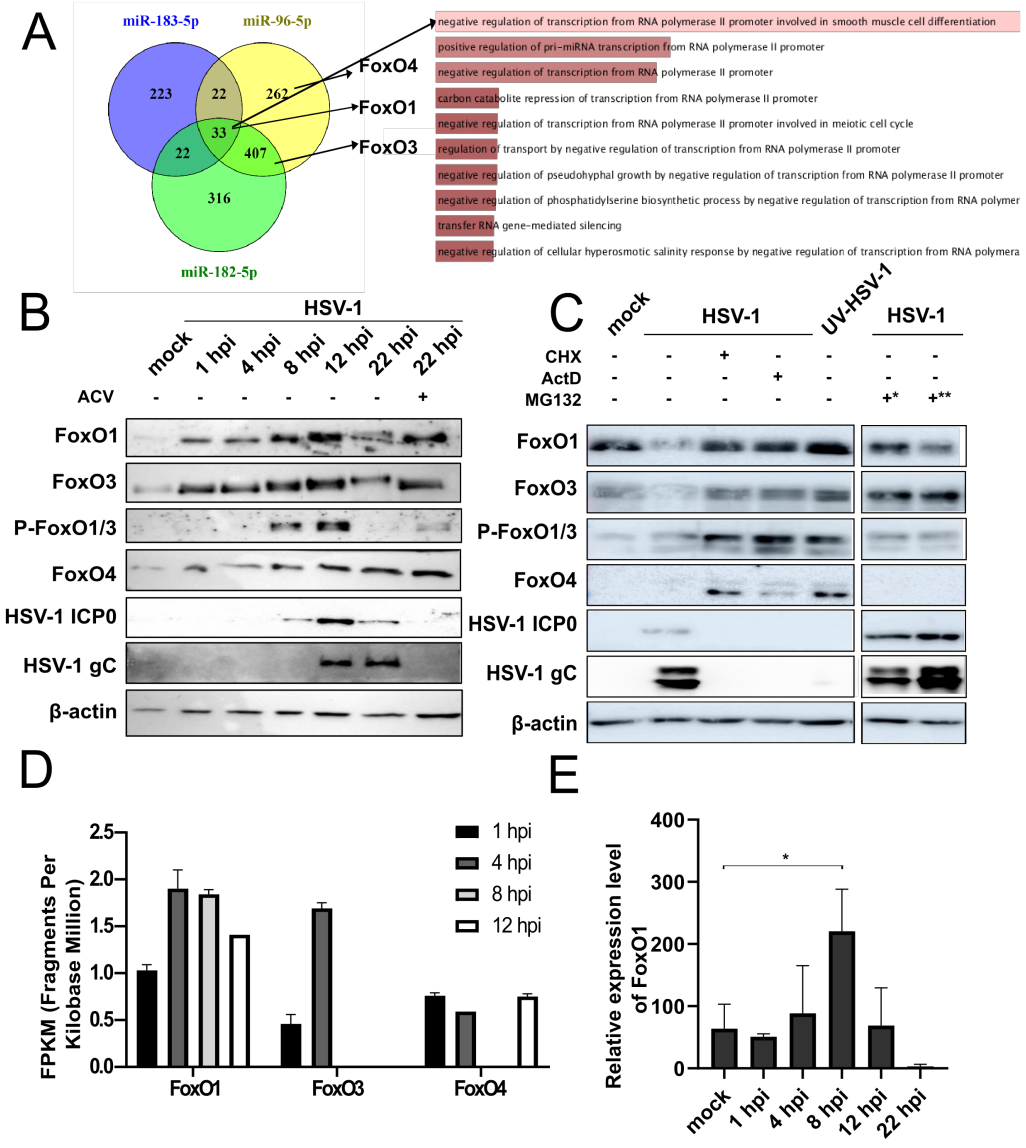

Figure 3. The members FoxO family are predicted targets of miRNAs of the miR-183/96/182 cluster and induced during productive HSV-1 infection. (A) The Venn diagram is showing the number of conserved targets for miR-183 (blue circle), miR-96 (yellow circle), and miR-182 (green circle). The predicted targets of the FoxO family are indicated with arrows. Gene ontology was determined by the Enrichr Gene Ontology analysis tool [68]. Genes with only poorly conserved sites are not shown. (B) RPE cells were mock-infected or infected with HSV-1, collected at indicated time points, and analyzed by western blot and RT-qPCR. ICP0, immediate-early viral gene, and gC, late viral gene. (C) As in (B), cells were mock-infected (mock), infected with HSV-1 (wt), or infected and pretreated with indicated inhibitors: cycloheximide (CHX), actinomycin D (ActD), proteasome inhibitor MG132 (*) 30 minutes before infection, or (**) 2 hours post-infection; or infected with UV-inactivated HSV-1 (UV-HSV-1). Proteins for the western blot were collected at 12 h.p.i. (D) Levels of FoxO1 and FoxO3 transcripts were obtained by total RNA sequencing and bioinformatics analysis. Results are normalized for sequencing depth and gene length and reported in Fragments Per Kilobase Million (FPKM). (E) The expression levels of FoxO1 mRNAs was determined by RT-qPCR and normalized

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to 18S rRNA expression. Statistical significance is indicated with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.4. Poly (I:C), IFN-β treatment, and infection with other herpesviruses increase the level of FoxO1 and FoxO3 proteins

We were intrigued by the increased levels of proteins of the FoxO family in cells infected with HSV-1, which indicates a possible transcriptional activation of FoxO genes. To address this, we analyzed the transcriptome of HSV-1 infected cells (Zubković et al. not published). Briefly, we infected HEK293 cells with strain KOS at MOI 10 and collected total RNA at 1, 4, 8, and 12 h.p.i. from two biological replicates for each time point. The sequencing libraries were prepared using the TruSeq Stranded Total RNA Sample Preparation Kit and (Illumina) and sequenced on the NextSeq 500 Illumina platform. The transcriptome analysis showed an increase of FoxO1 and FoxO3 mRNAs (Figure 3D) early in infection (4 h.p.i.), however, the results were somewhat inconclusive for the FoxO4 transcript due to the low number of the reads. The transcriptional activation of the FoxO family was additionally confirmed in a time-course experiment (Figure 3E) using RT-qPCR on the representative member of the family, FoxO1. Similar to the sequencing results we observed an increase early after infection (4 h.p.i.) and a sharp drop in mRNA level after 8 h.p.i.

