

HCMV UL24 and UL43 Genes may Facilitate Immune Evasion through Viral miR-UL59 Regulation

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Abstract

UL24 and UL43 are two tegument proteins of the US22 family of Human Cytomegalovirus (HCMV). The role of these two proteins is poorly understood, especially in host cellular interaction. Using co-immunoprecipitation and protein-identification by mass spectrometry, we characterized some intracellular proteins that are complex with viral UL24 and UL43 proteins. We identified that these two viral proteins could interact with each other and also with host cellular proteins, Dicer, and TRBP, which are important cofactors to regulate the biogenesis of the cellular miRNAs. The knockout of these two genes has significantly crippled the expression of HCMV miR-UL59 in the infected cells. Besides, the depletion of these viral genes has increased the mRNA expression of the UL16 binding protein 1 (ULBP1), a target gene of miR-UL59, which is a cell surface glycoprotein present on Natural Killer (NK) cells and other immune cells. These data indicate that UL24 and UL43 proteins may affect the expression of ULBP1 by regulating miR-UL59 to prevent the recognition of infected cells by the immune cells and thus may facilitate HCMV immune evasion. This study provides some theoretical basis for the future development of RNA-targeted small molecules to control HCMV infection.

Introduction

The structure of HCMV virus particles from the outside to the inside is Glycoprotein, Membrane, Tegument protein, Nucleocapsid, and Genome [1]. The HCMV viral genome encodes nearly 200 proteins. The US22 family is a member of its genome, including 12 viral genes, namely UL23, UL24, UL28, UL29, UL36, UL43, TRS1, IRS1, US22, US23, US24, and US26, for each gene

The encoded product has at least one of the four conserved amino acid sequences in the family [2]. The viral proteins encoded by these viral genes are mainly used as interlayer proteins and are important structural components of HCMV virus particles. Some of these viral genes are necessary for the growth of the virus, and their deletion will impair the virus growth or grow slowly. Some are not necessary for the growth of the virus, and their absence will not affect the replication and growth of the virus. In addition, some of these genes also play other important roles in the process of viral infection. For example, UL23 can regulate the immune response induced by IFN- γ , thereby enhancing the resistance of the virus and helping the virus escape [3]; UL29 /28 can activate early gene expression [4,5]; UL36 has anti-apoptotic activity [6]; TRS1 and IRS1 regulate the transcription of some genes, participate in regulating the Protein kinase RNA-activated (PKR) signaling pathway, and promote virus replication [7-9].

Among the members of the US22 family, TRS1 and IRS1 are mostly studied at present. Our understanding of most of the other family members is that they encode the interlayer protein, which is a structural component of the virus but their functions are still poorly understood. A research report using yeast two-hybrid to explore the interaction of HCMV virus proteins proved that pUL24 can interact with pUL43 [10]. Some recent studies have shown that the interlayer protein of HCMV plays various important roles in different stages of the virus life cycle. For example pp65 (immune escape), pp71 (Regulate gene expression), pp150 and pp28 (virus assembly and release) [1, 11, 12]. Therefore, we speculate whether these two genes cooperate in the process of viral infection to participate in some intracellular reactions and thus play some important functions. We constructed two overexpression plasmids of UL24 and UL43 and found through immunoprecipitation and silver staining that compared with the control group, these two proteins can co-precipitate some specific interacting proteins, and then through mass spectrometry analysis, the interactions with cellular proteins TRBP and Dicer were found. TRBP (Trans-activation response (TAR) RNA binding protein) is a double-stranded RNA binding protein, which can cooperate with Dicer to promote the processing of pre-miRNAs and load the processed miRNAs onto Ago2 (Argonaute 2) to form RNA induction. The RNA-induced silencing complex (RISC) ultimately regulates the expression of some genes [13-16].

Currently, the most studied HCMV miRNAs mainly include miR-UL112, miR-UL148D, miR-US5-1, miR-US5-2, and miR-US25-1, which play an important role in the process of viral infection, such as miR-UL112 regulates the latency and reactivation of CMV; miR-UL148D or miR-UL112 can regulate immune response and help virus escape; miR-US25 regulates the cell cycle; miR-US5-1 or miR-US5-2 can regulate inflammatory cytokines. These data indicate that HCMV-encoded miRNAs have a series of important roles in targeting host immune response, cell cycle, and vesicle transport [17-19]. In addition, there are many studies to investigate the functions of HCMV miRNAs to discover further novel miRNAs. The study of viral miRNAs became very important in therapeutic intervention, and recently several drug development strategies have been used to develop RNA-targeted small molecules. These strategies can be used to explore the role of HCMV miRNAs in pathogenesis and develop unique modes of action. Small molecule drugs as a new treatment against HCMV infection [20].

HCMV miRNAs play an important role in the process of virus infection of the host. It can not only regulate virus genes but also have some regulatory effects on host genes. Through different mechanisms, they participate in all aspects of virus infection and regulate some immune responses. Provide a suitable environment for growth and replication. Based on research reports, the viral UL24 and UL43 genes can encode viral interlayer proteins, which are not necessary for the growth of the virus, and the proteins encoded by these two viral genes can interact, therefore, we speculate that whether these two genes is similar to UL29/28 or TRS1/IRS1 in the US22 family, which are cooperating to help the HCMV growth and replication. According to mass spectrometry analysis, we found that the interacting proteins TRBP and Dicer of pUL24 and pUL43, these two host proteins are important components for processing mature miRNAs, and can load mature miRNAs onto RISC to regulate the expression of some genes. Therefore, we speculate whether these two viral genes can regulate some miRNAs, thereby affecting some immune responses in the host, helping the virus to escape the host's immune system, and promoting the immune escape of the virus. A lot of recent researches have focused on the development of RNA-targeted small molecule drugs. This project aims to explore the mechanism of miRNAs and provide some theoretical basis for the future development of RNA-targeted small molecule drugs to help HCMV disease control and treatment.

Materials And Methods

Plasmid

The overexpression plasmids constructed in this study are all based on the empty plasmid pLKO.DCMV.TetO [21]. pLKO-3×Flag-sf-GFP plasmid overexpresses GFP, pLKO-3×Flag-sf-UL43 qualitatively overexpresses pUL43, pLKO-3×Flag-sf-UL24 qualitatively overexpresses pUL24, pLKO-3×Flag-sf-US31 qualitatively overexpressed pUS31, pLKO-HA-UL43 qualitatively overexpressed pUL43, pLKO-TRBP-HA qualitatively overexpressed TRBP, cloning was constructed by enzyme digestion and ligation or in-fusion method, the primers required to construct the above plasmids are shown in **Table 1**

Antibodies, and reagents

The antibodies used in this topic are as follows: anti-Flag (Proteintech), anti-TRBP (Proteintech), anti-HA (Proteintech), anti-Dicer (Santa Cruz), anti-IE1 (a generous gift from Jay Nelson, Oregon Health & Science University), anti-UL44 (Virusys), anti-pp28, anti-pp150 (a generous gift from Thomas Shenk, Princeton University), anti-tubulin (Proteintech, 66031-1-Ig).

The reagents used in this subject are as follows: DNA ligase (TOYOBO), proteinase K (Beyotime Biotechnology), Anti-FLAG M2 Magnetic Beads (Sigma-Aldrich), Dynabeads™ Protein A (Invitrogen), Complete Protease Inhibitor Cocktail (I Roche), Phosphate buffer PBS (Hyclone); high-glycemic cell culture medium DMEM and fetal bovine serum from Gibco;

DNA polymerase PrimStar, In-fusion ligase, and restriction enzymes EcoRI, Sal I, Xba I, the I comes from Takara company; plasmid small-scale extraction kit, gel recovery kit, PCR product purification kit are all from Axygen company; high-purity plasmid small-scale medium-scale kit (Tiangen), MN large extraction kit, silver staining kit (Beyotime Biotechnology).

