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ATF1 Restricts Human Herpesvirus 6A Replication via Beta Interferon Induction

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ABSTRACT The stimulus-induced cAMP response element (CRE)-binding protein (CREB) family of transcription factors bind to CREs to regulate diverse cellular responses, including proliferation, survival, and differentiation. Human herpesvirus 6A (HHV-6A), which belongs to the *Betaherpesvirinae* subfamily, is a lymphotropic herpesvirus frequently found in patients with neuroinflammatory diseases. Previous reports implicated the importance of CREs in the HHV-6A life cycle, although the effects of the binding of transcription factors to CREs in viral replication have not been fully elucidated. In this study, we analyzed the role of the CREB family of transcription factors during HHV-6A replication. We found that HHV-6A infection enhanced phosphorylation of the CREB family members CREB1 and activating transcription factor 1 (ATF1). Knockout (KO) of CREB1 or ATF1 enhanced viral gene expression and viral replication. The increase in viral yields in supernatants from ATF1-KO cells was greater than that in supernatants from CREB1-KO cells. Transcriptome sequencing (RNA-seq) analysis showed that sensors of the innate immune system were downregulated in ATF1-KO cells, and mRNAs of beta interferon (IFN-β) and IFN-regulated genes were reduced in these cells infected with HHV-6A. IFN-β treatment of ATF1-KO cells reduced progeny viral yields significantly, suggesting that the enhancement of viral replication was caused by a reduction of IFN-β. Taken together, our results suggest that ATF1 is activated during HHV-6A infection and restricts viral replication via IFN-β induction.

IMPORTANCE Human herpesvirus 6A (HHV-6A) is a ubiquitous herpesvirus implicated in Alzheimer's disease, although its role in its pathogenesis has not been confirmed. Here, we showed that the transcription factor ATF1 restricts HHV-6A replication, mediated by IFN-β induction. Our study provides new insights into the role of ATF1 in innate viral immunity and reveals the importance of IFN-β for regulation of HHV-6A replication, which possibly impairs HHV-6A pathogenesis.

KEYWORDS ATF1, HHV-6, gene expression, herpesviruses, interferons, transcription factors

Human herpesvirus 6A (HHV-6A) is a lymphotropic virus which belongs to the *Roseolovirus* genus within the *Betaherpesvirinae* subfamily, together with the closely related virus HHV-6B (1–5). HHV-6A is frequently found in patients with neuroinflammatory diseases, although whether primary infection with HHV-6A has a causal role in these or other illnesses has not yet been determined (6–8). The double-stranded DNA genomes of herpesviruses are reported to encode approximately 70 to 200 proteins (9, 10). Most of these genes contain a collection of promoter/regulatory sequence elements that are regulated by host transcriptional machinery (11). Herpesvirus gene expression during productive infection represents a classic regulatory cascade (11). Immediate early (IE) genes are expressed first after infection. IE proteins activate the promoters of early genes whose

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transcription is totally independent of viral DNA synthesis, whereas the expression of late genes is augmented by (or in some cases totally dependent on) viral DNA synthesis (11).

The cyclic AMP (cAMP) response element (CRE)-binding protein 1 (CREB1), CRE modulator (CREM), and activating transcription factor 1 (ATF1) are members of the CREB family of transcription factors that are crucial for regulating several host genes mediating important cellular processes including cell proliferation, differentiation, and survival (12). Emerging evidence is revealing specific functions of CREB1 and ATF1 in immune responses through their regulation of T cell proliferation and cytokine production (12). CREB1 and ATF1 are phosphorylated by several different host cell kinases, including cAMP-dependent protein kinase A (PKA) (13, 14). In addition to cAMP, other signals such as Ca^{2+} and p38 mitogen-activated protein kinase (MAPK) have important roles for CREB1 and ATF1 activation, but these are not identical. For example, inhibition of Ca^{2+} signaling blocks the activation of CREB1 but still allows partial p38 MAPK-mediated activation of ATF1, while inhibition of p38 MAPK blocks ATF1 but not CREB1 in embryos (15, 16). Phosphorylated CREB1 or ATF1 translocates to the nucleus, where it dimerizes, forming homodimers or heterodimers, binds to its target sequences, such as the cAMP response element (CRE), and recruits p300/CREB-binding protein (CBP) to initiate transcription of numerous target genes (13). In addition to CREB1 and ATF1, other transcription factors such as ATF3, ATF4, and ATF6 are known to bind the CRE, but in contrast to CREB family members, they respond to other stresses and not to cAMP (17).

Previous reports suggested the importance of the CRE in the HHV-6A life cycle. First, the CRE site is the major regulatory element in the HHV-6A polymerase promoter which is activated in infected cells (18). Second, the HHV-6A IE2 protein transactivates a wide variety of promoters harboring CREs as well as those harboring NF-κB or nuclear factor for activated T cell (NFAT) response elements (19, 20). Third, HHV-6A infection induces CD4 gene transactivation in CD4-negative cells through the CRE in the CD4 promoter (21). These observations suggested that the CRE is activated in HHV-6A-infected cells possibly through CREB family transcription factors. However, the CRE and the CREB family transcription factors are also known to regulate the activation of T cells and the production of cytokines, both of which might impair viral replication. Thus, it is not clear whether CREB family transcription factors promote or inhibit HHV-6A replication. In the present study, we analyzed the role of the CREB family member ATF1 during HHV-6A infection.

RESULTS

HHV-6A activates CREB1 and ATF1 in infected cells. To investigate the role of CREB transcription factors, phosphorylation of CREB1 and ATF1 in cells infected with HHV-6A was analyzed. It is known that the cAMP-dependent protein kinase PKA phosphorylates CREB1 serine at position 133 (Ser-133) and ATF1 serine at position 63 (Ser-63). This promotes recruitment of transcriptional coactivators and results in target gene activation (13). T cell-derived JJhan cells were mock infected or infected with HHV-6A U1102 and harvested 72 h later. About 5% of these cells expressed viral proteins, as determined by immunofluorescence (data not shown). As shown in Fig. 1A, viral protein U14 was detected in the lysate of HHV-6A-infected cells but not mock-infected cells. In agreement with previous observations that the promoters harboring CRE were activated in infected cells (18), HHV-6A infection of T cell-derived JJhan cells significantly enhanced the phosphorylation of CREB1 Ser-133 and ATF1 Ser-63, as detected by immunoblotting (Fig. 1A and B). Treatment with the viral genome replication inhibitor phosphonoformic acid (PFA) impaired the accumulation of viral protein U14, although it had no effect on enhancement of CREB1 or ATF1 phosphorylation (Fig. 1C). This suggests that HHV-6A infection activates CREB1 and ATF1, although viral genome replication is not required for their activation.

Next, we analyzed the role of cAMP in the phosphorylation of CREB1 and ATF1 in HHV-6A-infected cells. We used the adenylate cyclase inhibitor SQ22536, which reduces the level of intracellular cAMP (22). We also analyzed the effect of SB203580, an inhibitor of p38 MAPK (23), because induction of the p38 MAPK cascade results in phosphorylation at the

