Veterinary Microbiology 239 (2019) 108494

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Bovine herpesvirus 1 tegument protein UL41 suppresses antiviral innate immune response via directly targeting STAT1

Wenqing Ma, Hongmei Wang*, Hongbin He*

Ruminant Diseases Research Center, Shandong Provincial Key Laboratory of Animal Resistance Biology, College of Life Sciences, Shandong Normal University, Jinan, 250014, China

ARTICLE INFO

Keywords:
BoHV-1
UL41
STAT1
Antiviral innate immune response

ABSTRACT

Bovine herpesvirus 1 (BoHV-1) has the ability to escape the host innate immune response and establish a long-term latent infection. The tegument protein UL41 causes the rapid degradation of existing and newly transcribed host mRNA and promote the expression of viral protein in the early stages of a productive infection. At present, how BoHV-1 UL41 evades JAK-STAT signaling pathway is unclear. In this study, we report that UL41 expression facilitates BoHV-1 replication and represses the generation of ISGs. Constitutive expression of UL41 inhibits the expression of STAT1, and treatment with drugs that inhibit the protein-degrading pathway did not restore STAT1 expression. Further study shows that UL41 binds and cleaves the mRNA of STAT1, thus blocks the formation of IFN-stimulated gene factor 3 complexes, and represses the activity of IFN response elements and the generation of ISGs. In brief, our results reveal a novel mechanism of BoHV-1 UL41 against natural innate immunity through direct targeting of the STAT1 transcript.

1. Introduction

Innate immunity responses can resist pathogen infection. Pathogens invasion triggers pattern recognition receptors (PRRs), the first line of host to resist virus infection, including toll-like receptors (TLRs), RIG-I-like receptors (RLRs), node-like receptors (NLRs) and cytosolic sensors for double-stranded DNA (Liu et al., 2018; Zhou et al., 2018; Zhu et al., 2016). These PRRs initiate signaling cascades to induce production of type I IFNs, in turn, actives the JAK-STAT pathway (Afroz et al., 2016). The activation of the JAK-STAT pathway is initiated upon binding of IFN-α/β to its receptors; IFNAR1 and IFNAR2 complex are respectively facilitating the transphosphorylation and the activation of Tyk2 and JAK1. IFNAR1 tyrosine 466 phosphorylated via activated Tyk2, and bind to the SH2 domain of STAT2. This facilitates the phosphorylation of STAT2 tyrosine 690 by activated Tyk2, thus making an anchor point to recruit STAT1. The newly recruited STAT1 is phosphorylated on residue tyrosine 701 via JAK1, and the phosphorylated STAT1 and STAT2 form a heterodimer, which separates from the receptor, and then binds with IRF9 and forms a heterotrimeric complex, the IFN-stimulated gene factor 3 (ISGF3) complexes. The complex translocates to the nucleus and binds to the IFN response elements (ISREs) for the induction of ISGs (Goodbourn et al., 2000), which have a central role in intracellular antiviral defenses (Schneider et al., 2014). Notably, many viruses can resist the antiviral natural immune response via antagonizing JAK-STAT signaling pathway (Nan et al., 2017, 2018). Like the tegument protein VP8 of BoHV-1 inhibits IFN-β signaling by reducing the nuclear accumulation of STAT1 via suppressing the nuclear translocation of STAT1 (Afroz et al., 2016).

The virus host shutoff protein (VHS), encoded by the *ul41* gene, which is an endoribonuclease with the activity of mRNA-specific RNase that triggers rapid degradation of host mRNAs to promote the sequential expression of viral proteins (Everly et al., 2002; Pasieka et al., 2008; Svobodova et al., 2012; Taddeo and Roizman, 2006). It has recently been reported that, UL41 is specific to its target substrate and has different cutting mechanisms for different types of viral and cellular mRNAs (Shu et al., 2013). For some viruses immediately early (IE) gene and stable host gene mRNAs are rapidly degraded 5′ to 3′ (Esclatine et al., 2004a, b; Zhang et al., 2017), this degradation mechanism has been attributed to removal of 5′ cap structure via the interaction between UL41 and eIF4H (Feng et al., 2005; Sarma et al., 2008), thus the uncapped mRNA is degraded 5′ to 3′ via the exonuclease Xrn1 (Karr and Read, 1999; Perez-Parada et al., 2004). As for the stress response mRNAs with the adenylate-uridylate (AU)-rich elements (AREs) in their 3′UTR, tristetraprolin (TTP) plays a major role in the degradation of these mRNA. TTP is a protein induced in infected cells and its mRNA is not degraded by UL41 (Shu et al., 2013). UL41 interacts with the AREs

* Corresponding authors at: Ruminant Diseases Research Center, College of Life Sciences, Shandong Normal University, 88 East of Wen-hua Road, Jinan, 250014, Shandong, China.

E-mail addresses: hongmeiwang@sdnu.edu.cn (H. Wang), hongbinhe@sdnu.edu.cn (H. He).

https://doi.org/10.1016/j.vetmic.2019.108494
Received 2 September 2019; Received in revised form 28 October 2019; Accepted 29 October 2019

Table 1
Primers used in this study.

Primer name	Primers Sequences (5′ to 3′)
UL41-EcoRI-F	CCGGAATTCGCCACCATGGGGCTCTTCAAGCTACT
UL41-BamHI-R	CGCGGATCCTTACTTGTCATCGTCGTCCTTGTAATCTTGGCGGGTTGTCCGTC
STAT1-NheI-F	CTAGCTAGCGCCACCATGTCCCAGTGGTATGAGCT
STAT1-XhoI-R	CCGCTCGAGCTAGGCATAATCTGGCACATCATAAGGGTAGACCGCATTCATCATAT
TBK1-XhoI-F	GCGGGCCCGCCACCATGCAGAGCACTTCTAATCATC
TBK1-ApaI-R	CCGCTCGAGGAAGACAGTCAACGTTGCGAAGGC
IRF3(5D)-SalI-F1	ACGGTCGACGCCACCATGGGAACCCCAAAGCCACG
IRF3(5D)-R1	GTCGTCGAGGTCGAGTGGGTGGTCGTTGTCAATGTGCAGGTCCACAGTATTCT
IRF3(5D)-F2	GACAACGACCACCCACTCGACCTCGACGACGACCAGTACAAGGCCTACCTG
IRF3(5D)-KpnI-R2	CGGGGTACCGCTCTCCCCAGGGCCCTGGAAAT
UL41-F	ACGACGACGAGGAGGCAACG
UL41-R	GCGGATGAGGCTCACGCACA
TYK2-F	GGTGAGATGGTAGCCGTGAA
TYK2-R	TGGTAGAGCTTGCGTAGGATGT
STAT1-F	TGAGGGTCCTCTCATCGTCACT
STAT1-R	GGCACAACTGGGTTTCAAAAC
STAT2-F	AACCTTGACAGCCCGTTTCA
STAT2-R	ATCGGCAGGAGGCTATTTGA
IFITM1-F	CAACATCCGCAGCGACAC
IFITM1-R	GTAGGCGAATGCCACGAA
IFITM2-F	CCTCTACCGCCAAGTGCCT
IFITM2-R	CCGCAAAGCCGTCTCGTA
IFITM3-F	CCTGACGACCACGGTGATC
IFITM3-R	CAGGCAGCACCAGTTCATGA
IRF9-F	TCACTCTTCCCAAAGTGTCAGTTC
IRF9-R	AGCGCGTCCTGCCTGAT
ISG15-F	GGAGGCCCATGGATGATG
ISG15-R	CCGAAGACGTAGATTCATGAACAC
MX1-F	AACACCTGACCGCGTACCA
MX1-R	GCACGAAGAACTGGATGATCAA
SOCS3-F	CCAGCCTGCGCCTCAA
SOCS3-R	CTTGCGCACTGCGTTCAC
TBK1-F	ACACCAAGCTGTTGAGACTTTCC
TBK1-R	CTATCTTTGGGATGAGTGCCTTCT
IRF3-F	GGAAGGAAGTGTTGCGTTTAGC
IRF3-R	CCCTGACTCCTGAGTTCACAAACT
cGAS-F	TAGCGGCGGAGGTGGTGAAC
cGAS-R	CCAGTGCGGAGCAGGTCTACG
STING-F	GGCGTCTGTGTCCTGGAATATGC
STING-R	GGTAGACAATGAGGCGGCAGTTG

region via binds to TTP, thus cleaving 3′UTR (Shu et al., 2015). The cleaved 3′ domain is not protected by a cap and is rapidly degraded. At present, UL41 is mainly through these two mechanisms abrogation of host protein translation, thereby freeing up host ribosomes to be recruited to viral mRNAs and translate virus proteins (Zhang et al., 2017). It has been confirmed that Herpes simplex virus 1 (HSV-1) UL41 suppresses IFN signal pathway by degrading host mRNA. For instance, HSV-1 UL41 was involved in counteracting the cGAS-STING-mediated DNA-sensing pathway by selectively degrading cGAS mRNA (Su and Zheng, 2017). However, so far little is known about the function of BoHV-1 UL41 in IFN signaling pathway.