Cells and viruses

The cells used in this study are as follows: human embryonic lung fibroblasts (MRC5) (were obtained from the American Type Culture Collection (ATCC), available at (<https://www.atcc.org/products/all/CCL-171.aspx>), and human embryonic kidney cells (HEK 293T) (SV40 large T transformed HEK293 cells, were provided by professor Jin Zhong at the Institut Pasteur of Shanghai, Chinese academy of sciences). These cells were cultured in a DMEM medium (Dulbecco modified Eagle medium) containing 10% fetal bovine serum. During this period, the cells were cultured in an incubator containing 5% carbon dioxide and saturated humidity at 37 degrees. MRC5 and HFFs cells were purchased from ATCC, and HEK 293T cells were a gift from Professor Li Bin, Shanghai Jiao tong University.

Wild-type HCMV carries the whole genome of HCMV lab strain AD169; HCMV-GFP is a recombinant virus derived from it, but the viral US4-US6 region of HCMV is replaced by the GFP under the control of a simian virus 40 early promoters (22, 23). We used HCMV-GFP to perform our experiments throughout this research; GFP indicates infected cells.

BAC mutagenesis and recombinant viruses

In this project, two wild-types HCMV Bacterial Artificial Chromosome (BAC) are used and modified based on requirements. The two wild-type virus BACs are pBAC-**AD**/Cre-GFP and pBAC-**TB40E**-Mcherry. The pBAC-AD/Cre-GFP is modified from pBAC-AD/Cre that carries the complete viral genome of the wild-type HCMV experimental virus strain. pBAC-AD/Cre-**GFP** is generated by replacing the pBAC-AD/Cre virus gene US4-6 with the green fluorescent protein gene (GFP), which is expressed under the control of the simian virus (SV40) early promoter. The clinical strain pBAC-**TB40E**-**Mcherry** is produced in the same way as the experimental strain. We used pBAC-AD/Cre-GFP and pBAC-TB40E-Mcherry to produce wild-type viruses as experimental controls.

For the construction of the recombinant mutant virus, the two-step Red Recombination System (Red Recombination System) is used as described previously [24], which can perform point mutation, deletion, and fragment insertion modifications to the BAC carrying the viral genome, and recombination of the modified BAC carrying the Kanna gene. The BAC is electroporated to the competent E. coli GS1783, after induction with L-arabinose, the Kanna gene is removed to obtain the target recombinant BAC, and finally, the BAC is electroporated to the MRC5 cells to promote the virus replicates in the cells and generation of virus particles. Cell lysis releases the virus into the media.

We constructed four viruses, three of which were modified based on the experimental virus strain pBAC-**AD**/Cre-GFP, named pBAC-**AD**-UL24-HA, pBAC-**AD**-UL43-Flag, and pBAC-**AD**-dd24-dd43 double deleted (knocked-out) virus, the other virus is in the clinical strain pBAC-**TB40E**-Mcherry. The recombinant BAC primers used in this experiment are shown in **Table 2**.

Virus growth analysis.

MRC5 cells were seeded in a 12-well plate. After 48 h, the cells were incubated with HCMV at a multiplicity of infection (MOI) of 0.1 or 1 in 300 µl of inoculum. Two hours later, the inoculum was removed, and replaced with a fresh medium. At different times post-infection, cell-free media from infected cultures were collected, and the virus titers in the media were determined by a 50% tissue culture infective dose (TCID₅₀) assay in human fibroblast cells.

Protein analysis

Protein interactions were analyzed by coimmunoprecipitation assay as previously described [25]. HEK293T cells were transfected with the indicated plasmids and collected after 48 h. Collected cells were lysed in 1 ml lysis buffer (40 mM HEPES [pH 7.4], 1 mM EDTA, 300 mM NaCl, and 0.5% NP-40) supplemented with 250 units of Benzonase nuclease and PIC, incubated at 4°C for 1 h, and centrifuged at 13,200 × *g* at 4°C for 15 min. The supernatant (40 µl) was saved as the input control and boiled in sodium dodecyl sulfate (SDS)-containing sample buffer and then, the rest of the supernatant was incubated with either:

FLAG M2 antibody-conjugated magnetic beads (Sigma-Aldrich) at 4°C for 2 h. Then, the beads were washed 5 times with 1 ml lysis buffer. The immunoprecipitants were eluted by 150 ng/µl FLAG peptide (Sigma-Aldrich), or with Protein A beads at 4°C for 1 hour. Then, the beads were washed 5 times with 1 ml lysis buffer. The immunoprecipitants were eluted by boiling in a heat block for 10 minutes/100 °C.

Finally, the input and elution were analyzed by immunoblotting with the indicated antibody.

Proteins were analyzed by immunoblotting as described in the previous study [1]. Cells were infected with HCMV at the MOI of 0.1 or 1, and cell lysates were collected in the sodium dodecyl sulfate (SDS)-containing protein sample buffer at different time points. Protein samples were separated by SDS-PAGE, transferred to PVDF membrane, and incubated with primary and secondary antibodies. After incubation with antibodies, the proteins were visualized by using Clarity Western ECL substrate (Bio-Rad).

Silver stain

Silver stain is performed as described previously [26-28]. After the electrophoresis, the gel was put into about 100 ml fixative and shaken at room temperature on a shaker for 20 minutes/60-70 rpm. After several steps of washing processes, and adding of the silver-stained chromogenic solution, the silver staining solution was discarded, silver staining stop solution (1×) was added and shaken at room temperature on a shaker for 10 minutes/60-70 rpm. Finally, discard the silver dye stop solution and washed by double-distilled water, and shake at room temperature on a shaker for 2-5 minutes/60-70 rpm.

RNA and DNA analysis

Intracellular relative mRNA levels were determined by reverse transcription-quantitative PCR (RT-qPCR) as previously described [29]. MRC5 cells were grown in 6-well plates for 48 hours and then infected with HCMV at MOI of 1. Total RNA was extracted using the TRIzol reagent (Invitrogen), and the cDNA was synthesized with a PrimeScript real-time (RT) reagent kit (TaKaRa) and quantified using SYBR Premix *Ex Taq* (TaKaRa) by quantitative PCR (RT-qPCR) with specific primer pairs (**Table 3**) according to previously described protocol [30]. All reactions were performed in two biological and two technical replicates. The amounts of viral transcripts were normalized to the Gapdh gene.

Intracellular DNA was measured by quantitative PCR as previously described [30]. For cellular and viral DNA analysis, cells in 12-well plates were infected with HCMV at an MOI of 0.1 and collected in 200 µl 2×digestion buffer (200mM NaCl, 20mM Tris-HCl [pH8.0], 50mM EDTA [pH8.0], 1% SDS) at indicated time points. And then, 200ul TE with 100 µg/ml proteinase K was added to lyse cells overnight at 55°C. DNA was extracted with phenol-chloroform. Supernatants were collected after centrifugation at 4°C, 400rpm for 10min, and treated with 100 µg/ml RNase A for an hour at 37°C. Supernatants were extracted again and precipitated with a half volume of ammonium acetate (7.5M), 1µl glycogen, and twice the volume of ethanol. DNA was resuspended in nuclease-free water. Viral or cellular DNA was quantified by qPCR with specific primers as shown in **Table 4**.

Statistical analysis in this study was performed using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, and ns, no significant).

Experimental Results And Analysis

1. Mass spectrometry analysis revealed that pUL43 or pUL24 proteins interact with TRBP and Dicer respectively

UL24 and UL43 are both members of the US22 family of HCMV, and there are reports in the literature that the viral proteins they encode have interactions [10]. Therefore, we hypothesize whether UL24 and UL43 can cooperate and regulate some of them like IRS1 and TRS1 members in the US22 family. The reaction within the cell also plays an important role in the process of viral infection. To explore this idea, we constructed Flag-tagged GFP, UL24, and UL43 overexpression plasmids and transfected them into HEK293T cells. After 48 hours of transfection, the cells were collected and lysed with cell lysate, and then added to the magnetic beads with Flag antibody. After incubation, the Flag-tagged protein was bound to the magnetic beads, and finally, the eluted product was competitively eluted with Flag peptide.