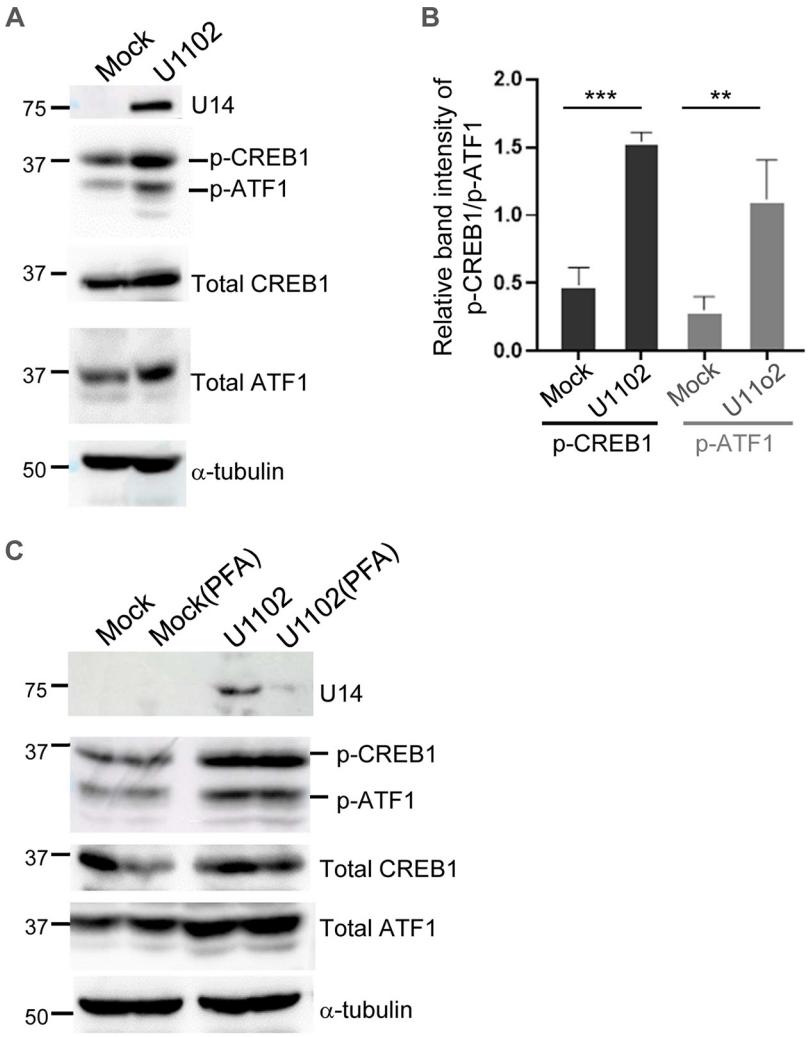

FIG 1 HHV-6A infection activates CREB1 and ATF1. (A) JJhan cells were mock infected or infected with U1102. At 72 h postinfection, cells were lysed and the extracts were immunoblotted using the indicated antibodies. (B) The intensities of p-CREB1 or p-ATF1 in each lane, normalized against α-tubulin, are shown as mean $\pm$ standard deviation from 3 independent experiments (***, $P < 0.001$; **, $P < 0.01$; unpaired Student's t test). (C) JJhan cells were mock infected or infected with U1102. At 24 h postinfection, the cells were divided into two aliquots and either treated with 200 μg/mL phosphonoformic acid (PFA) or left untreated. After 48 h of PFA treatment, the cells were lysed and analyzed by immunoblotting. The experiments were repeated at least 3 times. Numbers at left of panels A and C are molecular masses in kilodaltons.

same position in CREB1 and ATF1 (13). JJhan cells were infected with HHV-6A and mock treated or treated with these drugs for 72 h. As shown in Fig. 2, phosphorylation of CREB1 at Ser-133 and ATF1 at Ser-63 was decreased in the presence of SQ2256 or SB203580 in both mock-infected and virus-infected cells. The effect of SQ2256 was stronger than that of SB203580 (Fig. 2B). Of note, SQ2256 or SB203580 treatment each increased the accumulation of viral proteins (Fig. 2C). These results suggest that HHV-6A infection activates CREB1 and ATF1 mainly through a cAMP cascade but that p38 MAPK contributes to this activation to some extent.

The role of CREB1 and ATF1 in HHV-6A-infected cells. The observed increased accumulation of viral proteins in the presence of SQ2256 or SB203580 prompted us to investigate the role of CREB1 and ATF1 in HHV-6A-infected cells. We first generated JJhan cells expressing Cas9 and guide RNA (gRNA) targeting the CREB1 gene (JJhan-Cas9 CREB1-knockout [KO] cells), using a lentiviral CRISPR/Cas9 system. Control cells were generated by transduction of lentiviral vector expressing Cas9 only (JJhan-Cas9 CT cells). The expression of CREB1 and ATF1 in JJhan-Cas9 CREB1-KO or JJhan-Cas9 CT

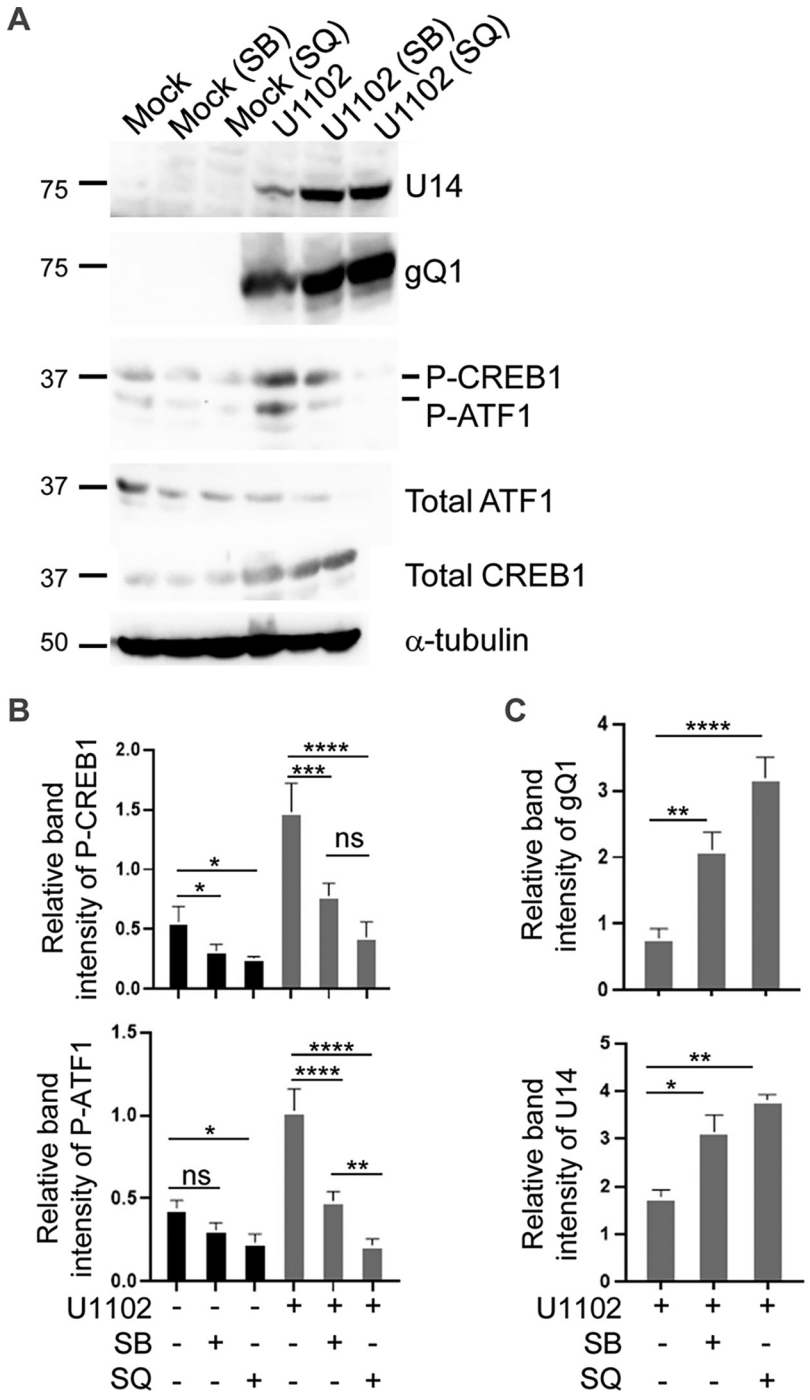

FIG 2 Effect of cAMP depletion on phosphorylation of CREB1 and ATF1 in HHV-6A-infected cells. (A) JJhan cells were infected with U1102 and mock treated or treated with either SB203580 (SB) or SQ22536 (SQ). After 72 h, infected cells were collected and analyzed by immunoblotting. Numbers at left are molecular masses in kilodaltons. (B and C) The intensities of p-CREB1 or p-ATF1 (B) and gQ1 and U14 (C) in each lane, normalized against α-tubulin, are shown as mean $\pm$ standard deviation from 3 independent experiments (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; Tukey's test).

cells was analyzed by immunoblotting. As expected, CREB1 was barely detected in lysates of JJhan-Cas9 CREB1-KO cells, whereas it was present in large amounts in lysates of JJhan-Cas9 CT cells. Of note, a small amount of CREB1 was detected in lysates of JJhan-Cas9 CREB-KO cells, possibly because of the presence of residual wild-type cells. The accumulations of ATF1 in JJhan-Cas9 CREB1-KO cells and JJhan-Cas9 CT cells