In the current study, we shows that the constitutive expression of BoHV-1 UL41 inhibits the expression of STAT1, thus repress the activity of ISRE and the generation of ISGs. In addition, our data shows that BoHV-1 UL41 could not suppress STAT1 expression via ubiquitination, lysosomal, or caspase pathways. Further study shows that UL41 binds and degrades STAT1 mRNA in an RNAse A-dependent manner. Thus, BoHV-1 UL41 abrogates the JAK-STAT-mediated signal pathway by down-regulating STAT1 via its RNase activity.

2. Materials and methods

2.1. Cells and viruses

HEK293 T (GDC0067) provide by China Center for Type Culture Collection (CCTCC) and Madin-Darby Bovine Kidney (MDBK) (ATCC CCL-22) were maintained at 37 °C in DMEM medium, supplemented with 25 mM HEPES buffer, 2 mM l-glutamine, 10% foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO2 incubator. The BoHV-1/BarthaNu/67 strain (HVRIIBRV0004) obtained from China Veterinary Culture Collection Center (CVCC) (Hou et al., 2017). For BoHV-1 infection, MDBK cells were infected with BoHV-1 at 0.1 MOI. After 1 h, cells were washed with PBS (Spark Jade) and cultured for further 4, 8, 12 and 24 h.

2.2. Plasmid constructs

All enzymes used for cloning procedures were purchased from Vazyme (Nanjing, China). UL41-Flag was cloned into pLVX-IRES-puro (Clontech Laboratories, Mountain View, CA). STAT1-HA was cloned into pcDNA3.1(+) (Clontech Laboratories, Mountain View, CA). IRF3(5D) and TBK1 were cloned into pCMV-HA (Clontech Laboratories, Mountain View, CA). ISRE promoter reporter plasmid was purchased from Beyotime Biotechnology (Haishang, China). IFN-β promoter reporter plasmid and pRL-TK plasmid was originally provided by Dr. W. Pan (Shandong Normal University, China). All constructs were subjected to restriction digestion profile and sequencing analysis for confirmation. The constructs generated in this study are listed in Table 1.

2.3. Electroporation and establishment of UL41 stable MDBK cells line

Plate the cells 18–24 h before electroporation at a density of 1–2 × 10^6 cells/mL to ensure that the cells are actively dividing and reach the appropriate cell density (generally 2–4 × 10^6 cells/mL) at the

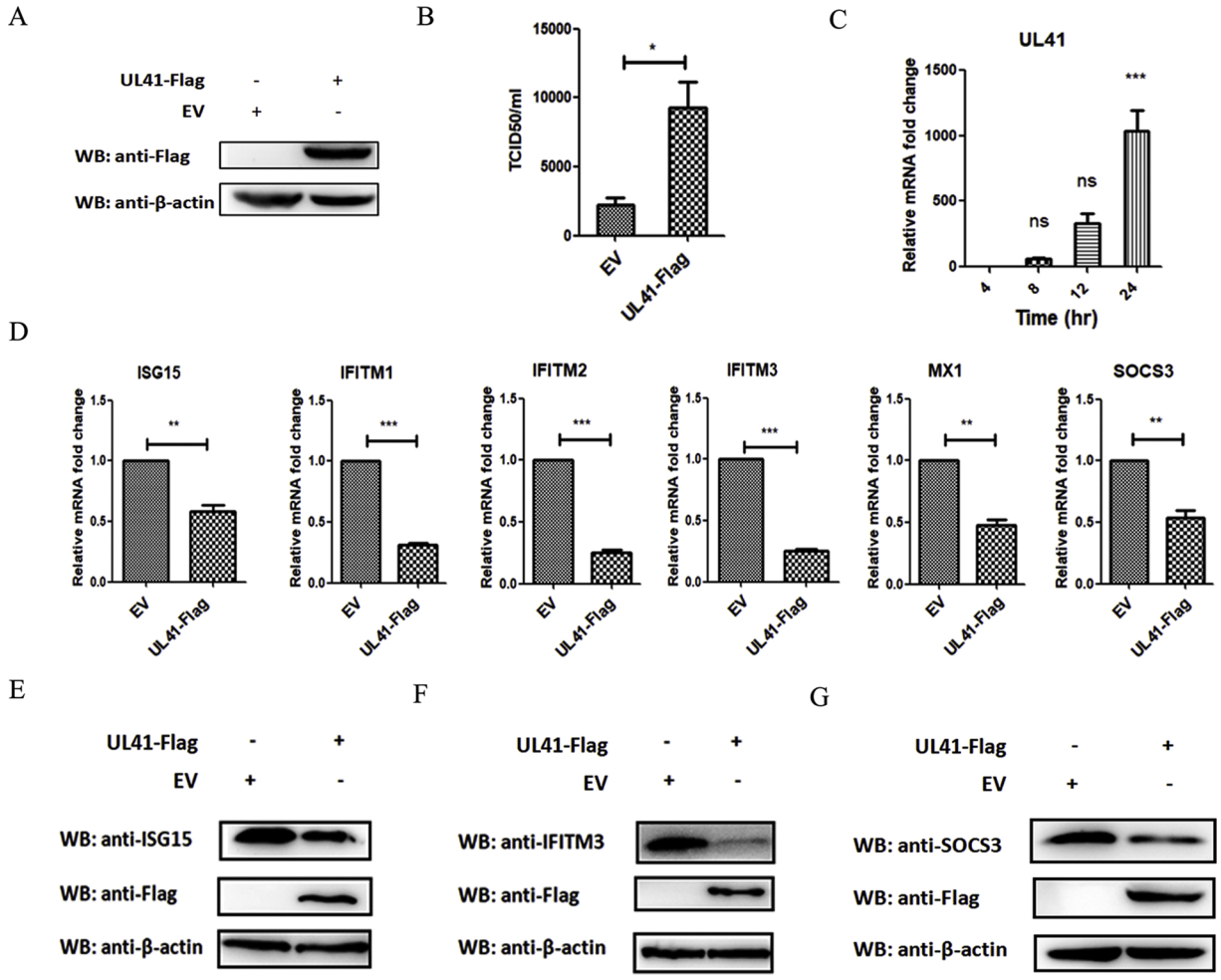

Fig. 1. BoHV-1 UL41 suppresses the natural immune response and promotes viral replication.
(A) Lysates of UL41-Flag cells and EV cells were analyzed by immunoblotting with anti-Flag and anti-β-actin antibodies. (B) UL41-Flag cells and EV cells were infected with 0.1MOI BoHV-1 at 4 h. TCID50 assay analysis the titer of BoHV-1. (C) Expression of UL41 mRNA in MDBKs infected with BoHV-1 (MOI: 0.1) for 4, 8, 12, 24 h. β-actin served as control. (D) Expression of ISG15, IFITM1, IFITM2, IFITM3, MX1 and SOCS3 mRNA in UL41-Flag cells and EV cells was measured by qRT-PCR. (E) Immunoblot analyzes the expression of ISG15 in UL41-Flag cells and EV cells. β-actin served as a loading control. (F) Immunoblot analyzes the expression of IFITM3 in UL41-Flag cells and EV cells. β-actin served as a loading control. (G) Immunoblot analyzes the expression of SOCS3 in UL41-Flag cells and EV cells. β-actin served as a loading control. Data were from three independent experiments and were analyzed by one-way ANOVA (B–D) and were presented as mean ± SD (*p < 0.05, **p < 0.01,***p < 0.001, ****p < 0.0001, ns means no significant difference).

time of harvesting for electroporation. Trypsinize and harvest adherent cells. Each transfection was performed with 10^6 cells. The cells were resuspended in 100 mL of Opti-MEM with 6 μg of pDNA. The cell suspension was transferred to electroporation cuvettes with two parallel plate electrodes spaced 2 mm apart. Cells were electrotransfected with the Entranster™-E Electroporation Solution (Engreen Biosystem, Beijing, China). MDBK cells were treated with 10 electric pulses at 180 V, 5-ms duration, and 1-Hz frequency. The cuvettes were kept at room temperature for 10 min following the pulse application to allow the cells to recover before pipetting them to a six-well plate with full cell culture medium.