The eluted products are separated by SDS-PAGE protein gel, and then the protein gel is silver-stained, as shown in **Figure 1A**

The UL43 and UL24 were shown many specific bands compared to GFP as shown in **Figure 1B**. therefore, we expanded the number of cells for immunoprecipitation and Coomassie brilliant blue staining and took specific bands for mass spectrometry. Based on the mass spectrometry results, the specific protein that interacts with pUL43 was TRBP, and the specific protein that interacts with pUL24 was Dicer. TRBP and Dicer can be combined, and they are important components that regulate the production of miRNAs. It is reported in the literature that pUL24 and pUL43 can interact. Therefore, we speculate whether UL24 and UL43 can coordinately regulate TRBP and Dicer, thereby affecting the production of miRNAs.

2. Overexpression to verify the protein-protein interaction detected by mass spectrometry

To prove that these two proteins can indeed interact specifically with pUL24 or pUL43, we perform immunoprecipitation experiments and detect whether they can interact with TRBP or Dicer by immunoblotting with specific antibodies. In the experiment, the HCMV virus gene US31 was used as a negative control, and a Flag-tagged US31 overexpression plasmid was constructed. We also constructed an HA-labeled UL43 overexpression plasmid to verify the interaction between pUL24 and pUL43. Because Dicer and TRBP are both important members of RISC, and the complex contains small nucleic acid molecules, to rule out that the interaction between proteins is mediated by nucleic acid rather than the direct action of protein, we treated the sample with RNase A enzyme, and then used Magnetic beads labeled with Flag antibody were used for immunoprecipitation. The results of western blotting showed that pUL24 or pUL43 can interact with Dicer and TRBP, and pUL24 can also interact with pUL43.

Next, we use TRBP as bait to test whether pUL24 and pUL43 can be co-precipitated. First, we constructed a TRBP overexpression plasmid with HA tag and transfected it with US31, UL24, or UL43 into HEK293T cells. Cells were lysed 48 hours after transfection and treated with RNase A enzyme as well. Then incubate the protein A magnetic beads with HA antibody to form a HA antibody-labeled magnetic bead, then add the processed cell supernatant to the magnetic beads, undergo immunoprecipitation, and finally separate the protein on the magnetic beads with the lysis solution. The product is eluted. The results of western blotting showed that TRBP could co-precipitate pUL24 and pUL43. In summary, pUL24 or pUL43 can interact with Dicer and TRBP, which are specific interactions between proteins and are not mediated by nucleic acids.

3. Verification of the interaction between cell endogenous proteins

Although we have demonstrated that pUL24 or pUL43 can interact with Dicer and TRBP through overexpression in HEK293T cells, this is consistent with our mass spectrometry results. However, in the case of viruses infecting cells, whether these two viral proteins can also interact with Dicer and TRBP needs further verification. We first carried out viral modification in the BAC containing the full genome of HCMV, respectively adding the HA tag to the C-terminal of the UL24 virus gene, and adding the Flag tag to the C-terminal of UL43 to construct two new BACs, namely pBAC-AD-UL24-HA and pBAC-AD-UL43-Flag. BAC was electroporated to MRC5 cells to obtain and infect the cells with these two viruses. After 48 hours of infection, the cells are collected and tested in related experiments.

As shown in **Figure 3A**, we respectively infected MRC5 cells with the viruses AD-GFP and AD-UL24-HA, collected cell samples 48 hours after infection, and then used HA antibody-coated magnetic beads for immunoprecipitation. The results of western blotting showed that compared with AD-GFP-infected cells, in AD-UL24-HA-infected cells, UL24-HA could co-precipitate TRBP and Dicer. In **Figure 3B**, we also used the AD-UL24-HA virus to infect cells, and Protein A magnetic beads were used and incubated with IgG (control) and TRBP antibodies.

The supernatant was added to the processed magnetic beads. The results of co-immunoprecipitation and immunoblotting showed that the antibody incubated with IgG could not co-precipitate pUL24, but the antibody incubated with TRBP antibody could co-precipitate pUL24 and Dicer. These two experimental results show that pUL24 expressed by the virus can interact with the endogenous TRBP and Dicer of the cell.

Similarly, to identify whether pUL43 can interact with endogenous TRBP and Dicer, like pUL24 in the infected cells. As shown in **Figure 3C**, MRC5 cells were infected with the viruses AD-GFP and AD-UL43-Flag, and cell samples were collected 48 hours post-infection, and then used Flag antibody-coated magnetic beads for immunoprecipitation. The results of western blotting showed that, in the cells infected with AD-UL43-Flag, UL43-Flag was able to co-precipitate TRBP and Dicer compared with the cells infected with AD-GFP. Furthermore, in **Figure 3D**, we infected cells with the AD-UL43-Flag virus and the Protein A magnetic beads were incubated with IgG or TRBP antibodies, then the supernatant of the infected cell lysate was added to the processed magnetic beads. The results of blotting showed that the antibody incubated with IgG could not co-precipitate pUL43, but the antibody incubated with TRBP could co-precipitate pUL43 and Dicer. These two experimental results show that pUL43 expressed by the virus can interact with the endogenous TRBP and Dicer in the cell.

In summary, the experimental results show that in the process of HCMV virus infection, the viral protein pUL24 or pUL43 can interact with the endogenous TRBP and Dicer.

4. Double knockout of UL24 and UL43 does not affect virus replication

According to the above experimental results, we found that pUL24 or pUL43 can interact with TRBP and Dicer, because TRBP and Dicer are two important components of RISC, which can regulate the production of miRNAs and thus regulate the expression of some genes, therefore; we speculate that whether these two viral proteins can work together to participate in the regulation of the production of miRNAs through the interaction with TRBP and Dicer that may ultimately play some important biological functions. Previous studies have shown that UL24 and UL43 are non-essential factors for the growth of the HCMV, any deletion of them will not affect virus replication [31, 32]. However, there is no related report of double deletion, we knocked out UL24 and UL43 in the wild-type virus genome at the same time to detect whether the replication of the virus will be affected. To perform viral gene knockout, the red recombination system was applied as reported in the previous literature [24].

We first electrotransformed the IScel-KanS containing the UL24 C-terminal homologous fragment into E. coli GS1783 containing wild-type pBAC-AD/Cre-GFP for homologous recombination, and then induced the enzyme to excise the IScel-KanS, and finally the BAC deleted UL24 was generated, named pBAC-AD-d24. Besides, using the same method as UL24 knockout, the IScel-KanS containing the C-terminal homologous fragment of UL43 was electroporated into E. coli GS1783 containing pBAC-AD-dUL24 for homologous recombination, and finally BAC double deleted UL24 and UL43 were obtained and named pBAC-AD-dd24-dd43. We finally obtained the double deletion virus AD-GFP-dd24-dd43 by electroporating BAC into MRC5 cells. We use the wild-type virus AD-GFP (or AD-WT) and the double deletion virus AD-GFP-dd24-dd43 (Or AD-Mut) with a low multiplicity of infection (MOI) of 0.1 and a high multiplicity of infection of 1. The MRC5 cells were incubated with the virus for 2 hours, and then removed the supernatant and replaced with a fresh medium. Finally, the cell supernatant was collected at a specific time point post-infection and used to test the virus titer. In **Figure 4A-B**, we found that the replication ability of the UL24/UL43 double-deleted virus was close to that of the wild-type virus.

Altogether, these results indicate that the double-knockout and single-knockout phenotypes of these two genes are the same and do not affect the virus replication and also show that these two viral genes are indeed non-essential genes for the growth of the HCMV virus.