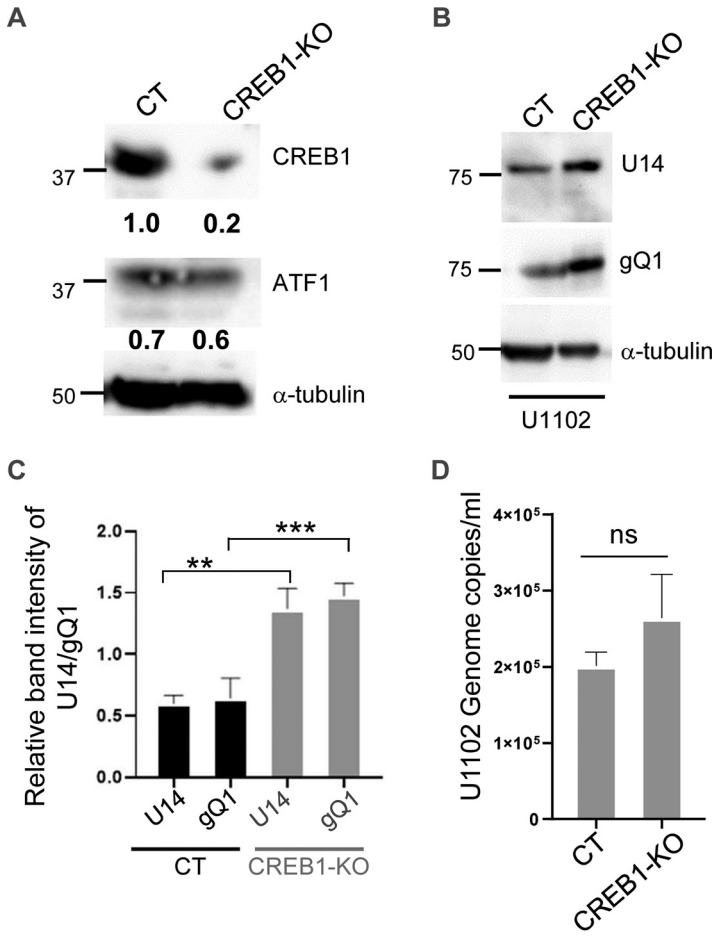

FIG 3 The role of CREB1 in HHV-6A-infected cells. (A) JJhan-Cas9 CT (CT) or JJhan-Cas9 CREB1-KO cells were harvested and analyzed by immunoblotting using the indicated antibodies. The intensities of CREB1 and ATF1, normalized against α-tubulin, are shown under each band. (B) JJhan-Cas9 CT or JJhan-Cas9 CREB1-KO cells were infected with U1102. At 72 h postinfection, cells were lysed and immunoblotted using the indicated antibodies. Numbers at left of panels A and B are molecular masses in kilodaltons. (C) The intensities of U14 or gQ1 in each lane, normalized against α-tubulin, are shown as mean $\pm$ standard deviation from 3 independent experiments (***, $P < 0.001$; **, $P < 0.01$; unpaired Student's *t* test). (D) The genome copies of U1102 were quantified by qPCR upon extraction of DNA from the supernatant of U1102-infected JJhan-Cas9 CT or JJhan-Cas9 CREB1-KO cells at 72 h postinfection. Data are shown as mean $\pm$ standard deviation from 3 independent experiments (unpaired Student's *t* test).

were identical (Fig. 3A). To analyze the effect of CREB1 on viral gene expression, these cells were infected with HHV-6A U1102 for 72 h and then analyzed by immunoblotting. About 5% of cells contained viral proteins, as determined by immunofluorescence (data not shown). As shown in Fig. 3B and C, the accumulation of viral proteins U14 and gQ1 in JJhan-Cas9 CREB1-KO cells was significantly increased relative to that in JJhan-Cas9 CT cells. We also analyzed yields of viral progeny in the supernatant of infected cells using quantitative PCR (qPCR). The number of copies of the HHV-6A genome in the supernatant of infected JJhan-Cas9 CREB1-KO cells was also slightly increased in comparison to JJhan-Cas9 CT cells, but the difference was not statistically significant (Fig. 3D). These observations suggest that CREB1 weakly promotes viral protein accumulation and viral replication, but the effect on the latter is not significant. Next, we analyzed the role of ATF1, another cAMP-responsive transcription factor, by generating ATF1-knockout (KO) JJhan (JJhan-Cas9 ATF1-KO) cells. As shown in Fig. 4A, ATF1 was detected in lysates of JJhan-Cas9 CT but not JJhan-Cas9 ATF1-KO cells. To analyze the effect of ATF1 on viral gene expression, these cells were infected with HHV-6A U1102 for 72 h and analyzed by immunofluorescence and immunoblotting.

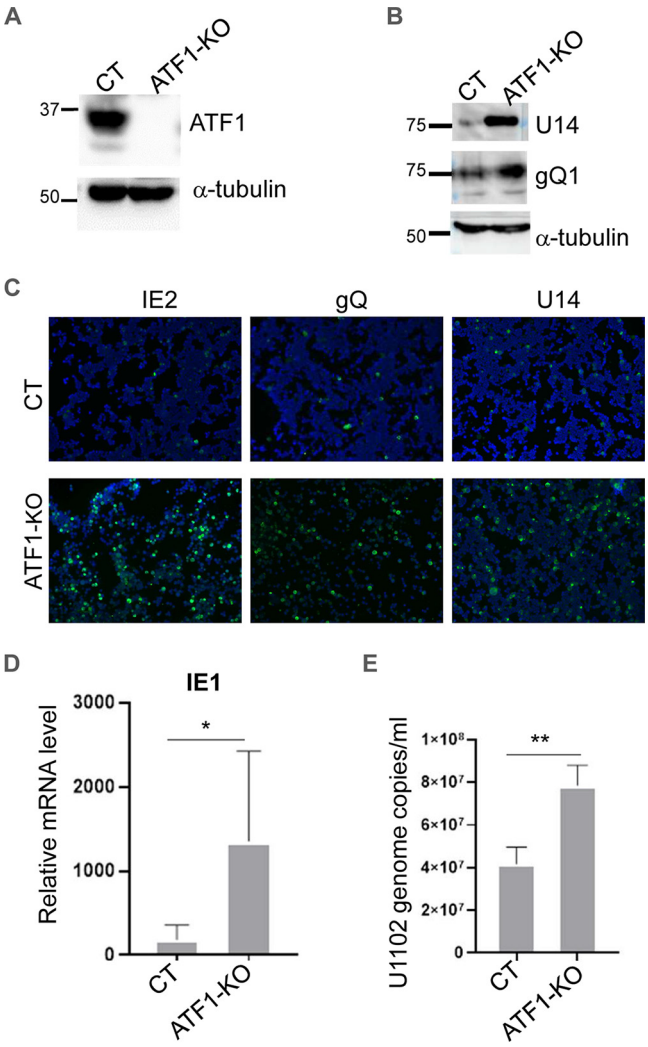

FIG 4 The role of ATF1 in HHV-6A-infected cells. (A) JJhan-Cas9 CT (CT) or JJhan-Cas9 ATF1-KO cells were harvested and analyzed by immunoblotting using the indicated antibodies. Numbers at left of panels A and B are molecular masses in kilodaltons. (B and C) JJhan-Cas9 CT or JJhan-Cas9 ATF1-KO cells were infected with U1102. At 72 h postinfection, the cells were immunoblotted (B) or assayed by immunofluorescence (C) using antibodies directed against the indicated virus proteins. (D) The RNA of U1102-infected JJhan-Cas9 CT or JJhan-Cas9 ATF1-KO cells was collected 72 h postinfection, and the level of the virus immediate early gene IE1 was quantified by RT-PCR. Data are shown as mean ± standard deviation from 3 independent experiments (*, $P < 0.05$; unpaired Student's *t* test). (E) Genome copy number of U1102 was quantified by qPCR upon extraction of DNA from the supernatant of virus-infected JJhan-Cas9 CT or JJhan-Cas9 ATF1-KO cells. Data are shown as mean ± standard deviation from 3 independent experiments (**, $P < 0.01$; unpaired Student's *t* test).

Accumulation of viral proteins was increased in JJhan-Cas9 ATF1-KO cells relative to JJhan-Cas9 CT cells (Fig. 4B and C). About 5% of JJhan-Cas9 CT cells and 20% of JJhan-Cas9 ATF1-KO cells contained viral protein, as determined by immunofluorescence. Real-time PCR (RT-PCR) also indicated significantly increased IE1 viral gene expression in JJhan-Cas9 ATF1-KO cells (Fig. 4D). Moreover, ATF1-KO significantly increased the number of copies of the HHV-6A genome in the supernatants of infected cells as detected by qPCR (Fig. 4E). These results show that ATF1 suppresses viral gene expression and replication in JJhan cells.