PLVX-UL41-Flag-IRES-puro was transfected into MDBK by electroporation, and empty vector pLVX-IRES-puro was transfected into MDBK as control. After 48 h, puromycin was added to cells at a concentration of 2.2 μg/mL and screened for 4 days until drug-resistant cell clones were obtained. Pick up drug-resistant cell clones and expand the culture. Western blot analysis was performed to detect the expression of UL41 through anti-Flag antibody (Cell Signaling Technology Inc, Danvers, MA, USA) (Ma et al., 2016).

2.4. qRT-PCR

Extraction of total RNA and quantifcation of mRNA or miRNA were performed as described previously (Hou et al., 2018). All real-time PCR values were normalized to β-actin expression to avoid system and random errors during sample processing, using the primers listed in Table 1.

2.5. Western blot

Western blotting analysis was performed as previously described (Lv et al., 2019).

2.6. Dual luciferase assay

HEK293 T cells were seeded in 96-well plates in DMEM containing 10% FBS and then co-transfected with reporter plasmids, such as IFN-β-Luc or IRES-Lus and internal control plasmid pRL-TK, with or without expression plasmids as indicated, by standard calcium phosphate precipitation transfected in triplicate with Attractene Transfection Reagent

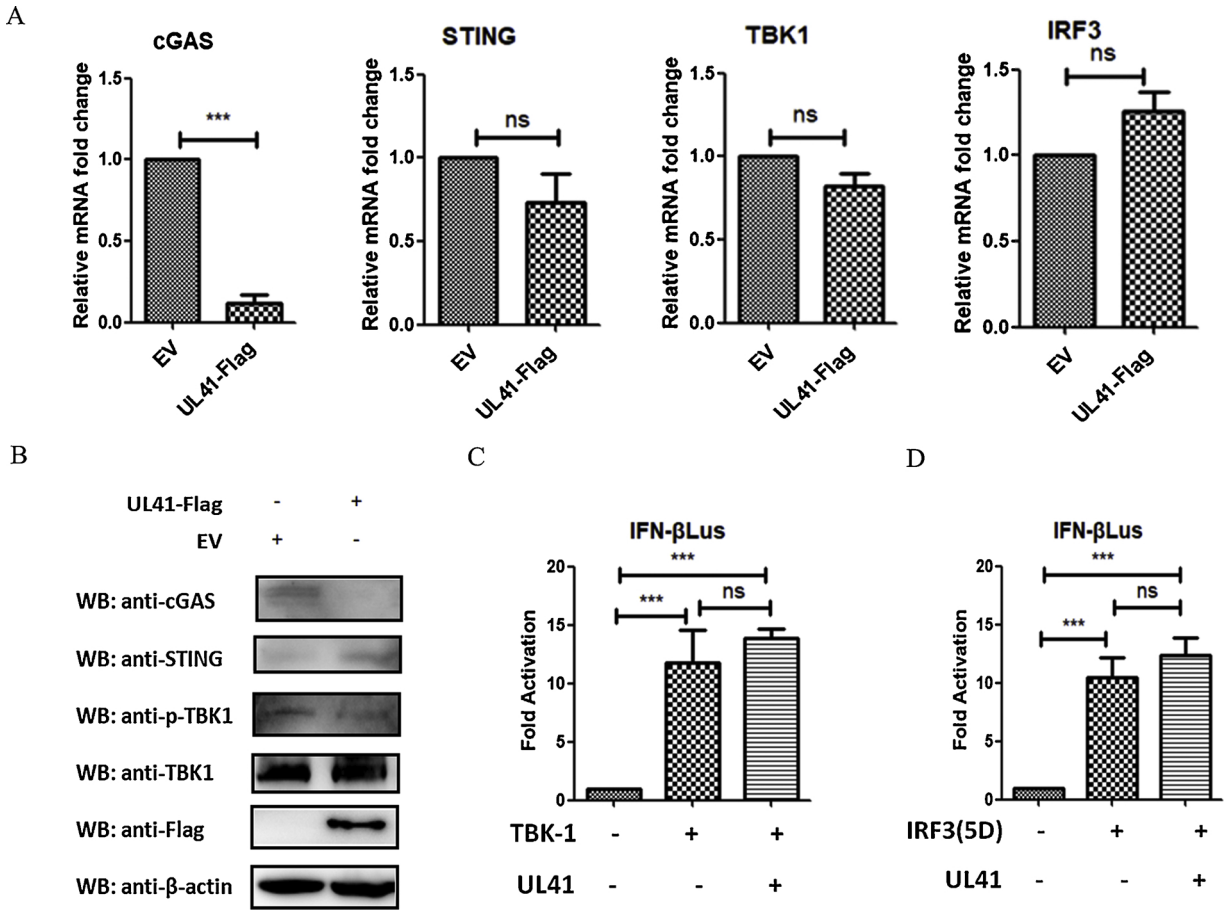

Fig. 2. BoHV-1 UL41 does not affect the induction of IFN-β by TBK 1 and **IRF3.**
(A) Expression of cGAS, STING, MAVS, TBK1, and IRF3 mRNA in UL41-Flag cells and EV cells was measured by qRT-PCR. (B) Immunoblot analyzes the expression of cGAS, STING, p-TBK1, and TBK1 in UL41-Flag cells and EV cells. β-actin served as a loading control. (C to D) HEK293 T cells were co-transfected with IFN-β-Luc reporter, pRL-TK, TBK1-HA (300 ng) (C), and IRF3(5D)-HA (D) along with empty vector (300 ng) or UL41-Flag plasmid. Luciferase activity was measured 24 h post-transfection in the cell lysates. Data were from three independent experiments and were analyzed by one-way ANOVA (A and E) and were presented as mean ± SD (*p < 0.05, **p < 0.01,***p < 0.001, ****p < 0.0001, ns means no significant difference).

(QIAGEN, Valencia, CA, USA). At 24 h after transfection, a dual-luciferase assay was performed using Dual-Luciferase Reporter Assay kit (Promega Corporation, Madison, WI, USA) and Spectra Max M5 microplate reader (Molecular Devices Instruments Inc, USA)(Hou et al., 2019).

2.7. RNA binding protein immunoprecipitation (RIP)

RIP assay was performed using the EZ-Magna RIP Kit (Millipore, Billerica, MA, USA) according to the manufacturer's protocol. UL41-Flag cell lines lysates of was prepared and incubated with RIP buffer containing magnetic beads conjugated with Flag antibody. Normal rabbit immunoglobulin G (IgG) (Solarbio, catalog no. SA131) was regarded as negative control. Samples were incubated in 55℃ with Proteinase K buffer for 30 min, and then immunoprecipitated RNA was isolated. The RNA concentration was measured by a Nanodrop (Thermo Fisher Scientifc, Waltham, MA). Furthermore, purified RNA was obtained and analyzed by RT-PCR and qRT-PCR (Guo et al., 2018).

2.8. Statistical analysis

Results are expressed as means ± SD. The comparisons between the difference groups were made by one-way ANOVA using GraphPad PRISM 5.0. Values of *p < 0.05 were considered statistically significant.

3. Results

3.1. UL41 facilitates BoHV-1 replication

To explore the role of UL41 in BoHV-1 infection, we constructed UL41-Flag lentivirus cell lines in MDBK. Western blot analysis was performed to detect the expression of UL41 by anti-Flag antibody. The UL41-Flag was detected in UL41-flag cell lines, but not in empty vector (EV)-expressed cells (Fig. 1A). The stably transfected UL41-Flag cells and EV cells were infected with BoHV-1 at an MOI of 0.1, and then harvested at the indicated time; TCID50 assay shows that UL41 facilitate the replication of BoHV-1 (Fig. 1B). After BoHV-1 infection, the expression of UL41 increased significantly (Fig. 1C). Then we examined the effect of UL41 expression on ISGs, the mRNA level of ISG15, IFITM1, IFITM2, IFITM3, MX1, and SOCS3 were lower in UL41-Flag cells than wild-type (Fig. 1D). UL41 also inhibits the expression of ISG15, IFITM3, and SOCS3 (Fig. 1E–G). The data shows that BoHV-1 UL41 promotes viral replication by suppressing the natural immune response.