To further investigate if the FoxO family induction is limited to HSV-1 infection or if it might represent a conserved host-response mechanism to infection with other herpesviruses, we studied the expression of FoxO members in cells infected with murine cytomegalovirus (MCMV). In brief, primary mouse embryonic fibroblasts (MEFs) were infected with MCVM strain C3X, and samples for protein and RNA analysis were collected at different times post-infection. Interestingly, we observed a strong upregulation of all tested members of the FoxO protein family (Figure 4A) during the time course of MCMV infection. Moreover, the analysis of RNA confirmed the transcriptional activation of FoxO genes (Figure 4B) similarly to HSV-1. Important to note, that although we observed an apparent increase in FoxO proteins phosphorylation, in contrast to HSV-1 infected human cells, we did not observe an obvious size shift (Figure 3B). At this point, we cannot explain this difference, but it might be related to the specificity of the antibodies or different activities of virus and/or cellular kinases.

Taken together, these results led us to a hypothesis that FoxO family members might be involved in intrinsic antiviral response and triggered in response to virus infection. For example, it has been found that FoxO1 promotes degradation of IRF3, and limits the IRF7 transcription [69], while FoxO3 has been found to target IRF7, and thus, negatively regulate virus-induced type I interferon (IFN) expression [70], [71], [72]. To test this hypothesis, we analyzed the expression of FoxO1 and FoxO3 in response to the immunostimulants poly (I:C) and IFN-β, which mimic viral infection and elicit an antiviral response, respectively. Indeed, stimulation of RPE1 cells with poly (I:C) resulted in gradual induction of both FoxO protein levels, with a peak of protein expression at 6 h post-stimulation (Figure 4C). On the other hand, stimulation with IFN resulted in a sharp, but very transient, increase in FoxO1 and FoxO3 immediately after stimulation (Figure 4D). The observed drop in FoxO protein levels 3h or 6h after stimulation with IFN-β is in agreement with the work of Litvak et al., showing that IFN-I limits the transcription of FoxO3 which is responsible for targeting IRF7, and thus, increasing the transcription of IRF7 for maximum antiviral response [70]. Regardless, the results indicate that FoxO family proteins are induced as a result of an immune response to viral infection.