Because the effect of ATF1-KO is more marked than the effect of CREB1 from the same family, we focused on JJhan-Cas9 ATF1-KO cells for further analysis by means of rescue experiments. JJhan-Cas9 ATF1-KO cells were transduced to express ATF1 under the control of a doxycycline (Dox)-inducible promoter (JJhan-Cas9 ATF1-KO/rescue

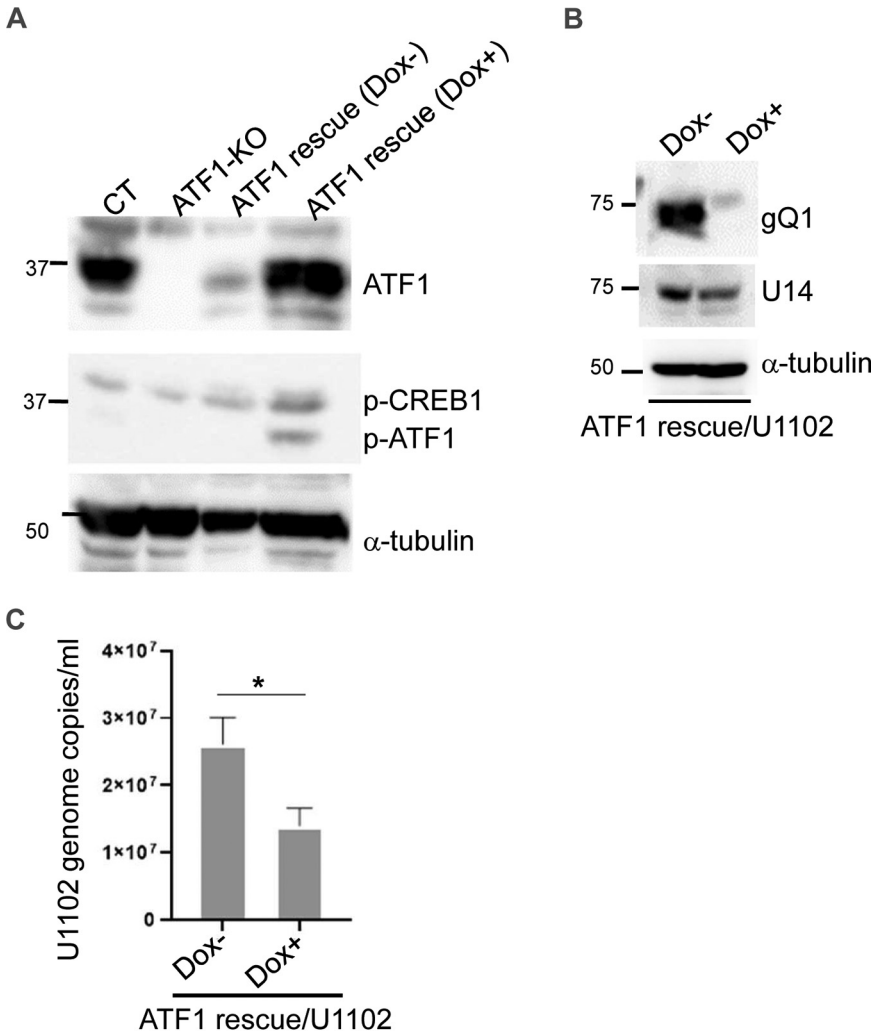

FIG 5 Exogenous expression of ATF1 in JJhan-Cas9 ATF1-KO cells restricts HHV-6A replication. (A) JJhan-Cas9 CT (CT), JJhan-Cas9 ATF1-KO, and JJhan-Cas9 ATF1-KO/rescue cells incubated with or without Dox for 24 h were analyzed by immunoblotting. Numbers at left of panels A and B are molecular masses in kilodaltons. (B and C) JJhan-Cas9 ATF1-KO/rescue cells were infected with U1102 in duplicate aliquots. At 24 h postinfection, the cells were either mock treated or treated with Dox. At 48 h posttreatment, these cells were analyzed using immunoblotting (B) with the indicated antibodies, while genome copy numbers were measured using qPCR (C) following DNA extraction from the supernatant of the infected cells. Data are shown as mean ± standard deviation from 3 independent experiments (*, $P < 0.05$; unpaired Student's *t* test).

cells). JJhan-Cas9 ATF1-KO/rescue cells barely expressed ATF1 in the absence of Dox, whereas ATF1 expression was rescued in the presence of Dox. Accumulation of ATF1 in Dox-treated JJhan-Cas9 ATF1-KO/rescue cells was even greater than that in JJhan-Cas9 CT cells (Fig. 5A). Low levels of phosphorylated ATF1 were detected in JJhan-Cas9 CT cells, but high levels were present in lysates from Dox-treated JJhan-Cas9 ATF1-KO/rescue cells. Next, JJhan-Cas9 ATF1-KO/rescue cells were infected with HHV-6A U1102 in the presence or absence of Dox for 72 h. As shown in Fig. 5B and C, Dox treatment of JJhan-Cas9 ATF1-KO/rescue cells impaired the accumulation of viral proteins and decreased yields of viral progeny. Taken together, these results suggest that ATF1 restricts viral gene expression and replication in JJhan cells.

Identification of ATF1-dependent gene regulation in HHV-6A-infected cells. The observation that ATF1 impaired viral replication is surprising because the polymerase promoter has been reported to be regulated by the CRE (18). To investigate whether an ATF1-dependent system regulates HHV-6 replication, we performed transcriptome

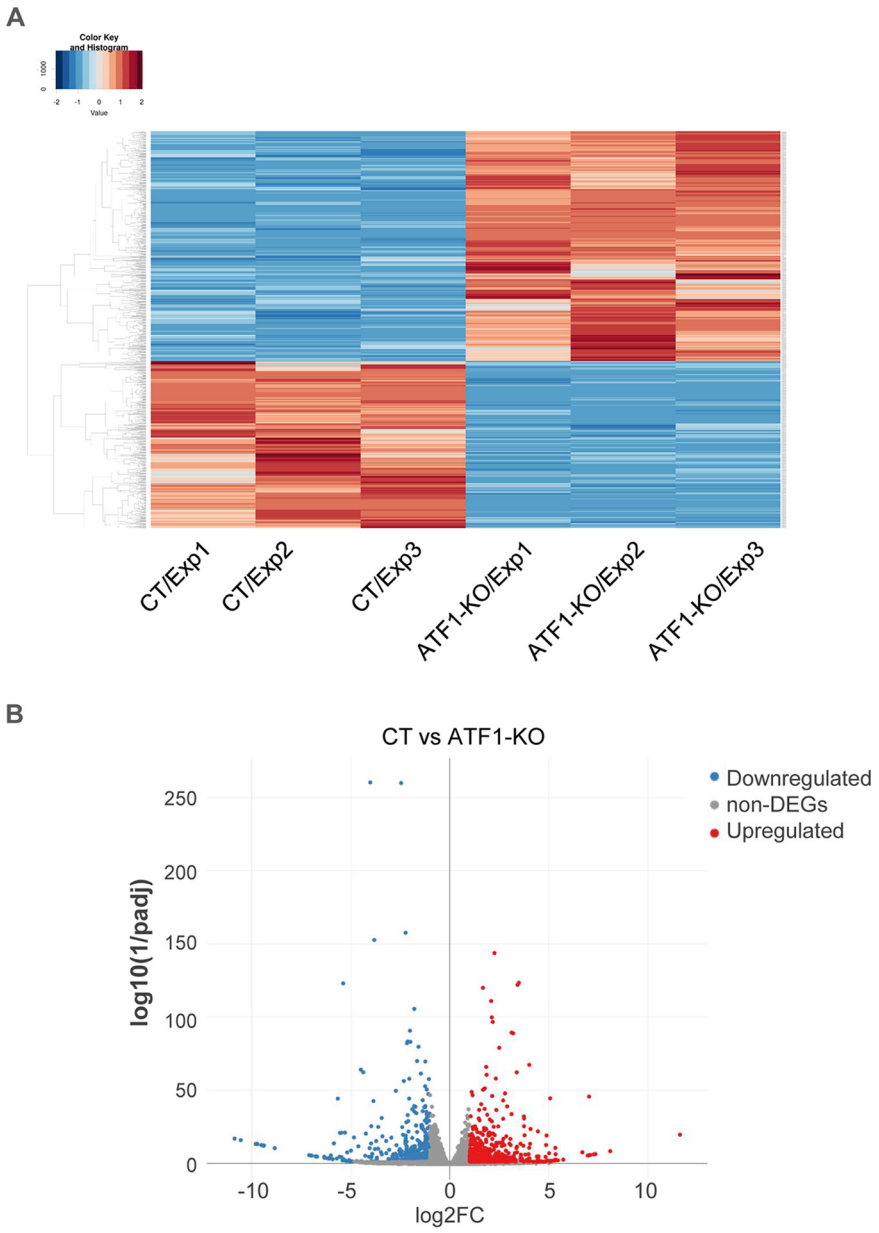

FIG 6 Gene expression signature of JJhan-Cas9 ATF1-KO cells infected with HHV-6A. JJhan-Cas9 CT (CT) or JJhan-Cas9 ATF1-KO cells infected with U1102 for 48 h were harvested to extract RNA, and gene expression was determined using RNA-seq. (A) Heatmap showing differentially expressed genes (DEGs) between JJhan-Cas9 CT (CT) and JJhan-Cas9 ATF1-KO cells determined using RNA-seq data. Normalized Z-score values were calculated for each differentially expressed gene (high, red; low, blue). The distribution of the expression level of the DEGs is shown on the color key legend (top left). (B) Volcano plot of the DEGs.