3.2. BoHV-1 UL41 does not affect the induction of IFN-β by TBK 1 and IRF3

To verify the effect of UL41 on the activation of IFN signaling pathway, we investigated the role of UL41 in cGAS-STING signaling pathway. Our data shows that BoHV-1 UL41 inhibits the mRNA

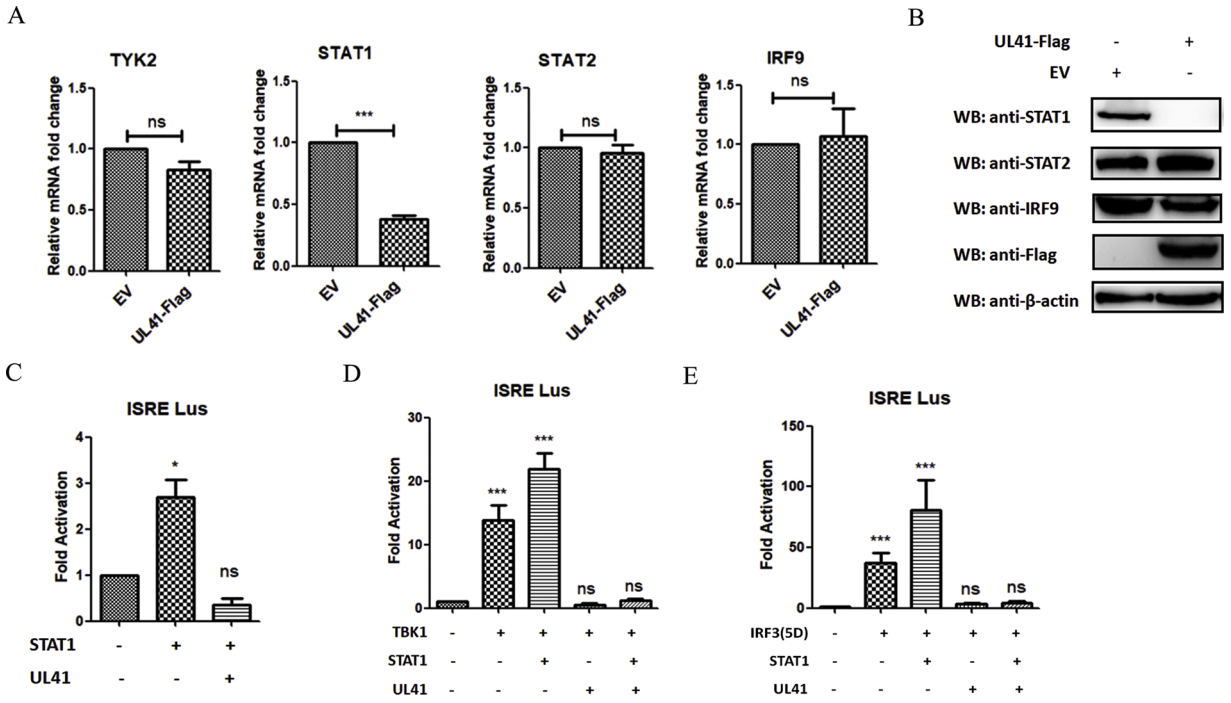

Fig. 3. BoHV-1 UL41 blocks JAK-STAT pathway via down-regulates the expression of STAT1.
(A) Expression of TYK2, STAT1, STAT2 and IRF9 mRNA in UL41-Flag cells and EV cells was measured by qRT-PCR. (B) STAT1, STAT2, and IRF9 expression in UL41-Flag cells and EV cells examined by western blot. β-actin was used as a loading control. (C) HEK293 T cells were co-transfected with ISRE promoter plasmid (ISRE-Luc; 300 ng), Renilla luciferase (pRL-TK; 100 ng) reporter plasmid, UL41-Flag plasmids (300 ng) and STAT1-HA plasmids (300 ng). Luciferase activity was measured 24 h post-transfection in the cell lysates. (D to E) HEK293 T cells were co-transfected with STAT1-HA, TBK1-Flag (D) or IRF3(5D)-HA (E) along with empty vector or UL41-Flag plasmid. Luciferase activity was measured 24 h post-transfection in the cell lysates. Data were from three independent experiments and were analyzed by one-way ANOVA (A, C and D) and were presented as mean ± SD (*$p < 0.05$, **$p < 0.01$,***$p < 0.001$, ****$p < 0.0001$, ns means no significant difference).

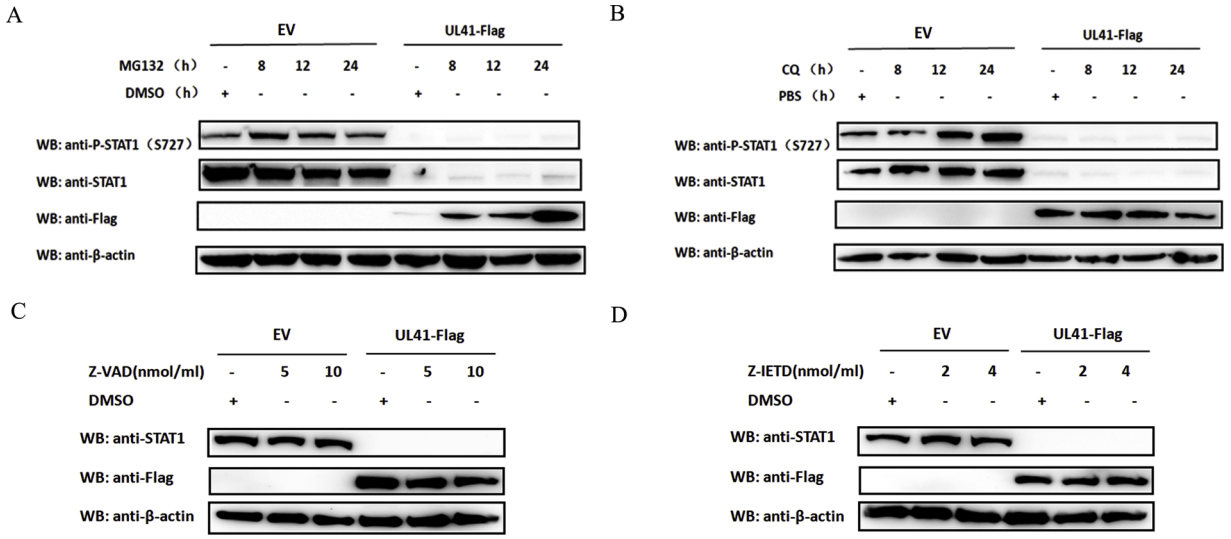

Fig. 4. BoHV-1 UL41 does not inhibit STAT1 expression by activating the degradation pathway.
(A) Immunoblot analysis of the phosphorylation and total protein level of STAT1 in UL41-Flag cells and EV cells treated with MG132 (5 μmol) for 8, 12, 24 h. β-actin served as control. (B) Phosphorylation and total protein level of STAT1 in UL41-Flag cells and EV cells treated with CQ at concentrations of 20 μmol for 8, 12, and 24 h and analyzed by Western blotting using p-STAT1 (s727) and STAT1 antibody. (C) Phosphorylation and total protein level of STAT1 in UL41-Flag cells and EV cells were treated with Z-VAD within 5, 10 nmol/mL for 24 h and harvested for Western blotting with the indicated antibodies. (D) Phosphorylation and total protein level of STAT1 in UL41-Flag cells and EV cells were treated with Z-IETD within 5, 10 nmol/mL for 24 h and harvested for Western blotting with the indicated antibodies.

expression of cGAS but not affect the expression of STING, TBK1, and IRF3 (Fig. 2A). Next, we investigated the protein level of cGAS, STING, TBK1, p-TBK1, and TBK1via immunoblotting. The data shows that UL41 inhibits the proteins level of cGAS but not inhibit the expression of STING, p-TBK1, and TBK1 (Fig. 2B). To detect the effect of UL41 on type I IFN production, we co-transfection of UL41, TBK1, and IFN-β-Luc report plasmids in 293 T cells, after 24 h, IFN-β activity was detected by dual luciferase assay, the result shows that UL41 does not affect TBK1-induced activation of IFN-β promoter (Fig. 2C). Similarly, the IFN-β activity induced by IRF3(5D) was not affected after transfection of