We also tested the HCMV early gene IE1, the early gene UL44, and the late gene UL99 (pp28) and UL32 (pp150) expression. We infected MRC5 cells with MOI of 1 and collected cells at a specific time point for western blotting. In **Figure 4C**, we found that in cells infected with a double-deletion virus, the expression of viral protein IE1 and even other tested genes were down-regulated. We further tested the transcription of early viral genes, infected MRC5 cells with an MOI of 1, and collected RNA from the cells 8 hours after infection, and performed RT-qPCR. As shown in **Figure 4D**, the mRNA level of IE1 was also down-regulated in the early stage of infection. These data suggest that UL24 and UL43 may affect the early stage of virus replication. Next, we had to explore whether it is the double knockout of these two viral genes that affect virus entry into cells or virus viability.

5. Double knockout of UL24 and UL43 does not affect the ability of the virus to enter cells and the stability of the virus

Based on the above experimental results, we speculate that the double knockout of UL24 and UL43 genes may affect the ability of the virus to enter the cell or the viability of the virus. To test that, MRC5 cells were infected with the wild-type or deleted viruses with MOI of 1 for 2 hours, then the fresh medium was replaced and the cells were collected at the indicated time points and data analyzed by quantitative PCR. The input was the virus stock solution. In **Figure 5A-C**, we find that there is almost no difference between the wild-type and the deleted virus genome. This shows that the double knockout of UL24 and UL43 (AD-Mut) does not affect the ability of the virus to enter the cell.

Next, we performed virus particle stability tests on wild-type (AD-WT) and mutant viruses (AD-Mut). We treated the wild-type virus and the deleted virus with the 37 °C for 4, 8, and 16 hours. Then, the untreated virus and the processed virus were used to infect MRC5 cells at an MOI of 1. After 24 hours of infection, we collected and incubated the cells with the virus IE1 antibody and the corresponding secondary antibody, and the IE1 positive cells were counted under the fluorescent microscope. As shown in **Figure 5D**, the number of IE1 positive cells gradually decreases with the extension of the 37 °C treatment time, but interestingly, we found that the number of IE1 positive cells in the mutated virus was almost similar to that of the wild-type virus regardless of whether it was processed with 37 °C or not. We express the stability of the virus in terms of infectivity, as shown in **Figure 5E**. The results were consistent with **Figure 5D**. It was clear that treatment with 37 °C has reduced the virus infectivity but there was no difference between the wild type and the deletion type in the reduction levels. In summary, these results indicate that the double knockout of UL24 and UL43 does not affect the ability of the virus to enter cells as well as the virus stability.

6. Double knockout of UL24 and UL43 does not affect the expression of 4 HCMV miRNAs related to immune escape

miRNAs are non-coding RNAs with a size of about 22 nucleotides and can participate in the regulation of many signaling pathways in cells. Initially, the Pfeffer study group were identified 9 HCMV-encoded miRNAs [33]. Later on, 26 miRNAs have been discovered through a series of studies, and they are all produced by RISC processing [34]. These miRNAs perform various functions after the virus infects cells, such as regulating the cell cycle, the expression of certain host or viral genes, viral DNA synthesis, the generation of virus assembly centers, and the production of immune regulation-related inflammatory factors [35-41].

According to our results that the UL24 and UL43 can interact with TRBP and Dicer and their knockout (AD-Mut) does not affect virus replication, entry, and stability. Also, based on previous studies that many of the miRNAs encoded by HCMV are involved in regulating the immune response and helping the virus escape. We speculate that pUL24 and pUL43 may work together to regulate some viral miRNAs. They may regulate some signaling pathways to escape the immune system's recognition of the virus. There are currently four known HCMV miRNAs that are mainly involved in immune regulation, which are miR-UL112-3p, miR-US5-1, miR-UL148D, and miR-US25-1-5p, therefore; we speculated that whether the knockout of UL43 and UL24 genes affect the expression of these miRNAs. To perform that, MRC5 cells were infected with wild-type viruses at an MOI of 1, and the cells were collected to detect the expression of these miRNAs. We extract RNA from cells and use the specific reverse transcription primers of these miRNAs, as shown in **Table 3**, to obtain their cDNA by the Stem-loop RT PCR method, and finally, qPCR was used for quantitative detection. As shown in **Figure 6A-D**, the expression of these miRNAs was very high at 48 hours post-infection (hpi). Therefore, we have used the 48 hpi as the time point to compare the differences between these miRNAs in wild-type and mutated virus-infected cells. As shown in **Figure 6E-H**, the expression levels of these four miRNAs under different virus infections have no significant difference, and this result indicates that the double knockout of UL24 and UL43 does not affect the production of these miRNAs.

7. RNA-seq found that the double knockout of UL24 and UL43 resulted in the down-regulation of miR-UL59

To further analyze the role of UL24 and UL43 in HCMV virus infection of host cells, and to find the target miRNAs regulated by these two genes, we performed RNA-seq. The MRC5 cells were infected with AD-WT and AD-Mut respectively with an MOI of 1. After 48 hours post-infection (hpi), cell collection and RNA extraction with 1ml Trizol were performed and sent for RNA-seq. Based on the results of RNA-seq, we found that the expression of miR-UL59 was different. For further verification, we infected the MRC5 cells with AD-WT and AD-Mut again at an MOI of 1. Each virus was treated with two identical treatments. After 48 hpi, we used Trizol to collect cells and extract RNA and the Stem-loop method to design miR-UL59 reverse transcription primers and quantitative qPCR primers (**Table 4**) [42-43]. Experimental results showed that the double knockout of UL24 and UL43 resulted in the downregulation of miR-UL59.

At present, there are limited research reports on miR-UL59, and it is described that the target gene of miR-UL59 is ULBP1 (UL16-binding protein 1) [44]. ULBP1 is one of the ligands of NKG2D, and NKG2D is an activating receptor expressed on immune effector cells, which can recognize different MHC I related ligands, including MIC and ULBP proteins. Infection or stress response can induce the expression of the NKG2D ligand, leading to the activation of effector cells and ultimately killing the ligand-related target cells [45-48]. Besides, it has previously been reported that the membrane glycoprotein UL16 of HCMV can bind to three NKG2D ligands, namely MICB, ULBP1, and ULBP2, and UL16 is also very important for the immune escape of HCMV.

To check the impact of these two genes on ULBP1 mRNA levels, we similarly infected the MRC5 cells with AD-WT and AD-Mut at an MOI of 1. After 48 hpi, we used Trizol to cells were collected and the ULBP1 mRNA levels were measured

by quantitative qPCR using specific primers as shown in **Table 4** [42, 43]. Experimental results showed that the double knockout of UL24 and UL43 has resulted in up-regulation of ULBP1 mRNA levels

Altogether, RT-qPCR results indicated that the double knockout of UL24 and UL43 resulted in the down-regulation of miR-UL59 and the up-regulation of ULBP1 mRNA levels. This suggests that UL24 and UL43 may regulate the expression of miR-UL59, which in turn affects the expression of ULBP1, and thus regulating anti-virus immune response, this will need further experimental confirmation in the future.

8. Double knockout of UL24 and UL43 in clinical virus strains does not affect virus replication, entry, and virus stability

In addition to the HCMV experimental virus strain, we also want to know whether the double knockout of UL24 and UL43 genes in the HCMV clinical virus strain (TB40E-Mcherry = TB-WT), can similarly affect the virus replication and its cellular entry. At first, we generated UL24/UL43 double deletion virus named TB40E-Mcherry-dd24 -dd43 (TB-Mut). Then to analyze the growth ability of this clinical mutant virus (TB-Mut), we infected the MRC5 cells with the wild type and the deleted virus for 2 hours at an MOI of 0.1 or 1, and then the medium was replaced with a fresh one. Finally, the cell supernatant was collected at a specific time point post-infection and was used to infect MRC5 cells and the virus titer was measured by tissue culture infectious dose 50% (TCID50) assay. As shown in **Figures 8A-B**, the growth ability of the Mut virus was almost similar to that of the wild-type virus. These results indicate that the double knockout of UL24 and UL43 in clinical virus strain, similar to the experimental virus strain, does not affect the replication of the virus, thus confirming that the UL24 and UL43 are non-essential genes for clinical virus strains too.