sequencing (RNA-seq) analysis. JJhan-Cas9 CT cells or JJhan-Cas9 ATF1-KO cells were infected with HHV-6A U1102, and RNA was extracted 48 h thereafter. We then conducted a global RNA sequencing analysis of changes in whole-genome expression in JJhan-Cas9 ATF1-KO versus JJhan-Cas9 CT cells. We generated a heatmap that depicts the segregation of up- versus downregulated RNA transcripts in infected cells (Fig. 6A). We found 521 upregulated and 402 downregulated genes in the JJhan-Cas9 ATF1-KO cells (Fig. 6B). Of these, "inflammatory response-," "leukocyte migration-," and "response to lipopolysaccharide"-related genes were significantly downregulated in JJhan-Cas9 ATF1-KO cells. In contrast, genes involved in "cell adhesion" and "positive regulation of cytosolic calcium ion concentration" were upregulated (Fig. 7). Of note, altered gene profiles observed here are

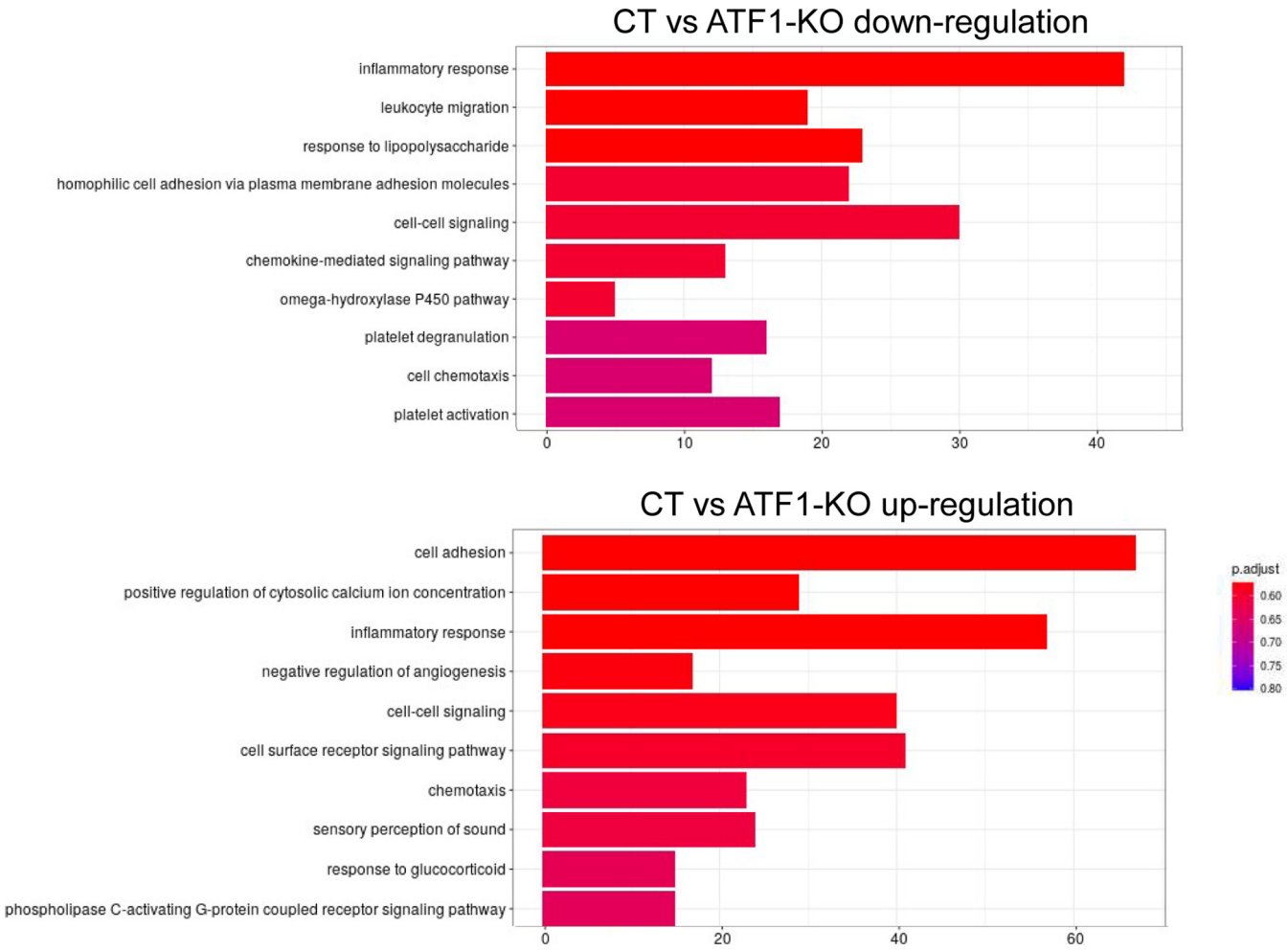

FIG 7 GO classifications of JJhan-Cas9 CT and JJhan-Cas9 ATF1-KO DEGs. The top 10 GO-enriched terms for the up- and downregulated genes in JJhan-Cas9 ATF1-KO cells compared to JJhan-Cas9 CT cells (CT) are shown in the bar graphs. p.adjust values of the GO terms are shown on the color key legend (bottom right).

possibly due to the high level of viral replication in these cells in addition to a direct transcriptional role of ATF1 itself. In JJhan-Cas9 ATF1-KO cells, some of the genes involved in "innate immune response," including cyclic GMP-AMP synthase (cGAS), Toll-like receptor 3 (TLR3), and TLR10, or genes involved in "regulation of inflammatory response" including caspase 1 (CASP1) and CASP4 were downregulated. To determine whether ATF1 is involved in the expression of these genes, we quantified the mRNA in mock-infected and HHV-6A-infected cells by qPCR. Consistent with the RNA-seq analysis, expression of cGAS, TLR3, TLR10, CASP1, and CASP4 was significantly reduced in JJhan-Cas9 ATF1-KO relative to JJhan-Cas9 CT cells, when mock infected or infected with HHV-6A for 72 h (Fig. 8). As loss of TLRs and cGAS mRNA possibly impairs the type I IFN production which is triggered by viral infection, we analyzed the levels of IFN-β and IFN-β-regulated gene mRNAs in infected cells by qPCR. As shown in Fig. 9, HHV-6A infection dramatically increased levels of IFN-β mRNA in JJhan-Cas9 CT but not JJhan-Cas9 ATF1-KO cells. A similar trend was observed for IFN-regulated genes, such as MX-1 and IFI-27 (Fig. 9). These results suggest that impairment of the IFN-β response contributes to promotion of viral replication in JJhan-Cas9 ATF1-KO cells.

We further analyzed the role of ATF1 for IFN-β production during viral infection. JJhan-Cas9 CT or JJhan-Cas9 ATF1-KO cells were infected with herpes simplex virus 1 (HSV-1), and mRNA was extracted for quantification 24 h later. As expected, HSV-1 infection increased the amount of mRNA for beta interferon (IFN-β) and MX-1 in