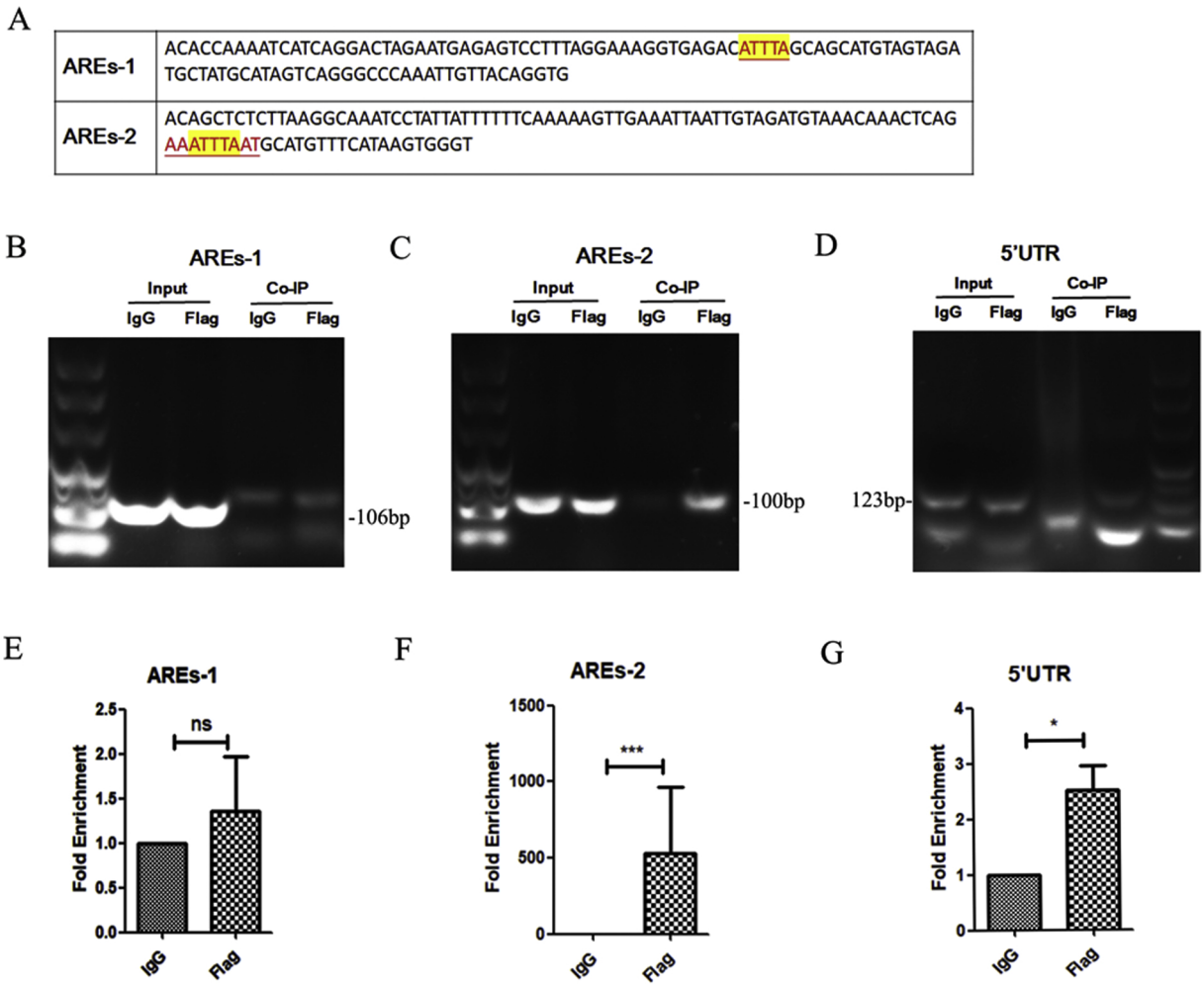

Fig. 5. UL41 binds to 5′UTR cap and 3′UTR AREs of the STAT1 transcript.
(A) RIP was performed using UL41-Flag cell lines and either anti-Flag or Normal Rabbit IgG as the immunoprecipitating antibody. Purified RNA was then analyzed by RT-PCR using RIP Primers specific for the AREs-1, PCR product was not observed both in the anti-Flag RIP (Co-IP Flag) and the Normal rabbit IgG RIP (Co-IP IgG). (B) Purified RNA was then analyzed by RT-PCR using RIP Primers specific for the AREs-2, PCR product was observed in the anti-Flag RIP (Co-IP Flag) and substantially less was detected in the Normal rabbit IgG RIP (Co-IP IgG). (C) Purified RNA was then analyzed by RT-PCR using RIP Primers specific for the 5′cap, PCR product was observed in the anti-Flag RIP (Co-IP Flag) and substantially less was detected in the Normal rabbit IgG RIP (Co-IP IgG). AREs and 5′cap specific cDNA was also observed in the Input. (D) RIP was performed using UL41-Flag cell lines and either anti-Flag or Normal Rabbit IgG as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for the AREs-1(E), AREs-2 (F), and 5′cap (G). Data were from three independent experiments and were analyzed by one-way ANOVA (E–G) and were presented as mean ± SD (*$p < 0.05$, **$p < 0.01$,***$p < 0.001$, ****$p < 0.0001$, ns means no significant difference).

UL41 (Fig. 2D). These indicated that UL41 blocked the cGAS-STING pathway, but not affect the induction of IFN-β by TBK 1 and IRF3.

3.3. BoHV-1 UL41 abrogates the JAK-STAT pathway via inhibits STAT1

Next, we detected the mRNA levels of critical genes in the JAK-STAT pathway in both UL41-Flag cells and EV cells by qRT-PCR assay. We found that the expression of STAT1 mRNA was lower in UL41-Flag cells as compared to control cells. TYK2, STAT2, and IRF9 were not affected (Fig. 3A). To evaluate whether the VHS shut the synthesis of the key proteins in the JAK-STAT pathway, we detected the expression of STAT1, STAT2, and IRF9 via immunoblotting assay. The data shows that UL41 overexpression inhibits the STAT1, not affect the expression of STAT2 and IRF9 (Fig. 3B). Therefore, these results suggest that the expression of UL41 inhibits the production of STAT1. To verify whether UL41 can inhibit downstream ISGs expression by regulating JAK-STAT1 signaling pathway, we co-transfection of UL41 and STAT1 plasmids in 293 T cells, after 24 h, ISRE activity was detected by dual luciferase assay, the result shows that UL41 inhibits STAT1-induced ISRE promoter activity (Fig. 3C). However, the ISRE promoter activity induced by STAT1 transfection alone was low, thus we activated the IFN-signaling pathway by transfection of TBK1 in HEK293 T cells. Transfection of TBK1 enhanced the ISRE promoter activity induced by STAT1, and the ISRE-Lus activity was inhibited after transfection of UL41 (Fig. 3D). Similarly, the ISRE promoter activity induced by IRF3(5D) and STAT1was inhibited after transfection of UL41 (Fig. 3E). These results suggest that UL41 inhibited the activation of ISRE via suppressing JAK-STAT signal pathway.

3.4. BoHV-1 UL41 does not inhibit STAT1 expression by activating the degradation pathway

To verify whether the expression of UL41 inhibits the expression of STAT1 by triggering the degradation mechanisms, we treated UL41-Flag overexpressed cell lines and EV cells with MG132, CQ, Z-IETD-FMK (caspase 8 inhibitor) and Z-VAD-FMK (pan-caspase inhibitor), respectively. Our data shows that none of the drug treatments had any effect on the recovery of STAT1 (Fig. 4A–D). These experiments demonstrated that there may be other regulatory mechanisms for UL41 down-regulation of STAT1.