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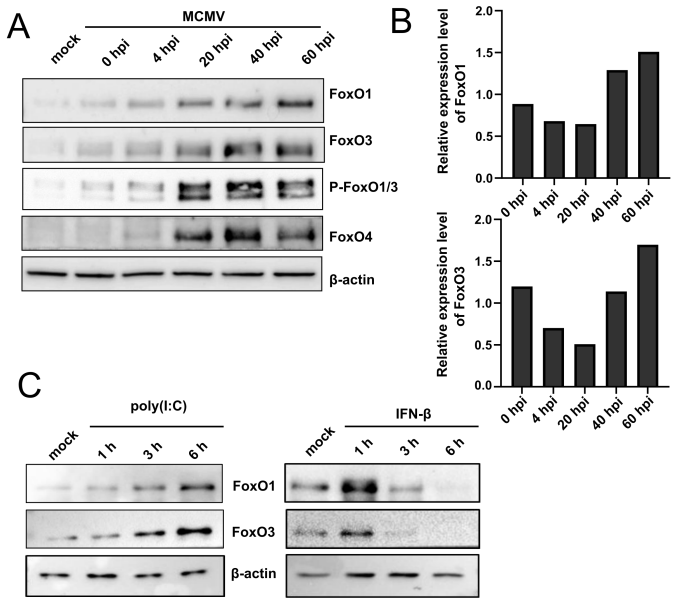

Figure 4. MCMV infection, IFN, and poly (I: C) induce the FoxO family of proteins. (A) Mouse embryonal fibroblasts (MEF) were mock-infected or infected with MCMV and samples for protein analysis were collected at indicated time points after infection (h.p.i.). (B) Cells were infected as in (A) and RNA samples were collected. The expression levels of FoxO1 and FoxO3 mRNAs were determined by RT-qPCR and normalized to 18S rRNA expression. (C) Proteins samples were collected from untreated RPE1 (MOCK) or cells treated with poly (I: C) (left panel) or IFN-β (right panel) at indicated time points after treatment and analyzed by western blot.

3.5. FoxO1 and FoxO3 are not required for efficient replication of HSV-1

To address the biological relevance of the miR-183/96/182 cluster upregulation in HSV-1 infection on the one hand, and the upregulation of FoxO family members on the other, we performed several functional assays. Firstly, we asked if the increased levels of miRNAs of the miR-183/96/182 cluster affect the virus replication. To test this, we transfected HFFs with mimics of miR-96, miR-182, and miR-183 or negative control mimics (NC), and 24 post-transfection we infected cells with HSV-1 at MOI of 5 (Figure 5A) and 0,001 (Figure 5B). To our surprise, we did not observe any obvious difference in virus replication (Figure 5A-B). Similarly, these miRNAs only had minor effects on viral infection in Neuro-2a cells using a high throughput assay in which cells were transfected with individual miRNAs and infected with HSV-1-luciferase-reporter virus (Pan et al., not published). These results led us to the conclusion that the upregulation of the miR-183/96/182 cluster has, at best, a minor role in HSV-1 replication in cultured cells.

Next, to investigate the role of FoxO proteins in HSV-1 infection we inactivated FoxO1, Foxo3, and FoxO4 (FoxO1 -/-, FoxO3 -/-, FoxO4 -/-) genes in SH-SY5Y cell using the CRISPR-Cas9 technology. Briefly, we generated guides using the GPP sgRNA Designer tool **[35], [36]** and selected the top 3 different candidate guides to target each of the genes. Several individual clones were analyzed for the expression of targeted genes and only the complete knock-down cells were used in further experiments. We successfully knocked out FoxO1 and FoxO3 proteins (a representative western blot Figure 5C); however, we were not able to generate FoxO4 deficient cells, or cells deficient for all FoxO proteins (not shown). Next, FoxO1 and FoxO3 deficient cells were infected at high and low MOIs, and virus replication was monitored through the time course of infection. Interestingly, we observed a slight decrease in ICP0 and gC expression in a high MOI experiment (Figure

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5C) at 18 h.p.i. However, we observed, at best minor, a difference in virus yield between individual FoxO deficient cells and negative control, at high (Figure 5D) and low (Figure 5E) MOIs. These results led us to the conclusion that individual FoxO proteins are not required for efficient HSV-1 infection. It is possible that FoxO family protein members have, at least to some extent, redundant functions and compensate for individual depletion.