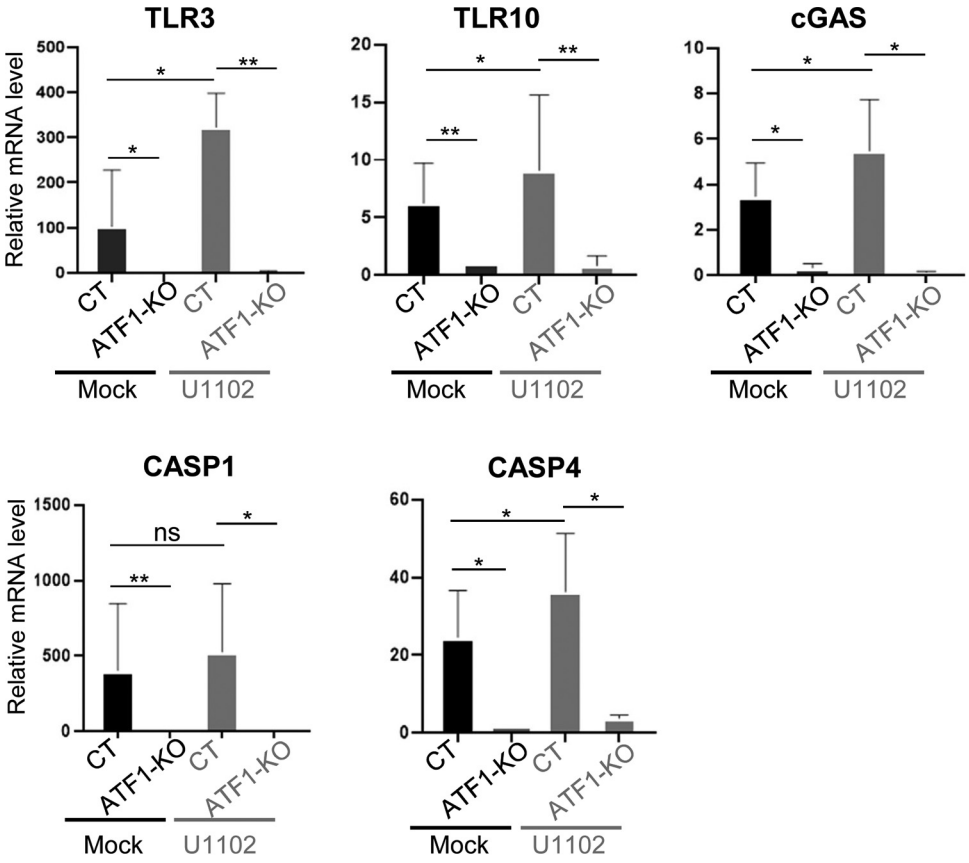

FIG 8 ATF1 is required for expression of genes involved in innate immunity. JJhan-Cas9 CT (CT) or JJhan-Cas9 ATF1-KO cells were mock infected or infected with U1102. At 72 h postinfection, RNA was extracted from the cells. The expression of TLR3, TLR10, cGAS, CASP1, and CASP4 was quantified using qRT-PCR. Relative amounts of these mRNAs were normalized to β-actin. Data are shown as mean ± standard deviation from 3 independent experiments (*, $P < 0.05$; **, $P < 0.01$; Tukey's test).

JJhan-Cas9 CT cells (Fig. 10). In contrast to the results from HHV-6A-infected cells, however, the amounts of these mRNAs were identical in JJhan-Cas9 ATF1-KO cells and JJhan-Cas9 CT cells. These observations suggest that the role of ATF1 for IFN-β production is not conserved in herpesviruses.

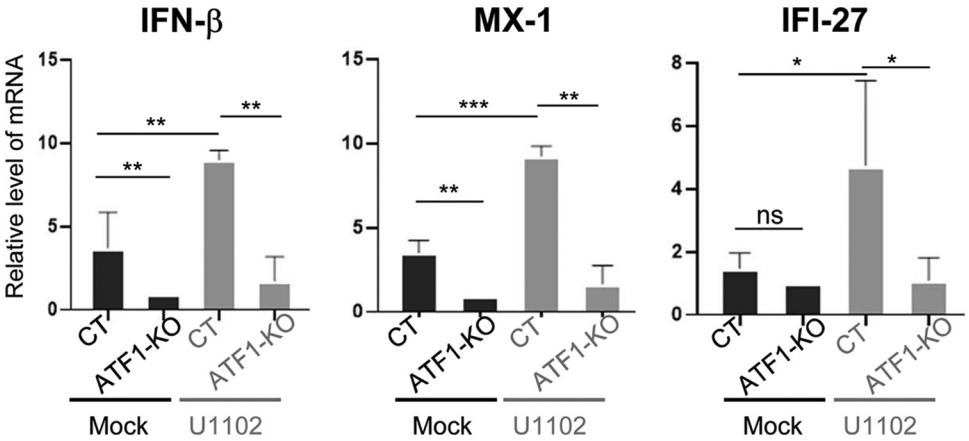

FIG 9 ATF1 is required for expression of IFN-β and IFN-regulated genes in HHV-6A-infected cells. JJhan-Cas9 CT (CT) or JJhan-Cas9 ATF1-KO cells were mock infected or infected with U1102. At 72 h postinfection, RNA was extracted from the cells. The expression of IFN-β, MX-1, and IFI-27 was quantified using qRT-PCR. Relative amounts of these mRNAs were normalized to β-actin. Data are shown as mean ± standard deviation from 3 independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Tukey's test).

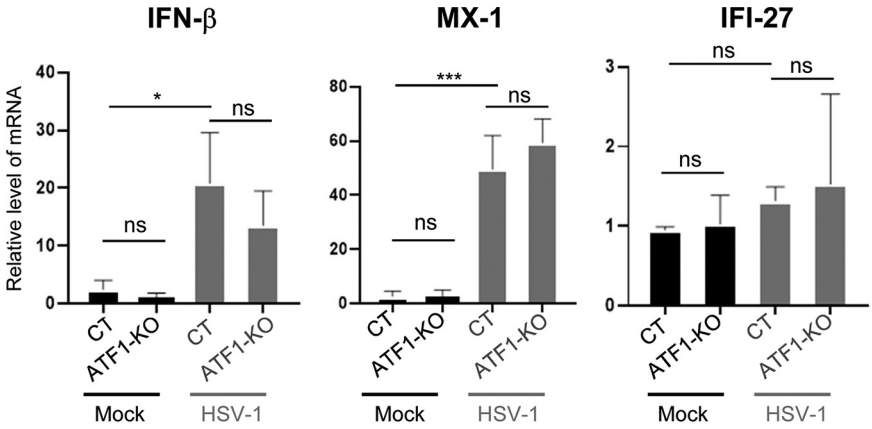

FIG 10 ATF1 is not required for expression of IFN-β and IFN-regulated genes in HSV-1-infected cells. JJhan-Cas9 CT (CT) or JJhan-Cas9 ATF1-KO cells were mock infected or infected with HSV-1. At 24 h postinfection, RNA was extracted from the cells. The expression of IFN-β, MX-1, and IFI-27 was quantified using qRT-PCR. Relative amounts of these mRNAs were normalized against β-actin. Data are shown as mean $\pm$ standard deviation from 3 independent experiments (*, $P < 0.05$; ***, $P < 0.001$; Tukey's test).

Importance of IFN-β in ATF1-KO cells for HHV-6A replication. As we showed above, IFN-β was downregulated in these cells before and after infection with HHV-6A. We hypothesized that ATF1 is important for IFN-β synthesis under normal conditions, which impacts viral infection. Thus, we analyzed the effects of IFN-β in JJhan-Cas9 ATF1-KO cells during HHV-6A infection. JJhan-Cas9 CT or JJhan-Cas9 ATF1-KO cells were infected with HHV-6A U1102 in the presence or absence of exogenous IFN-β. At 72 h postinfection, cells and supernatants were analyzed. As shown in Fig. 11A, the accumulation of viral proteins U14 and gQ1 was dramatically reduced in JJhan-Cas9 CT cells following IFN-β treatment, in agreement with previous observations that type I IFN treatment suppressed HHV-6A replication (24). Accumulation of U14 and gQ1 was increased in JJhan-Cas9 ATF1-KO cells relative to JJhan-Cas9 CT cells, but IFN-β treatment of the former reduced the accumulation of these proteins to levels similar to those in the latter. Furthermore, the number of copies of the HHV-6A genome in the supernatant of JJhan-Cas9 ATF1-KO cells treated with IFN-β was significantly reduced compared to mock-treated cells, whereas IFN-β did not have any significant effect on

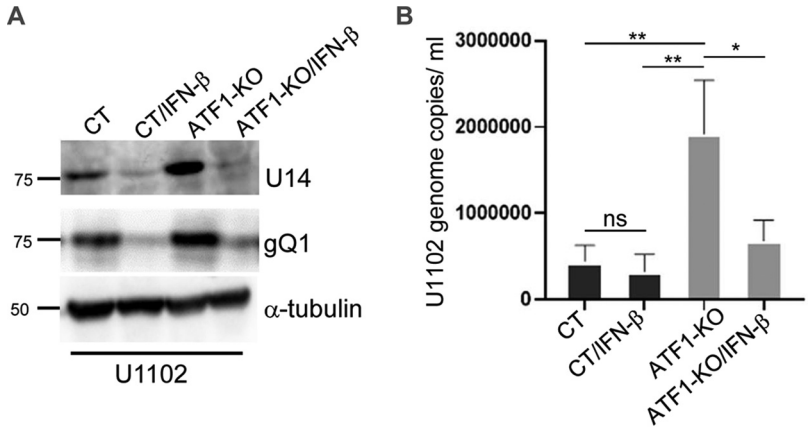

FIG 11 The effects of IFN-β treatment on HHV-6A replication in JJhan-Cas9 ATF1-KO cells. JJhan-Cas9 CT (CT) or JJhan-Cas9 ATF1-KO cells were mock treated or treated with 0.02 ng/mL of IFN-β for 1 h. The cells were then infected with U1102. At 72 h postinfection, they were analyzed by immunoblotting using the indicated antibodies (A), or genome copy numbers were quantified using qPCR following DNA extraction from the supernatant of the infected cells (B). Data are shown as mean $\pm$ standard deviation from 3 independent experiments (*, $P < 0.05$; **, $P < 0.01$; Tukey's test). Numbers at left of panel A are molecular masses in kilodaltons.