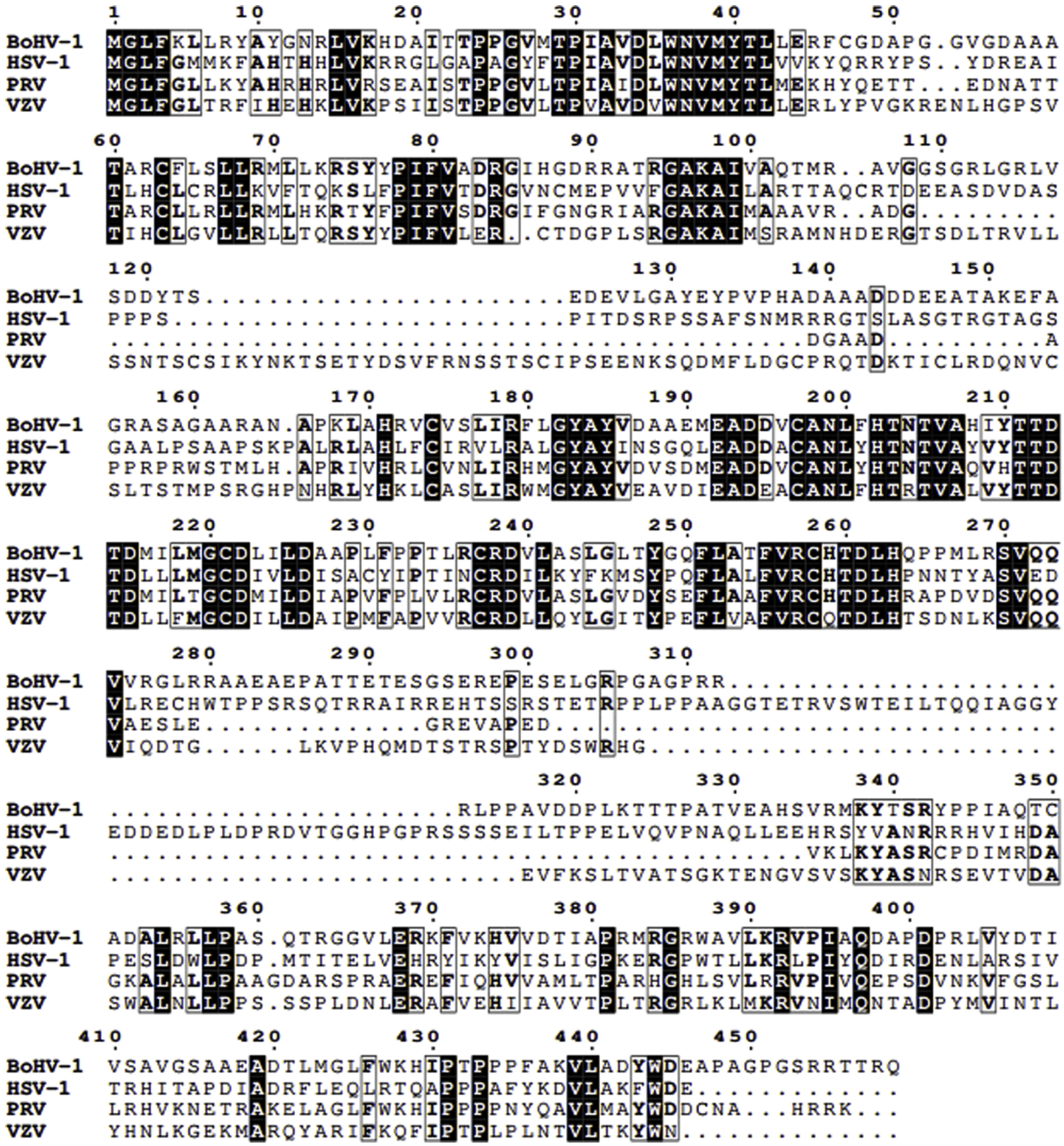

Fig. 6. Comparison of the amino acid sequences of BoHV-1 UL41 and its homologues.
The sequence labels are as follows: BoHV-1, bovine herpesvirus 1; HSV-1, human herpesvirus 1; VZV, varicella-zoster virus; PRV, pseudorabies Virus. Invariant residues are highlighted in bold letters with white boxes, and highly conserved residues are in white bold text surrounded by black boxes. The alignment was generated using MEGA 6. 06, and the figure was generated using the ESPript server (Robert and Gouet, 2014).

3.5. BoHV-1 UL41 binds and cleaves STAT1 mRNA

As mentioned above VHS protein shutoff of host protein synthesis, disruption of preexisting polyribosomes, and the degradation of cellular mRNAs in the absence of de novo viral gene expression (Doepker et al., 2004). There are two ways for UL41 to degrade mRNA, one is to bind and remove the 5′ cap of the gene, and the other is to bind and cleaved the AREs region of 3′UTR. To verify whether the UL41 degradation of STAT1 mRNA by binding to the 5′cap structure or the ARES region in 3′UTR of STAT1, we used anti-Flag antibody to precipitate UL41-binding RNA in UL41-Flag MDBK cells by RIP assay. Next, we compared four splices of STAT1 in MDBK cells, designed four pairs of primers from 5′UTR and 3′UTR (containing AREs) with two potential sites (Fig. 5A), and then amplified the UL41 bound RNA by RT-PCR. The sequencing results showed that UL41 mainly binds to second AREs not the first AREs of STAT1 3′UTR, but slightly to 5′ caps (Fig. 5B–D). qRT-PCR was performed to measure RNA levels in immunoprecipitates also shows that UL41 mainly binds to second AREs of STAT1 mRNA (Fig. 5E–G). The data shows that UL41 degrades STAT1 mRNA by binding to the second AREs region and 5′ caps of STAT1, thus blocking JAK-STAT signaling pathway during viral infection.

4. Discussion

Viral infection induces the production of type 1 IFN, thus activates the antiviral response. IFN-α/β induces ISGs production by activating