Next, we checked whether the knockout of these two genes affects virus entry into cells. To perform that, the MRC5 cells were infected with wild-type and deleted clinical viruses at an MOI of 1 for 2 hours, then the supernatant was removed and replaced with fresh ones. The cells were collected at the specified time point post-infection, and the viral genome (viral DNA) was extracted and analyzed by qPCR. The virus stock solution was used as Input. As shown in **Figure 8C-E**, there was almost no difference in the amount of wild-type virus genome and deleted virus genome, which shows that the double knockout of UL24 and UL43 does not affect the ability of clinical strains to enter cells as well.

Furthermore, we tested the virion stability of wild-type and deleted viruses in the clinical strain. The wild-type virus and the deleted virus were treated with 37 °C for 4, 8, and 16 hours, then the MRC5 cells were infected with the untreated and the treated virus separately with MOI of 1. At 24 hours post-infection (hpi), the virus IE1 gene was incubated with primary and the corresponding fluorescent secondary antibody, finally the IE1 positive cells were calculated under the fluorescence microscope. As shown in **Figure 8F**, the number of IE1 positive cells gradually decreases with the extension of the 37°C treatment time but there was no significant difference between the number of IE1 positive cells with both the wild type and mutant virus. Also, to check the stability of the virus in terms of its infectivity. As shown in **Figure 8G-F**, the treatment with 37 °C has reduced the infectivity of the virus without a significant difference between the wild type and the deletion type. In summary, these results indicate that HCMV clinical viruses were consistent with experimental strain in that the double knockout of UL24 and UL43 does not affect the entry of clinical strains into cells and the stability of virus particles.

9. Double knockout of UL24 and UL43 in clinical viruses also leads to down-regulation of miR-UL59

The previous results showed that in the experimental virus strain, the double knockout of UL24 and UL43 would inhibit the expression of miR-UL59, and the expression of its target gene ULBP1 was also affected, so we wanted to observe whether this phenotype is in clinical virus strains unanimous.

We spread MRC5 cells in a 12-well plate and then infected the cells with wild-type virus and deletion mutant virus with MOI of 1. After 48 hours of infection, the cells were harvested with Trizol, and RNA was extracted. Next, quantitative PCR

primers were used to detect the expression of miR-UL59. In Figure 3.9A, we detected that the expression of miR-UL59 was down-regulated after the virus-infected cells. In 3.9B, we also found that the target gene of miR-UL59, ULBP1, was down-regulated. The mRNA level was higher in cells infected with the deletion virus than in cells infected with the wild-type virus, and the results were consistent with the results of the experimental virus strains, indicating that knocking out UL24 and UL43 in clinical strains can also inhibit the expression of miR-UL59 and upregulate the mRNA of ULBP1 Level. As one of the ligands of NKG2D, ULBP1 is essential for cellular immunity. Therefore, we speculate that although UL24 and UL43 of the HCMV virus do not affect virus replication, they may participate in the regulation of the expression of some miRNAs in the cell and thus participate in immune regulation to promote the immune escape of the virus.

Discussion

UL24 and UL43 are the interlayer proteins that make up HCMV virus particles. The previous studies have shown that these two genes are not necessary for virus replication, and their deletion does not affect virus growth [2, 32]. A recent study proved that the pUL24 and pUL43 proteins encoded by these two viral genes can interact [10]. It is reported in the literature that some genes in the US22 family can play a synergistic effect during viral infection. For example, UL29/28 can activate the expression of early genes, and TRS1 and IRS1 can regulate gene transcription to promote viral replication. Moreover, even if some genes expressing interlayer protein are not necessary for virus growth, they can inhibit the expression of antiviral genes, thereby inhibiting the immune response and helping the virus escape, such as UL82, UL83, etc., so we speculate whether UL24 and UL43 are also. During viral infection, it plays a synergistic role in regulating certain intracellular reactions. First, we constructed the overexpression plasmids of these two viral genes and transferred them into cells for expression. Through co-immunoprecipitation and mass spectrometry analysis, we found that the Dicer and TRBP proteins can interact with these two viral proteins. (pUL24) and (pUL43) respectively in HEK293T cells. To prove this interaction, we added the HA tag to the C-terminus of UL24 or the Flag tag to the C-terminus of UL43 in the HCMV virus genome. Then these two viruses were used to infect MRC5 cells separately. Based on immunoprecipitation assay, the data showed that pUL24 or pUL43 can also interact with Dicer and TRBP. These results indicate that endogenous interactions also exist in the infected cells.

Furthermore, to check whether they have a synergistic effect, we knocked out UL24 and UL43 genes together (double deletion) to observe the phenotype. First, through viral genome modification, these two viral genes were knocked out to construct a mutant virus. The results of virus growth analysis showed that knockout of these two genes at the same time did not affect virus growth, but affected the transcription and expression of early viral genes. Besides, we checked whether the knockout of these two genes affected virus entry. To test that we incubated the virus with the MRC5 cells for a certain period, then the cells were collected and the extracted genomes were measured by quantitative PCR. The results showed that the knockout of these two genes did not affect the virus entry, as well as the infectivity and the stability of the virus was also not affected. In addition to the experimental HCMV strains, we also obtained the same results with the clinical HCMV strains.

Furthermore, Dicer and TRBP are two important cellular proteins for processing pre-miRNAs and composing RNA-induced silencing complex (RISC), we checked whether the encoded pUL24 and pUL43 can regulate the production of some miRNAs through the interaction with Dicer and TRBP, and thereby regulating some cellular immune responses to facilitate the virus escape. Since studies have been reported that HCMV tegument proteins can inhibit the expression of some cellular proteins or surface antigens to escape the immune system, for example, a study has been reported that UL82, the interlayer protein of HCMV, can inhibit STING-mediated signaling pathways to avoid antiviral immune responses [49]. Also, the protein pp65 encoded by the viral gene UL83 can inhibit the expression of antiviral genes [50]. This raises the question of whether these two genes may involve in the regulation of the antiviral immune response. Based on the results of previous experiments, we expected that UL24 and UL43 may regulate the production of

downstream miRNAs. There are about 26 miRNAs encoded by HCMV, of which the most studied antiviral related are miR-UL112-3p, miR-US5-1, miR-UL148D, and miR-US25-1-5p. We identified that the expression of these four miRNAs was not affected in cells infected with the mutant-type virus compared to wild-type one.

To find the target HCMV miRNAs of these two viral genes, we performed RNA-seq and identified that UL24 and UL43 regulate the expression of miR-UL59. Experimental data showed that compared with wild-type virus, the double deletion virus type has resulted in the down-regulation of miR-UL59 expression, which is a newly discovered HCMV miRNA. A study has been reported that the target gene of miR-UL59 is ULBP1. ULBP1 is one of the ligands of NKG2D, which is an activating receptor expressed on immune effector cells that can recognize different MHC I-related ligands, including MIC and ULBP proteins. Infection or stress response can induce the expression of the NKG2D ligand, leading to the activation of effector cells and ultimately killing the ligand-related target cells. Therefore, we tested the expression of ULBP1 in infected cells. Experimental data showed that UL24 and UL43 double-deletion virus-infected cells had significantly higher levels of ULBP1 mRNA than wild-type virus-infected cells, and UL24 and UL43 double-deletion clinical virus infections also got same As a result, the deletion of UL24 and UL43 resulted in the down-regulation of miR-UL59 and the increase of ULBP1 mRNA levels.

In summary, our current research results show that when the two non-essential genes UL24 and UL43 are knocked out at the same time, they have no effect on the growth, entry, and stability of the virus, but lead to the down-regulation of virus miR-UL59, and its corresponding target the mRNA level of ULBP1 gene was up-regulated. Based on the existing results, we speculate that UL24 and UL43 are related to the expression of miR-UL59. UL24 and UL43 reduce the expression of ULBP1 on the cell surface, which will reduce the cytotoxicity mediated by infected cells to NK cells, thereby avoiding NK cell mediation. This leads to apoptosis and helps the immune to escape infected cells. Therefore, further experiments need to explore whether UL24 and UL43 are related to NK cell-mediated cytotoxicity in the future.