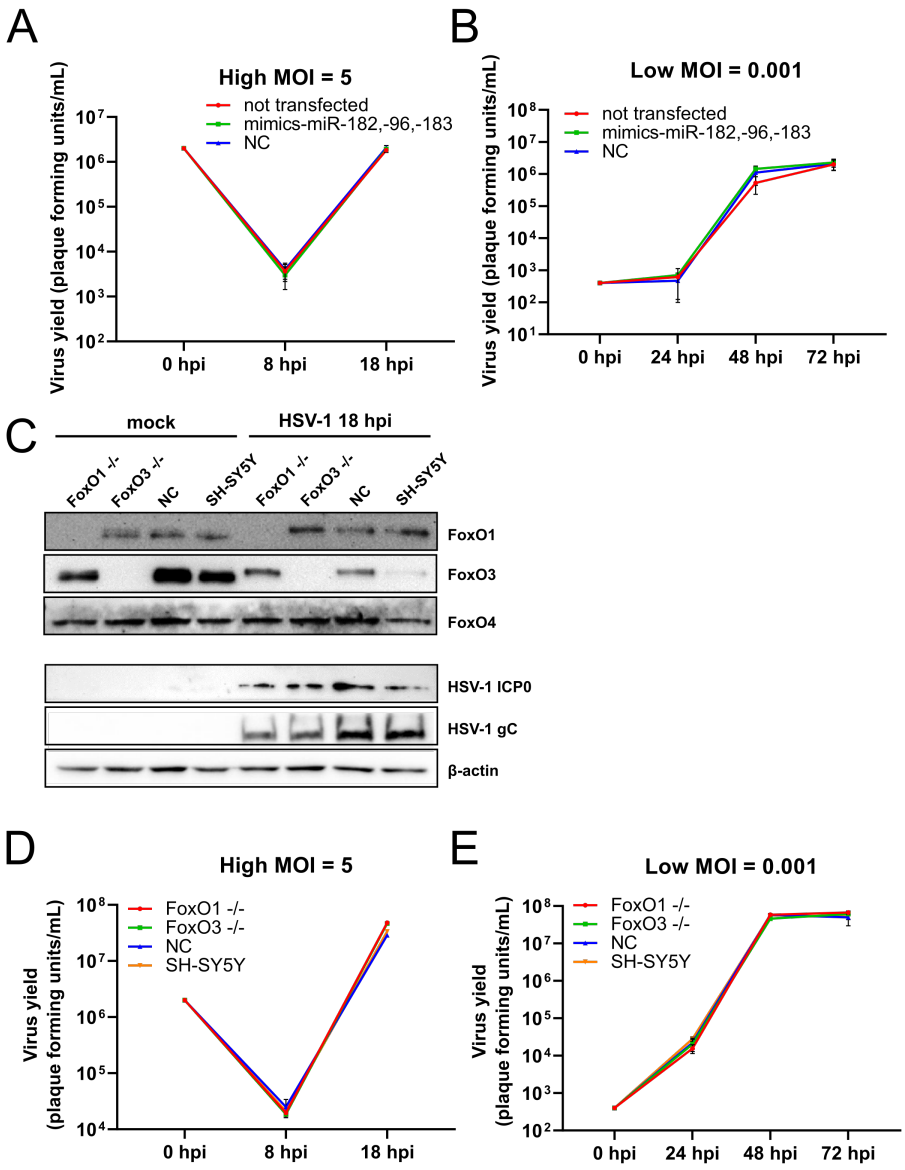

Figure 5. FoxO proteins are not required for HSV-1 replication. (A) HFFs were transfected with miR-182, -96, and -183 mimics or negative control mimic (NC) and after 24h infected with HSV-1 at an MOI of 5 (A) and 0,001 (B). Samples for titration were collected at the indicated time points and titrated on Vero cells. The virus yield is shown as plaque-forming units per ml (Pfu/ml). (C) Cells were mock-infected (mock) or infected with HSV-1 at an MOI of 5 and proteins were extracted 18 h.p.i. for western blot analysis. SH-SY5Y – parental cells, NC- negative control gRNA, FoxO1-/- and FoxO3-/- cells. (D) Cells were infected at an MOI of 5 and 0,001 MOI (E) and the supernatants were collected at the indicated time points after infection (h.p.i.). Virus titer was determined by titration on Vero cells and shown as plaque-forming units per ml (Pfu/ml). SH-SY5Y – parental cells, NC- negative control gRNA, FoxO1-/- and FoxO3-/- cells.

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4. Discussion

Deregulation of host miRNA has been observed in a number of infections, including studies involving HSV-1 [39], [23], [40], [41], [21], [42], [43], [26], [44], [29], [45], [46], [47]. However, a discrepancy between different studies (i.e. miRNAs that were deregulated) was quite significant, indicating a strong dependence on the miRNAome of the cells used in the experiments. In this study, we aimed to investigate the deregulation of miRNAs in primary cells, and in two completely unrelated experiments to identify a genuine miRNA response of infected cells. We show that miR-183, -96, -182, and -375, are the only reproducibly deregulated miRNAs in a number of primary cells, but not in all cancer-derived cells. The observed discrepancy with cancer cell lines and different studies can be explained by the fact that cancer cell lines can have dramatically altered miRNAomes [73], which probably masks the potential upregulation. Another possibility is that the pathways leading to the transcriptional activation of the cluster are lost in cancer cells (i.e. not responsive to the ICP0-mediated upregulation). A recent study by Kim et al. in which authors analyzed miRNA expression in tears of patients with herpes epithelial keratitis and found that among many, miR-182 and miR-183 were upregulated, supports the idea that activation of the miR-183/96/182 represents the innate antiviral response to HSV-1 infection [47]. Similarly, the