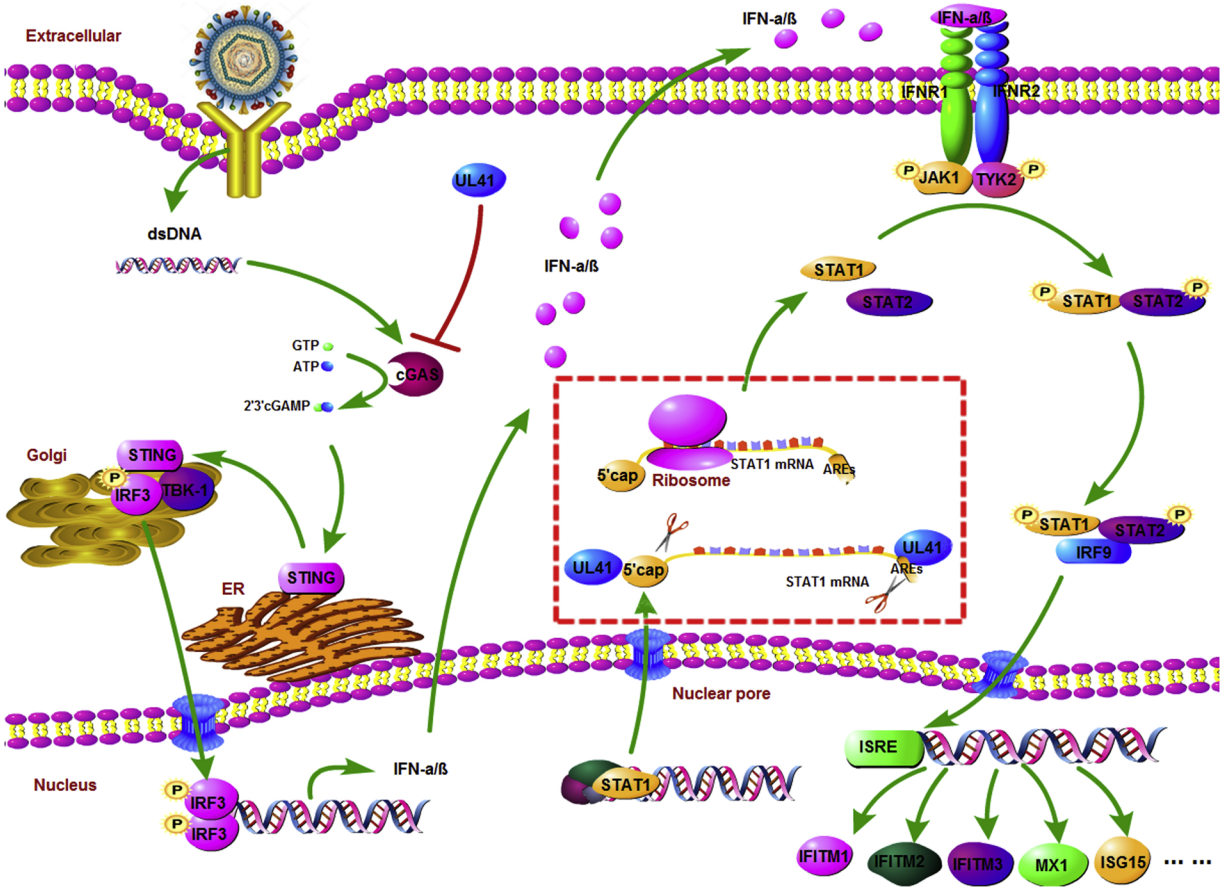

Fig. 7. A model for the UL41 protein suppression of the innate immune response via targeting the STAT1 transcript.
BoHV-1 nucleic acids were recognized by cGAS, cGAS undergoes oligomerization and catalyzes the synthesis of the second messenger molecule cGAMP from ATP and GTP, which in turn binds to and activates the adaptor STING. STING recruits the kinase TBK1 and the transcription factor IRF3, leading to their phosphorylation and activation as well as induction of type I IFNs. IFN-α/β binds to their receptors IFNAR-1 and IFNAR-2 and activates the JAK-STAT pathway. The phosphorylated STAT1 and STAT2 form heterodimer, followed by interaction with IRF9 to form ISGF3.The ISGF3 binds to ISRE in DNA to activate transcription of ISGs. BoHV-1 UL41 blocking JAK-STAT signaling pathway by binding to 3′UTR AREs region and 5′UTR cap of STAT1, cleavage STAT1 mRNA, inhibit the production of ISGs, thus resists the antiviral natural immune response and facilitate viral infection.

the JAK-STAT pathway through its receptor. Some of ISGs have been shown to possess direct antiviral effect (Schneider et al., 2014). The JAK-STAT pathway plays a crucial role in combating viral infection. However, viruses have evolved strategies to escape from antiviral host defenses evoked by IFN-activated JAK-STAT signaling (Afroz et al., 2016). Viral proteins antagonize the IFN-induced host antiviral effects through the JAK-STAT signaling pathway in different ways. For example, human parainfluenza virus 2 (HPIV2) and simian virus 5 (SV5) induce polyubiquitination and degradation of STAT1 via the P and V proteins, respectively (Ulane and Horvath, 2002). The V proteins of Nipah virus and Hendra virus, members of the Henipavirus genus, prevent both STAT1 phosphorylation and nuclear translocation (Rodriguez et al., 2002, 2003). BoHV-1 VP8 protein prevents nuclear accumulation of STAT1 by interacting with STAT1 (Afroz et al., 2016). HSV-1 UL41 inhibits the expression of STAT1 via inducing SOCS3 (Yokota et al., 2004). However, our data show that overexpression of BoHV-1 UL41 inhibits SOCS3 mRNA levels, suggesting that UL41 does not inhibit STAT1 expression by inducing SOCS3. BoHV-1 UL41 consists of 459 amino acids; four conserved functional domains still exist (Berthomme et al., 1993), indicating that UL41 is highly conserved in α-herpesvirus, and that BoHV-1 UL41 also has RNase A activity. But sequence analysis reveals that the amino acid identity is approximately 33.7% compared to that of HSV-1 (Fig. 6). There may be different mechanisms for UL41 of HSV-1 and BHV-1 to inhibit STAT1 expression.

To clarify how UL41 interacts with host genes to promote viral replication, we examined the effects of UL41 on upstream and downstream signaling pathways of type I IFNs. cGAS-STING signal pathway plays an important role in identifying DNA virus infection and cellular innate immune response. Our data shows that constitutive expression of BoHV-1 UL41 can down-regulate the expression of cGAS, but did not affect the expression of STING, p-TBK1, and TBK1, and the production of IFN-β induced by TBK 1 and IRF3. This suggests that BoHV-1 UL41 suppresses upstream signaling pathways of type I IFNs via inhibition of cGAS expression. Next, the crucial genes of the JAK-STAT pathway were detected; our data shows that BoHV-1 UL41 can block the JAK-STAT pathway via inhibited the expression of STAT1.

Some viral proteins use host degradation pathways to control the JAK-STAT signal. Such as the HIV protein Vif uses the “classical” Elongin-Cul-SOCS box (ECS)-E3 ligase complex to promote STAT1 and STAT3 degradation (Gargan et al., 2018). Similarly, The Respiratory Syncytial Virus (RSV) protein NS1 interacts with Elongin C and Cul2, thereby forming an E3 ligase complex, which specifically targets STAT2 for proteasomal degradation (Elliott et al., 2007). Does the UL41 inhibit the expression of STAT1 by triggering the degradation mechanism? Our data showed drug treatment does not restore the expression of STAT1 inhibited by UL41, suggesting that there may be other mechanisms for UL41 to inhibit STAT1 expression.

UL41 as an endoribonuclease with substrate specificity similar to that of RNase A, and mediates selective degradation of both viral and cellular mRNAs (Su and Zheng, 2017). It has been demonstrated previously that the UL41 cleaves off the cap structure of stable host mRNAs (Feng et al., 2005; Page and Read, 2010), thus the mRNA is the rapidly

degraded 5′ to 3′ (Perez-Parada et al., 2004), or selectively degrades stress-response mRNAs containing an AREs in its 3′UTR via its RNase activity (Esclatine et al., 2004a; Shen et al., 2014; Su et al., 2015; Zenner et al., 2013). We found that there are two AREs regions in the bovine STAT1 3′UTR, and the second one has a higher AU abundance than the first one. Our results show that UL41 mainly binds to the second AREs region of STAT1. Why UL41 prefers to combine with the second AREs region remains to be further studied.

In this study, we reported the mechanism of BoHV-1 UL41 protein abrogates antiviral innate immunity and facilitates the viral replication (Fig. 7). Our data showed that BoHV-1 tegument protein UL41 blocks the JAK-STAT signaling pathway by binding the AREs region and 5′ caps of STAT1 mRNA for degradation, thus inhibiting the expression of ISGs, thereby blocking the transmission of antiviral response signals. The findings of this study provide a new direction for the study of the molecular mechanism of BoHV-1 resistance to antiviral innate immunity.

Author contributions

HH and HW developed the concept of the study; HH and WM designed experiments; WM performed experiments; HW and WM collected and analyzed data; WM wrote the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

This work was partially supported by grants from the National Primary Research & Development Plan (2018YFD0501600), Primary Research & Development Plan of Shandong Province (2018GNC113011) and the earmarked fund for the Taishan Scholar Project (Hongbin He).