JJhan-Cas9 CT cells (Fig. 11B). Thus, the antiviral role of IFN-β during HHV-6A infection is very pronounced in ATF1-deficient cells. These observations strongly suggest that enhancement of viral gene expression and replication by knocking out ATF1 was mediated by impairment of IFN-β production, if not its complete blockade.

DISCUSSION

Members of the CREB family of transcription factors are stimulated by cAMP signaling and are crucial for the activation of T cells. Although host cells use stimulus-inducible transcription factors as a defense mechanism against invading pathogens, at the same time the pathogens may rely on them for their growth. Here, we report a novel interaction between HHV-6A and the ATF1 transcription factor. We found that ATF1 and CREB1 are activated in HHV-6A-infected cells. Knocking out ATF1 increases viral gene expression and replication. In ATF1-KO cells, mRNA for type I IFN sensors was reduced relative to controls. Furthermore, we showed that mRNAs for IFN-β and IFN-regulated genes were upregulated in HHV-6A-infected JJhan cells via ATF1. IFN-β treatment prevented the increased viral replication in ATF1-KO cells. Taken together, our results suggest that ATF1 is activated during viral infection and restricts HHV-6A replication through IFN-β production.

Although both CREB1 and ATF1 are activated by cAMP elevation and bind to the CRE, the roles of these transcriptional factors are not identical. For example, inactivation of the ATF1 gene in mice does not cause any obvious phenotypic abnormalities, whereas CREB1-deficient mice die perinatally due to atelectasis of the lung (25, 26). Interestingly, complete inactivation of both ATF1 and CREB1 results in embryonic death before implantation, suggesting that these two proteins can compensate for each other's function, although they are not equivalent (26). Furthermore, the mechanisms regulating CREB1 and ATF1 by intracellular signaling are not the same (15, 16). These differences are possibly responsible for the predominant effect of ATF1 relative to CREB1 during HHV-6A infection.

The positive effect of CREB1 or ATF1 on the type I IFN system has not been fully investigated thus far. However, it has been reported that CREB family transcription factors target TLR genes (27). In agreement with this observation, expression of TLR3 and TLR10 was also reduced in the ATF1-deficient cells that we created. Similarly, mRNA of another sensor, cGAS, was also reduced in these cells. A role of TLR3 and cGAS in the type I IFN response to herpesviruses has been reported by many groups (28, 29). Our results suggest that ATF1 induces IFN-β production and restricts HHV-6A replication via various different target genes in T cells. In the present study, we did not determine how HHV-6A infection activates CREB1 and ATF1 but found that it depended on cAMP but was independent of viral genome replication. ATF1 is possibly the sensor for various different stimuli including viral infection, which regulates IFN-β induction in T cells, but the precise mechanisms regulating ATF1 activation require further analysis. In addition, we did not observe any effect of ATF1 on IFN-β induction in HSV-1-infected JJhan cells (Fig. 10). Thus, both viruses activate the IFN-β pathway in T cells, but the mechanisms involved are not the same.

Similar to CREB1 and ATF1, other transcription factors such as ATF3, ATF4, and ATF6 are ubiquitously expressed and bind to the CRE in response to various different stimuli (17). Earlier work has shown that ATF3 and ATF4 negatively regulate antiviral interferon signaling (30, 31). Opposing effects of these transcriptional factors have been observed during gluconeogenesis (17). In response to fasting, CREB1 is activated by glucagon-induced cAMP signaling and promotes the expression of key gluconeogenic enzymes, whereas ATF3 and ATF6 bind to the CRE and block the recruitment of CREB1 to the promoter of gluconeogenic genes, leading to reduced gluconeogenesis (17). Taken together with these findings, our results possibly suggest a novel global role for the CREB and ATF transcription factors in the antiviral response.

CREs have been identified in the promoters and enhancers of many T cell-specific genes, including T cell receptor α (TCRα), TCR Vβ, CD3δ, CD8α, interleukin-2 (IL-2),

CD25/IL-2Rα, and IL-2Rγ, suggesting that CRE-binding transcription factors play an important role in T cell functionality (12). Experiments using a mouse strain expressing a dominant negative form of CREB1 under the control of a T cell-specific promoter showed that proliferation and function of activated T cells depend on CREB family transcriptional factors (32, 33). Their activation also increases the release of cytokines through the regulation of IFN-γ production (34). In addition, CREB1 and ATF1 regulate IL-1β expression, which mediates inflammation (35, 36). Our RNA-seq analysis showed that knocking out ATF1 reduced the expression of CASP1 and CASP4 at the mRNA level, both of which regulate maturation and secretion of IL-1β during pyroptosis (37). Thus, CREB family transcription factors may play important roles in the regulation of viral infection through various different pathways *in vivo*. At the same time, CREB family transcription factors are considered to be important for viral gene expression because CREs can be found in viral promoters. For example, five copies of the CRE can be found in the human cytomegalovirus (HCMV) major immediate early (MIE) promoter. The role of these sites for IE expression is not obligate in most cells, but their effects vary with cell type, cellular differentiation state, and activity of certain signaling pathways (38). The CRE in the HCMV MIE promoter is optimal for lytic replication in fibroblasts but is critical during HCMV IE gene expression from latency (39), suggesting a role for appropriate stimuli during reactivation. In the case of varicella-zoster virus (VZV), viral infection induced CREB1 phosphorylation both in tonsil T cells and in fibroblasts (40). Activation of CREB1 is important for VZV replication because treatment with specific inhibitors reduced viral replication and pathogenesis in *in vivo* models (40, 41). Unlike the HCMV MIE promoter, no CRE is present in the HHV-6A MIE promoter (42), and our experiments did not identify any positive role of CREB1 or ATF1 for viral gene expression. However, we cannot exclude the possibility of a redundant role for CREB1 and ATF1 for viral gene expression because functional CREs are present in the HHV-6A polymerase promoter (21).

Our RNA-seq analysis of HHV-6A-infected cells revealed that genes involved in cell adhesion were highly enriched in JJhan-Cas9 ATF1-KO cells relative to controls, possibly due to the high level of viral replication in these cells in addition to a direct transcriptional role of ATF1 itself. The roles and significance of these genes and underlying systems in the viral life cycle require further study in future. Our current findings provide important insights into the novel interaction between HHV-6A and the CREB family of transcription factors, which might offer new targets for antiviral therapy.

MATERIALS AND METHODS

Cells and viruses. HEK293T and Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS). The human T-lymphoblastoid cell line JJhan was cultured in RPMI 1640 medium containing 8% FBS. Umbilical cord blood mononuclear cells (CBMCs) were cultured as described previously (43, 44). CBMCs were purchased from the Cell Bank of the RIKEN BioResource Center, Tsukuba, Japan. The use of CBMCs in this study was approved by the Ethics Committee of Kobe University Graduate School of Medicine (approval number 1209). HHV-6A strain U1102 was propagated in activated CBMCs until the cytopathic effect was maximal. Cells were lysed by freezing and thawing once at −80°C. Cell debris was removed by centrifugation at 1,500 × *g* for 5 min, and supernatants were used for virus infections. For infecting the target cell line JJhan with HHV-6A virus stock, 1×10^6 cells were collected by centrifugation and resuspended in an appropriate volume containing 5×10^6 genome copies of the virus or in medium alone (for mock infection). These cells were centrifuged at 35°C for 30 min at 1,700 × *g*, followed by culturing in RPMI medium supplemented with 2% FBS (44, 45). After 72 h the cells were collected and analyzed by immunofluorescence as described below, and infectivity was determined. HSV-1 strain F was kindly provided by Y. Kawaguchi and propagated and titrated in Vero cells (46–49). For HSV-1, 1×10^6 cells were infected with HSV-1 at a multiplicity of infection of 5 for 24 h. Transfection experiments were performed using Lipofectamine 3000 (Thermo Fisher Scientific) as previously described (50, 51).