Declarations

Future Work: Also, we will try to apply western blotting and flow cytometry to detect whether the protein expression of ULBP1 on the cell surface is affected in the case of double knockout of UL24 and UL43. And we will also test whether there is a difference in the toxic effects of NK cells on HCMV-infected cells in the case of wild-type and deleted virus infections.

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Authors' contributions

Conceptualization and Writing, S. S.; Data analysis, S. S, and N. M. S. Authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no conflict of interest.

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Tables

Table 1 Primers for Plasmids Construction

Plasmid	Sequences (5'-3')
pLKO-3×Flag-sf-GFP	F: 5' ACGCGTCGACGTGAGCAAGGGCGAGG-3'
	R: 5'-GGAATTCCTTGTACAGCTCGTCC-3'
pLKO-3×Flag-sf-UL43	F: 5' ACGCGTCGACGAGAAAACGCCGGCGGAG-3'
	R: 5'-GGAATTCTCACCTTCGAGCAAAGAGCC-3'
pLKO-3×Flag-sf-UL24	F: 5'-TGATGATAAAGTCGACGAGGAGACCCGGGCGGG-3
	' R: 5'-TCGAGGTCGAGAATTCTCAACGGTGCTGACGTC-3'
pLKO-3×Flag-sf-US31	F: 5'-TGATGATAAAGTCGACTCGCTCTTGGAGCGCGAAG-3
	' R: 5'-TCGAGGTCGAGAATTCTTAGTGTTGTTCCACCCC-3'
pLKO-HA-UL43	F: 5'-ACGCGTCGACCATGTACCCTTATGACGTGCC-3'
	R: 5'-GCGGCCGCGGATCCGAGAAAACGCCGGCGGAGAC
pLKO-TRBP-HA	F: 5'-ACGCGTCGACATGAGTGAAGAGGAGCAA-3'
	R: 5'-GTCATAAGGGTACTTGCTGCCTGCCAT-3'

F; Forward, R; Reverse

Table 2 Primers for BAC Recombination

BAC Plasmid	Sequences (5'-3')
Δ UL24 BAC (AD/TB)	F: 5'TCTGCTGAGGTGCGTTTCGGCTCAGTTGCTTACCTGCATACCTGTGACGCCACGAGTGACGTAGG GATAACAGGGTAATCGATTT '3
	R: 5'TATGCCCCAAGCAGCGTCGTCGTCCTCGTGGCGTCACAGGTATGCAGGTAAGCAACTGAGCCA GTGTTACAACCAATTAACC '3
Δ UL43 BAC (AD/TB)	F: 5'GGCCGCGTGCCTGGGAACGCGCACGGCGCGGTCCCGTGACCGCGGACGCCGT CGGTACAGGAT GACGACGATAAGTAGGG '3
	R: 5'TGGTAACTGTGGTGGAGACGGTACCGACGGCGTCCGCGGTACGGGACCGCGCCGTGCGCCAACCA ATTAACCAATTCTGATTAG '3
UL24-HA BAC (AD)	F: 5'AAAGGACGTCAGCACCGTGCCACCATGGC '3 5'CTCGTGGCGTCACAGTCAGAATTCAGCATAATC '3
	R: 5'ACGGCATCTACGATCGCGTGCCCGACTGCCCAAAGGACGTCAGCACCGT '3 5'GGTGTTTTATGCCCAAGCAGCGTCGTCGTCCTCGTGGCGTCACAGTCA '3
UL43- Flag BAC (AD)	F: 5'GGGCTCTTTGCTCGAAGGGACTACAAAGACC '3 5'GACGGCGTCCGCGTTTCAGAATTCCTTGTC '3
	R: 5'AACTGTTTTGCCGTGTTTTGCGCAAAAAGAAGGGGCTCTTTGCTCGAAGG '3 5'GCGACGGTGGTAACTGTGGTGGAGACGGTACCGACGGCGTCCGCGTTCA'3

F: Forward, R: Reverse

Table 3. Stem-loop Primers for RT-PCR

Primers	sequences (5'-3')
miR-UL112-3p	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGCCTGGA
miR-US5-1	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGACGCTCTC
miR-UL148D	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCGGTGAAG
miR-US25-1-5p	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGGGTCCGAG
miR-UL59	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGACGGCATG

Table 4. Primers for quantitative PCR

Plasmid	sequences (5'-3')
IE1 qF	CAAGTGACCGAGGATTGCAA
IE1 qR	CACCATGTCCACTCGAACCTT
ULBP1 qF	GTACTGGGAACAAATGCTGGAT
ULBP1 qR	AACTCTCCTCATCTGCCAGCT
miR-UL112-3p qF	AAGTGACGGTGAGATCCA
miR-US5-1 qF	TGACAAGCCTGACGAG
miR-UL148D qF	TCGTCCTCCCCTTCTTC
miR-US25-1-5p qF	AACCGCTCAGTGGCTC
miR-UL59 qF	GTTCTCTCGCTCGTCATG
Universal Rev	CTCAACTGGTGTCTGGGA

Table 5. Primers for quantitative PCR

Plasmid	sequences (5'-3')
IE genome qF	TCTGCCAGGACATCTTTCTCG
IE genome qR	GGAGACCCGCTGTTTCCAG
Actin qF	CTCCATCCTGGCCTCGCTGT
Actin qR	GCTGTACCTTCACCGTTCC