References

Afroz, S., Brownlie, R., Fodje, M., van Drunen Littel-van den Hurk, S., 2016. VP8, the major tegument protein of bovine herpesvirus 1, interacts with cellular STAT1 and inhibits interferon beta signaling. J. Virol. 90, 4889–4904.
Berthomme, H., Jacquemont, B., Epstein, A., 1993. The pseudorabies virus host-shutoff homolog gene: nucleotide sequence and comparison with alphaherpesvirus protein counterparts. Virology 193, 1028–1032.
Doepker, R.C., Hsu, W.L., Saffran, H.A., Smiley, J.R., 2004. Herpes simplex virus virion host shutoff protein is stimulated by translation initiation factors eIF4B and eIF4H. J. Virol. 78, 4684–4699.
Elliott, J., Lynch, O.T., Suessmuth, Y., Qian, P., Boyd, C.R., Burrows, J.F., Buick, R., Stevenson, N.J., Touzelet, O., Gadina, M., Power, U.F., Johnston, J.A., 2007. Respiratory syncytial virus NS1 protein degrades STAT2 by using the Elongin-Cullin E3 ligase. J. Virol. 81, 3428–3436.
Esclatine, A., Taddeo, B., Evans, L., Roizman, B., 2004a. The herpes simplex virus 1 UL41 gene-dependent destabilization of cellular RNAs is selective and may be sequence-specific. Proc. Natl. Acad. Sci. U. S. A. 101, 3603–3608.
Esclatine, A., Taddeo, B., Roizman, B., 2004b. The UL41 protein of herpes simplex virus mediates selective stabilization or degradation of cellular mRNAs. Proc. Natl. Acad. Sci. U. S. A. 101, 18165–18170.
Everly Jr, D.N., Feng, P., Mian, I.S., Read, G.S., 2002. mRNA degradation by the virion host shutoff (Vhs) protein of herpes simplex virus: genetic and biochemical evidence that Vhs is a nuclease. J. Virol. 76, 8560–8571.
Feng, P., Everly Jr, D.N., Read, G.S., 2005. mRNA decay during herpes simplex virus (HSV) infections: protein-protein interactions involving the HSV virion host shutoff protein and translation factors eIF4H and eIF4A. J. Virol. 79, 9651–9664.
Gargan, S., Ahmed, S., Mahony, R., Bannan, C., Napoletano, S., O'Farrelly, C., Borrow, P., Bergin, C., Stevenson, N.J., 2018. HIV-1 promotes the degradation of components of the type 1 IFN JAK/STAT pathway and blocks anti-viral ISG induction. EBioMedicine 30, 203–216.
Goodbourn, S., Didcock, L., Randall, R.E., 2000. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J. Gen. Virol. 81, 2341–2364.
Guo, J., Cai, H., Liu, X., Zheng, J., Liu, Y., Gong, W., Chen, J., Xi, Z., Xue, Y., 2018. Long non-coding RNA LINC00339 stimulates glioma vasculogenic mimicry formation by regulating the miR-539-5p/TWIST1/MMPs Axis. Mol. Ther. Nucleic Acids 10, 170–186.
Hou, P., Wang, H., Zhao, G., He, C., He, H., 2017. Rapid detection of infectious bovine Rhinotracheitis virus using recombinase polymerase amplification assays. BMC Vet. Res. 13, 386.
Hou, P., Zhao, G., He, C., Wang, H., He, H., 2018. Biopanning of polypeptides binding to bovine ephemeral fever virus G1 protein from phage display peptide library. BMC Vet. Res. 14, 3.
Hou, P., Zhao, M., He, W., He, H., Wang, H., 2019. Cellular microRNA bta-miR-2361 inhibits bovine herpesvirus 1 replication by directly targeting EGR1 gene. Vet. Microbiol. 233, 174–183.
Karr, B.M., Read, G.S., 1999. The virion host shutoff function of herpes simplex virus degrades the 5' end of a target mRNA before the 3' end. Virology 264, 195–204.
Liu, Z., Qin, Q., Wu, C., Li, H., Shou, J., Yang, Y., Gu, M., Ma, C., Lin, W., Zou, Y., Zhang, Y., Ma, F., Sun, J., Wang, X., 2018. Downregulated NDR1 protein kinase inhibits innate immune response by initiating an miR146a-STAT1 feedback loop. Nat. Commun. 9, 2789.
Lv, L., Zhao, G., Wang, H., He, H., 2019. Cholesterol 25-hydroxylase inhibits bovine parainfluenza virus type 3 replication through enzyme activity-dependent and -independent ways. Vet. Microbiol., 108456.
Ma, W., Wang, Y., Yao, X., Xu, Z., An, L., Yin, M., 2016. The role of Exo70 in vascular smooth muscle cell migration. Cell. Mol. Biol. Lett. 21, 20.
Nan, Y., Wu, C., Zhang, Y.J., 2017. Interplay between janus kinase/signal transducer and activator of transcription signaling activated by type I interferons and viral antagonism. Front. Immunol. 8, 1758.
Nan, Y., Wu, C., Zhang, Y.J., 2018. Interferon independent non-canonical STAT activation and virus induced inflammation. Viruses 10.
Page, H.G., Read, G.S., 2010. The virion host shutoff endonuclease (UL41) of herpes simplex virus interacts with the cellular cap-binding complex eIF4F. J. Virol. 84, 6886–6890.
Pasieka, T.J., Lu, B., Crosby, S.D., Wylie, K.M., Morrison, L.A., Alexander, D.E., Menachery, V.D., Leib, D.A., 2008. Herpes simplex virus virion host shutoff attenuates establishment of the antiviral state. J. Virol. 82, 5527–5535.
Perez-Parada, J., Saffran, H.A., Smiley, J.R., 2004. RNA degradation induced by the herpes simplex virus vhs protein proceeds 5' to 3' in vitro. J. Virol. 78, 13391–13394.
Robert, X., Gouet, P., 2014. Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res. 42, W320–324.
Rodriguez, J.J., Parisien, J.P., Horvath, C.M., 2002. Nipah virus V protein evades alpha and gamma interferons by preventing STAT1 and STAT2 activation and nuclear accumulation. J. Virol. 76, 11476–11483.
Rodriguez, J.J., Wang, L.F., Horvath, C.M., 2003. Hendra virus V protein inhibits interferon signaling by preventing STAT1 and STAT2 nuclear accumulation. J. Virol. 77, 11842–11845.
Sarma, N., Agarwal, D., Shiflett, L.A., Read, G.S., 2008. Small interfering RNAs that deplete the cellular translation factor eIF4H impede mRNA degradation by the virion host shutoff protein of herpes simplex virus. J. Virol. 82, 6600–6609.
Schneider, W.M., Chevillotte, M.D., Rice, C.M., 2014. Interferon-stimulated genes: a complex web of host defenses. Annu. Rev. Immunol. 32, 513–545.
Shen, G., Wang, K., Wang, S., Cai, M., Li, M.L., Zheng, C., 2014. Herpes simplex virus 1 counteracts viperin via its virion host shutoff protein UL41. J. Virol. 88, 12163–12166.
Shu, M., Taddeo, B., Roizman, B., 2015. Tristetraprolin recruits the herpes simplex virion host shutoff RNase to AU-rich elements in stress response mRNAs to enable their cleavage. J. Virol. 89, 5643–5650.
Shu, M., Taddeo, B., Zhang, W., Roizman, B., 2013. Selective degradation of mRNAs by the HSV host shutoff RNase is regulated by the UL47 tegument protein. Proc. Natl. Acad. Sci. U. S. A. 110, E1669–1675.
Su, C., Zhang, J., Zheng, C., 2015. Herpes simplex virus 1 UL41 protein abrogates the antiviral activity of hZAP by degrading its mRNA. Virol. J. 12, 203.
Su, C., Zheng, C., 2017. Herpes simplex virus 1 abrogates the cGAS/STING-mediated cytosolic DNA-sensing pathway via its virion host shutoff protein, UL41. J. Virol. 91.
Svobodova, S., Bell, S., Crump, C.M., 2012. Analysis of the interaction between the essential herpes simplex virus 1 tegument proteins VP16 and VP1/2. J. Virol. 86, 473–483.
Taddeo, B., Roizman, B., 2006. The virion host shutoff protein (UL41) of herpes simplex virus 1 is an endoribonuclease with a substrate specificity similar to that of RNase A. J. Virol. 80, 9341–9345.
Ulane, C.M., Horvath, C.M., 2002. Paramyxoviruses SV5 and HPIV2 assemble STAT protein ubiquitin ligase complexes from cellular components. Virology 304, 160–166.
Yokota, S., Yokosawa, N., Okabayashi, T., Suzutani, T., Miura, S., Jimbow, K., Fujii, N., 2004. Induction of suppressor of cytokine signaling-3 by herpes simplex virus type 1 contributes to inhibition of the interferon signaling pathway. J. Virol. 78, 6282–6286.
Zenner, H.L., Mauricio, R., Banting, G., Crump, C.M., 2013. Herpes simplex virus 1 counteracts tetherin restriction via its virion host shutoff activity. J. Virol. 87, 13115–13123.
Zhang, P., Su, C., Jiang, Z., Zheng, C., 2017. Herpes simplex virus 1 UL41 protein suppresses the IRE1/XBP1 signal pathway of the unfolded protein response via its RNase activity. J. Virol. 91.
Zhou, P., Ding, X., Wan, X., Liu, L., Yuan, X., Zhang, W., Hui, X., Meng, G., Xiao, H., Li, B., Zhong, J., Hou, F., Deng, L., Zhang, Y., 2018. MLL5 suppresses antiviral innate immune response by facilitating STUB1-mediated RIG-I degradation. Nat. Commun. 9.
Zhu, Y.Y., Xing, W.X., Shan, S.J., Zhang, S.Q., Li, Y.Q., Li, T., An, L., Yang, G.W., 2016. Characterization and immune response expression of the Rig-I-like receptor mda5 in common carp Cyprinus carpio. J. Fish Biol. 88, 2188–2202.