Plasmids. To produce lentiCRISPR-CREB1 and lentiCRISPR-ATF1, sense and antisense oligonucleotides were designed for insertion into the Esp3I site in the lentiCRISPR v2 (a gift from F. Zhang, Addgene plasmid no. 52961) (52), which expresses Cas9 and a synthetic single-guide RNA described in Table 1. The DNA oligonucleotides were annealed and incorporated into the lentiCRISPR v2 vector linearized with the Esp3I restriction enzyme. pInd20-ATF1, a lentivirus vector that expresses ATF1 under the control of a Dox-inducible promoter, was constructed by PCR amplification of the ATF1 sequence from the cDNA synthesized from the total RNA of HEK293T cells, as described previously (53), and was cloned into pInducer20 (a gift from S. Elledge, Addgene plasmid no. 44012) (54).

TABLE 1 Primer sequences

Target	Sequence
CREB1 gRNA	5′-CACCGTGGAGTTGGCACCGTTACAG-3′
	5′-AAACCTGTAACGGTGCCAACTCCAC-3′
ATF1 gRNA	5′-CAACCGCAGGACTCATCCGACAGCAT-3′
	5′-AAACATGCTGTCGGATGAGTCCTGC-3′
TLR3 mRNA	5′-GCGCTAAAAAGTGAAGAACTGGAT-3′
	5′-GCTGGACATTGTTCAGAAAGAGG-3′
TLR10 mRNA	5′-GGTTAAAAGACGTTCATCTCCACG-3′
	5′-CCTAGCATCCTGAGATACCAGG-3′
cGAS mRNA	5′-GGGAGCCCTGCTGTAACACTTCTTAT-3′
	5′-CCTTTGCATGCTTGGGTACAAGGT-3′
CASP1 mRNA	5′-GCTGAGGTTGACATCACAGGCA-3′
	5′-TGCTGTCAGAGGTCTTGTGCTC-3′
CASP4 mRNA	5′-GGGATGAAGGAGCTACTTGAGG-3′
	5′-CCAAGAATGTGCTGTCAGAGGAC-3′
IFN-β mRNA	5′-CTTGGATTCCTACAAAGAAGCAGC-3′
	5′-TCCTCCTTCTGGAACTGCTGCA-3′
MX-1 mRNA	5′-GGCTGTTTACCAGACTCCGACA-3′
	5′-CACAAAGCCTGGCAGCTCTCTA-3′
IFI-27 mRNA	5′-CGTCCTCCATAGCAGCCAAGAT-3′
	5′-ACCCAATGGAGCCCAGGATGAA-3′
β-Actin mRNA	5′-GCACCCAGCACAATGAAGA-3′
	5′-CGATCCACACGGAGTACTTG-3′
HHV-6A IE1 mRNA	5′-AATCAATCTTCTGGGTGGGAAGAAAATCCAGCAATGTAATAATTGATGGGTGCAATCG-3′
	5′-ACTATTCTCAAGAAGTGGCTCCGGAGAACATTCTCATCACAGACATTCTTTCTTATATCG-3′
HHV-6A genome	5′-CGCTAGGTTGAGAATGATCGA-3′
	5′-CAAAGCCAAATTATCCAGAGCG-3′

Construction of cell lines. HEK293T cells were transfected with lentiCRISPR v2, lentiCRISPR-CREB1, or lentiCRISPR-ATF1 along with packaging plasmids pCAG-HIV-gag and pCMV-VSV-G-RSV-Rev as previously described (50, 55, 56). At 48 h posttransfection, the supernatants of the transfected cells were harvested, and JJhan cells were transduced with these supernatants and selected with 1 μg/mL of puromycin. Resistant cells were designated JJhan-Cas9 CT, JJhan-Cas9 CREB1-KO, or JJhan-Cas9 ATF1-KO, respectively. JJhan-Cas9 ATF1-KO/rescue cells were constructed similarly from JJhan-Cas9 ATF1-KO cells using pInd20-ATF1 and selected with 500 μg/mL of G418. For induction of ATF1, JJhan-Cas9 ATF1-KO/rescue cells were treated with 1 μg/mL Dox for 24 h.

Antibodies and reagents. For immunoblotting and immunofluorescence analysis, we used mouse monoclonal antibody against α-tubulin (DM1A; Sigma), rabbit monoclonal antibody against CREB (D76D11; Cell Signaling Technology) and phospho-CREB (Ser133) (87G3; Cell Signaling Technology), and rabbit polyclonal antibody against ATF1 (11946-1-AP; Proteintech). Monoclonal antibodies to HHV-6A IE2 (AIE2-1), U14 (AU14), and gQ1 (AgQ1-119) were produced and used as previously described (43, 45, 57). Rabbit monoclonal antibody against phospho-CREB (87G3) detects both p-CREB (Ser133) and p-ATF1 (Ser63). Adenylate cyclase inhibitor SQ22536, p38 MAPK inhibitor SB203580, and recombinant human IFN-β were purchased from Selleck, Tokyo Chemical Industry, and Wako, respectively.

SQ22536 and SB203580 treatment. JJhan cells were either mock infected or infected with HHV-6A U1102 in triplicate aliquots. One aliquot of both mock- and HHV-6A-infected cells was treated with 50 μM SQ22536, and another aliquot was treated with 10 μM SB203580, while the other was left untreated. After 72 h, the cells were analyzed by immunoblotting.

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated from the cells using NucleoSpin RNA kits (Macherey-Nagel) according to the manufacturer's instructions, and cDNA was synthesized from the isolated RNA with SuperScript III (Thermo Fisher Scientific). The amount of cDNA of specific genes was quantified using the SYBR Select master mix (Thermo Fisher Scientific) according to the manufacturer's instructions. The primer sequences are given in Table 1. The amount of these mRNAs was normalized to the amount of β-actin mRNA. The relative abundance of each gene product was calculated using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method (58, 59).

Immunoblotting and immunofluorescence. Immunoblotting and immunofluorescence were performed as described previously (46, 53, 60–62). The band intensities of indicated bands were evaluated using Image J software and normalized to those of α-tubulin. For immunofluorescence, the cells were fixed with methanol-acetone and stained with the indicated antibodies. Nuclear DNA was stained with Hoechst 33342, and specific signals were detected using a fluorescence microscope (BZ-X810; Keyence).

Calculation of viral genome copy numbers. JJhan cells (1×10^6) were infected with U1102 (5×10^6 genome copies/mL). At 72 h postinfection, DNA was extracted from the supernatant of the cells using the DNeasy blood and tissue kit (Qiagen), and the genome copy number per milliliter was quantified by qPCR using SYBR Select master mix (Thermo Fisher Scientific). The sequences of primers used for this purpose are given in Table 1 (63).

Statistical analysis. For the comparison of two groups, the unpaired Student *t* test was used. Tukey's test was used for multiple comparisons. A *P* value of >0.05 was considered not significant (n.s.).

RNA-seq library preparation and sequencing. JJhan-Cas9 CT or JJhan-Cas9-ATF1-KO cells were infected with HHV-6A U1102 for 48 h. Total RNA was isolated from the cells using NucleoSpin RNA kits (Macherey-Nagel) according to the manufacturer's instructions. Under the same conditions, normally, 5% of JJhan-Cas9 CT cells and 20% of JJhan-Cas9 ATF1-KO cells were found to be infected as determined by immunofluorescence at 72 h after infection, but infectivity was not determined at 48 h in this experiment. Sequencing libraries were generated using the NEBNext Poly(A) mRNA magnetic isolation module and NEBNext UltraTMII directional RNA library prep kits (New England Biolabs [NEB]) following the manufacturer's recommendations. After cluster generation, the library preparations were sequenced on a NovaSeq 6000. Differentially expressed genes (DEGs) were detected using DESeq2 (version 1.24.0) (64) with the threshold set at |log2FC (fold change)| of >1 and a p.adjust of <0.05 calculated by the Benjamini and Hochberg (BH) method. Gene set enrichment analysis was conducted by DAVID (version 1.22.0) (65, 66), and gene ontology (GO) terms with p.adjust of <1 by the BH method were extracted. DEGs were analyzed by Ingenuity Pathway Analysis (IPA) (version 51963813; Qiagen Inc.). Heatmaps were created by calculating Z-scores of the count data using stats (version 3.6.1) and gplots (version 3.0.1.1) R packages.

Data availability. RNA sequencing data have been registered in the DNA Data Bank of Japan under accession number DRA014797.

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