same cluster has been found upregulated in other herpesvirus infections [27], and also in completely distant RNA viruses [74], [75], [76]), which indicates a conserved innate antiviral response mechanism. Indeed, although the exact role of this cluster in antiviral defense is yet to be elucidated, there are multiple lines of evidence indicating an important role in the control of innate and adaptive immunity (reviewed in [77]). miRNAs of the cluster target protein phosphatase 2 catalytic subunit alpha (PPP2CA) and tripartite motif-containing 27 (TRIM27), a negative regulator of IRF3, STAT1, and TBK1 [76], promoting IFN production and signaling. The enhanced IFN signaling can lead to the reduction of virus replication, as it has been shown for vesicular stomatitis virus [76]. Moreover, Stittrich et al. have shown that the signal transducer and activator of transcription 5 (STAT5) -mediated activation of miR-182 by interleukin 2 receptor (IL-2R) initiates a positive feedback response by targeting FoxO1 and promotes clonal expansion of activated helper T lymphocytes [66]. Taking together, one might expect that the upregulation of the miR-183/96/182 cluster will affect HSV-1 infection; however, our results show that this is not the case, which is not surprising. In fact, HSV-1 has developed numerous mechanisms to circumvent the host antiviral defense of the cell, including various functions of incoming virion/tegument proteins and proteins expressed after infection (reviewed in [78], [79]). For example, only the TBK-1/IRF3/IFN axis is targeted by at least five different viral proteins, ICP0, ICP27, ICP34.5, US3, and vhs (reviewed in [78], [79]). This indicates that activation of the miR-183/96/182 cluster might be part of an early cellular response to virus infection, preceding the adaptation of large DNA viruses to it. However, Oussaief et al. found that the expression of the miR-183/96/182 cluster is repressed in cells latently infected with Epstein-Barr virus (EBV mediated by EBV-encoded latent membrane protein 1 (LMP-1)) [28]. These results show that, for the virus, the roles of the miR-183/96/182 cluster in the latency program might be opposite to productive infection and that their control might be required to enable efficient latent infection. It is difficult to predict a role for these miRNAs in the establishment and/or maintenance of latent HSV-1, particularly in the absence or limited ICP0 expression, however, this is yet to be revealed.