qF, qPCR forward; qR, qPCR reverse

Figures

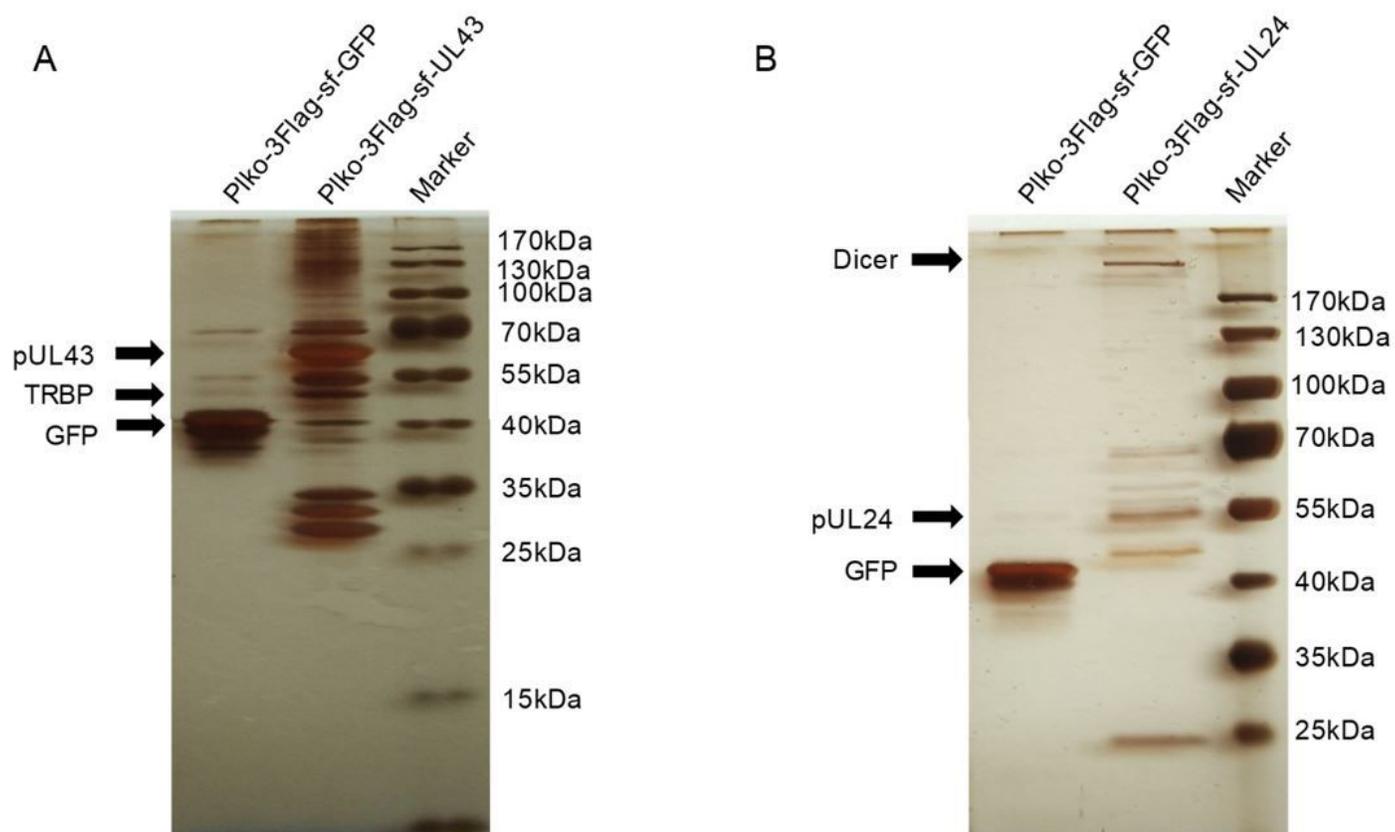


Figure 1

The results of silver staining. (A) pLKO-3×Flag-sf-UL43 or (B) pLKO-3×Flag-sf-UL24 was transfected into HEK293T cells, after 48h, cells were collected and the cell lysate was detected by immunoprecipitation assay. The results of proteogel electrophoresis and silver staining showed that pUL24 and pUL43 could pull down more specific bands compared with GFP (control).

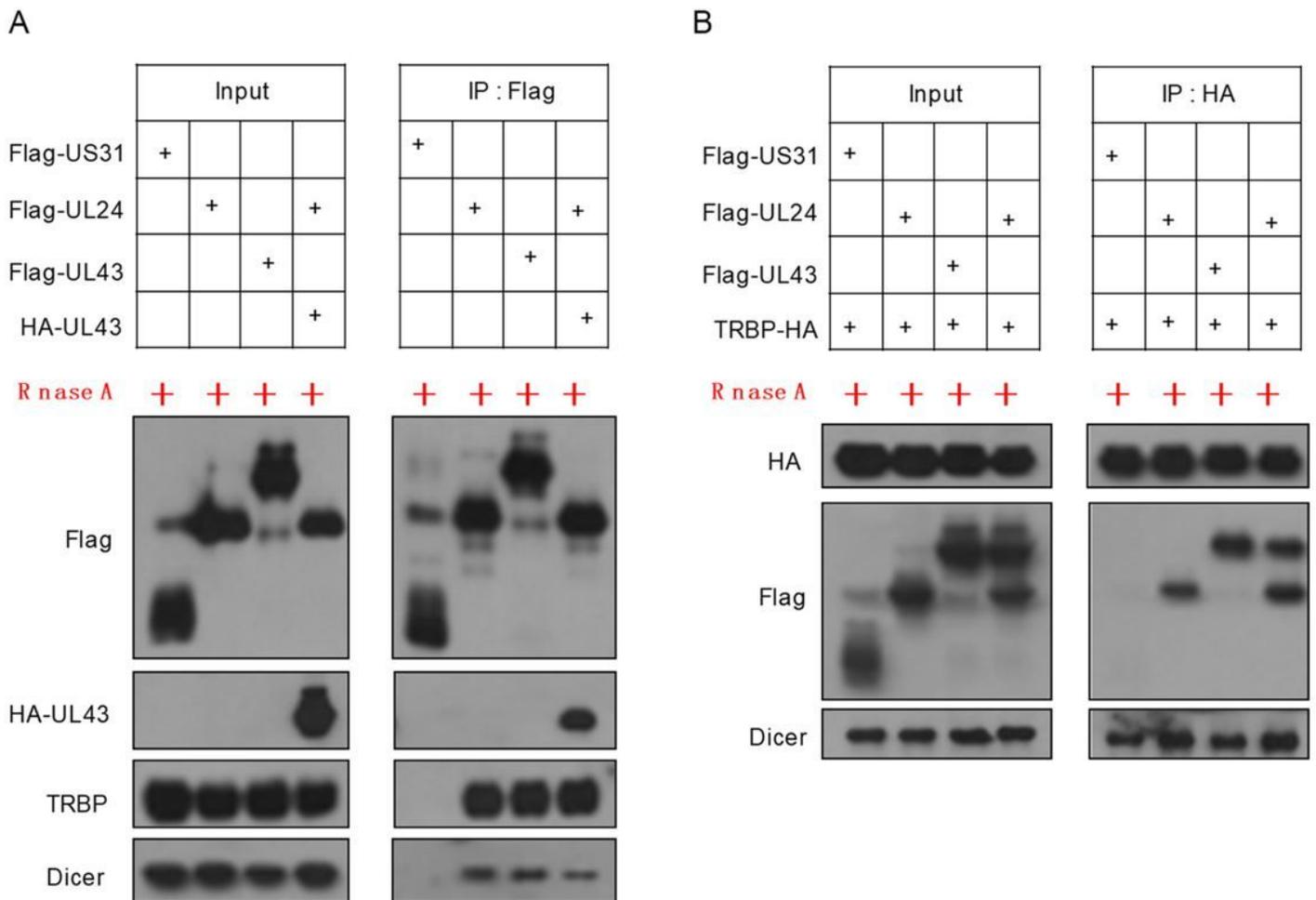


Figure 2

pUL24 and pUL43 interact with Dicer and TRBP specifically. (A) pUL24 and pUL43 plasmids were transfected into HEK293T cells. After 48 hours post-transfection, cells were collected and the cell lysate was processed by immunoprecipitation assay using Flag beads. And the immunoprecipitated proteins were detected by immunoblotting. The results of the immunoblotting showed that pUL24 and pUL43 were associated together, and they both could interact with Dicer and TRBP specifically. (B) These plasmids were also transfected with TRBP respectively and protein A beads labeled by HA antibody were used to conduct immunoprecipitation assay. And the results were consistent with figure A.