On the other hand, one can argue that miRNAs cannot have a meaningful role during productive HSV-1 infection. First, productive HSV-1 infection is rather rapid, in contrast to protracted CMV replication, and inevitably leads to the destruction of the infected cell. Second, it has been shown that HSV-1 interferes with miRNA function and biogenesis at

the stage of nuclear export [80], [81], which limits the potential of miRNA regulation in general. Thus, it would be rather surprising that miRNAs, which accumulate late in infection, can exert a relevant control of genes. Indeed, in our study, we show that protein levels of members of the FoxO protein family, the confirmed targets of miRNAs of the upregulated cluster, are increased early in infection followed by a decrease late in infection. The late phenomenon might be contributed to the regulation of miRNAs; however, the same observation was true in cells in which we did not detect an increase of miRNAs after infection. Thus, we can conclude that miR-183, -96, and -182, at best, have a minor contributing role in this process. Nonetheless, although we were not able to establish the hypothesized regulatory feedback loop, we were very intrigued with the expression of the FoxO protein family members.

In this study, we show that in cells devoid of FoxO1 or FoxO3 expression HSV-1 replication is as efficient as in WT cells, indicating that individual FoxO proteins are not required for efficient replication. Potential compensatory/overlapping functions of other members might explain the lack of a phenotype, or these proteins might exert their role in latency. Important to mention, that we were not able to generate cells in which FoxO4 or all FoxO proteins were inactivated. In contrast to our observation, roles for individual FoxO proteins have been shown for EBV and KSHV. For example, it has been shown that in EBV-positive advanced nasopharyngeal carcinoma cells, which exhibit type II latency, EBV LMP1 modulates PI3K/AKT/Foxo3 pathway resulting in the accumulation of FoxO3 phosphorylation. This inactivation of FoxO3 led to the induction of miR-21 which in turn downregulated programmed cell death 4 (PDCD4) and Fas ligand and reduced apoptosis [82]. In addition, Liu et al. have shown that in EBV-associated gastric cancer cells which show type I/II latency FoxO1, FoxO3, and FoxO4 protein levels were significantly lower compared to the control cells. Interestingly, FoxO1 mRNA was downregulated, but not FoxO3 or FoxO4 mRNAs, which might suggest diverse regulatory mechanisms by various EBV latent genes [57]. Similarly, FoxO1 has been shown sufficient to suppress Kaposi's sarcoma-associated herpesvirus (KSHV) lytic replication and maintain latency by controlling levels of reactive oxygen species (ROS), since disruption of FoxO1 could trigger KSHV reactivation and induce lytic infection by increasing cellular ROS level [60]. On the other hand, a recent study investigating human cytomegalovirus (HCMV) has shown that FoxO1 and FoxO3 positively regulate virus lytic gene expression by activating alternative major immediate-early gene promoters and promoting IE re-expression and virus reactivation [61]. Taken together, our study shows that individual FoxO proteins are not required for efficient virus replication; however, more research, and likely, an adequate *in vitro* latency model, is needed to understand their roles in HSV-1 latency.

Supplementary Materials: The following supporting information can be downloaded at: .....

Figure S1: HSV-1 miRNAs represent only a small fraction of miRNAs in infected cells;
Figure S2: miRNAs found reproducibly deregulated between two data sets;
Figure S3: miRNAs miR-182, -96, -183 target 3'UTRs of FoxO genes;
Table S1: Primers for CRISPR-Cas9 constructs used for generating FoxO family knockout cells and negative control;
Table S2: Human (hsa-) and herpes simplex virus 1 (hsv1-) mature miRNAs detected in mock samples, and at 8 h.p.i., and 18 h.p.i.;
Table S3: Human (hsa-) and herpes simplex virus 1 (hsv1-) mature miRNAs detected in mock samples, and at 10 h.p.i., and 24 h.p.i.;
Table S4: Overlap between TargetScan and MiRDB search.

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