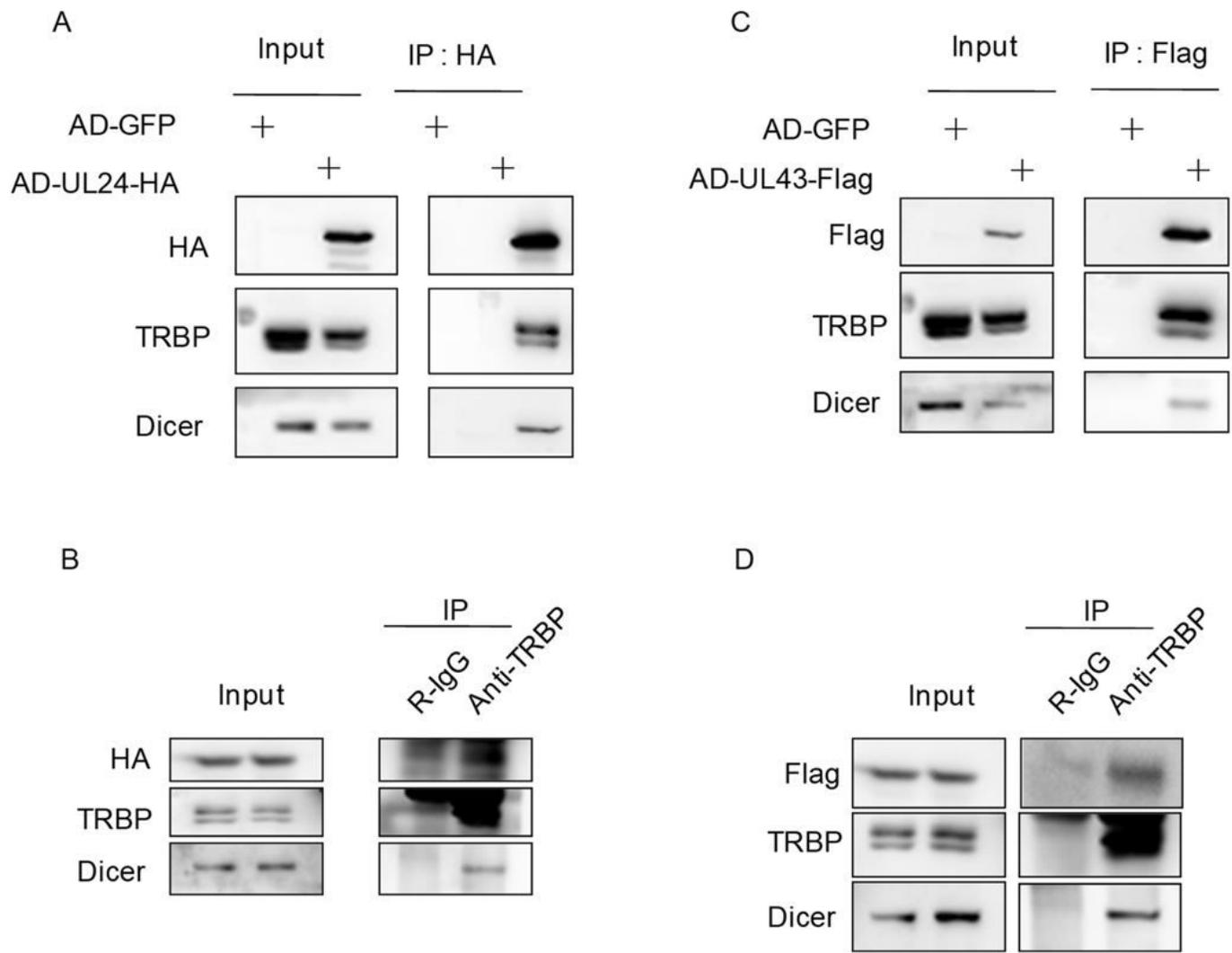


Figure 3

Endogenous pUL24 or pUL43 can also interact specifically with Dicer and TRBP. (A) MRC5 cells were infected with virus AD-GFP and AD-UL24-HA respectively and then conducted immunoprecipitation with HA beads. (B) AD-UL24-HA virus was used to infect MRC5 cells, and protein A beads were used for immunoprecipitation. In the control group, beads were incubated with IgG antibodies, and in the experimental group, beads were incubated with TRBP antibodies. (C) MRC5 cells were infected with AD-GFP virus and AD-UL43-Flag virus respectively and then conducted immunoprecipitation with Flag beads. (D) MRC5 cells were with AD-UL43-Flag virus, and protein A beads were used for immunoprecipitation. In the control group, beads were incubated with IgG antibodies, and in the experimental group, beads were incubated with TRBP antibodies.

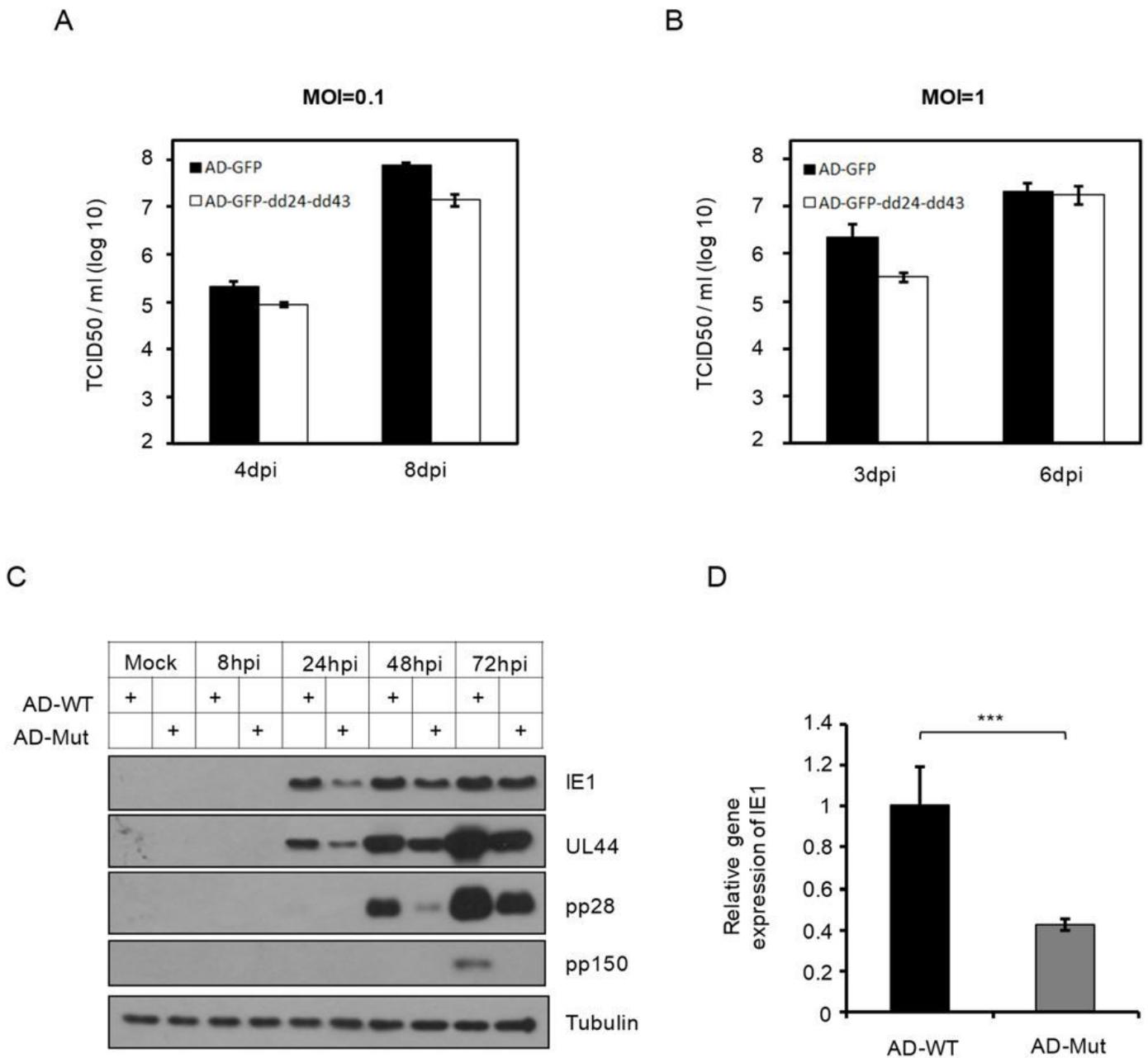


Figure 4

Double knockout of UL24 and UL43 did not affect viral replication. (A) MRC5 cells were infected with virus AD-GFP or AD-GFP-dd24-dd43 at an MOI of 0.1 or (B) MOI of 1. The supernatant was collected at indicated time points post-infection and the viral growth titer was measured by tissue culture infectious dose 50% (TCID50) assay. A representative result of two biological and two technical replicates for a single experiment is shown. (C) MRC5 cells were infected with two viruses referred above at an MOI of 1, and cells were collected at indicated time points and the expression of viral genes was detected. (D) MRC5 cells were infected with two viruses referred above at an MOI of 1, and cells were collected with Trizol at 8hpi, and after RNAs extraction, the mRNA levels of IE1 were analyzed by quantitative qPCR. The relative gene expression levels were normalized against that of GAPDH, and the normalized gene expression of AD-GFP infected cells was set equal to 1, ***, $P < 0.001$. A representative result of two biological and two technical replicates for a single experiment is shown.

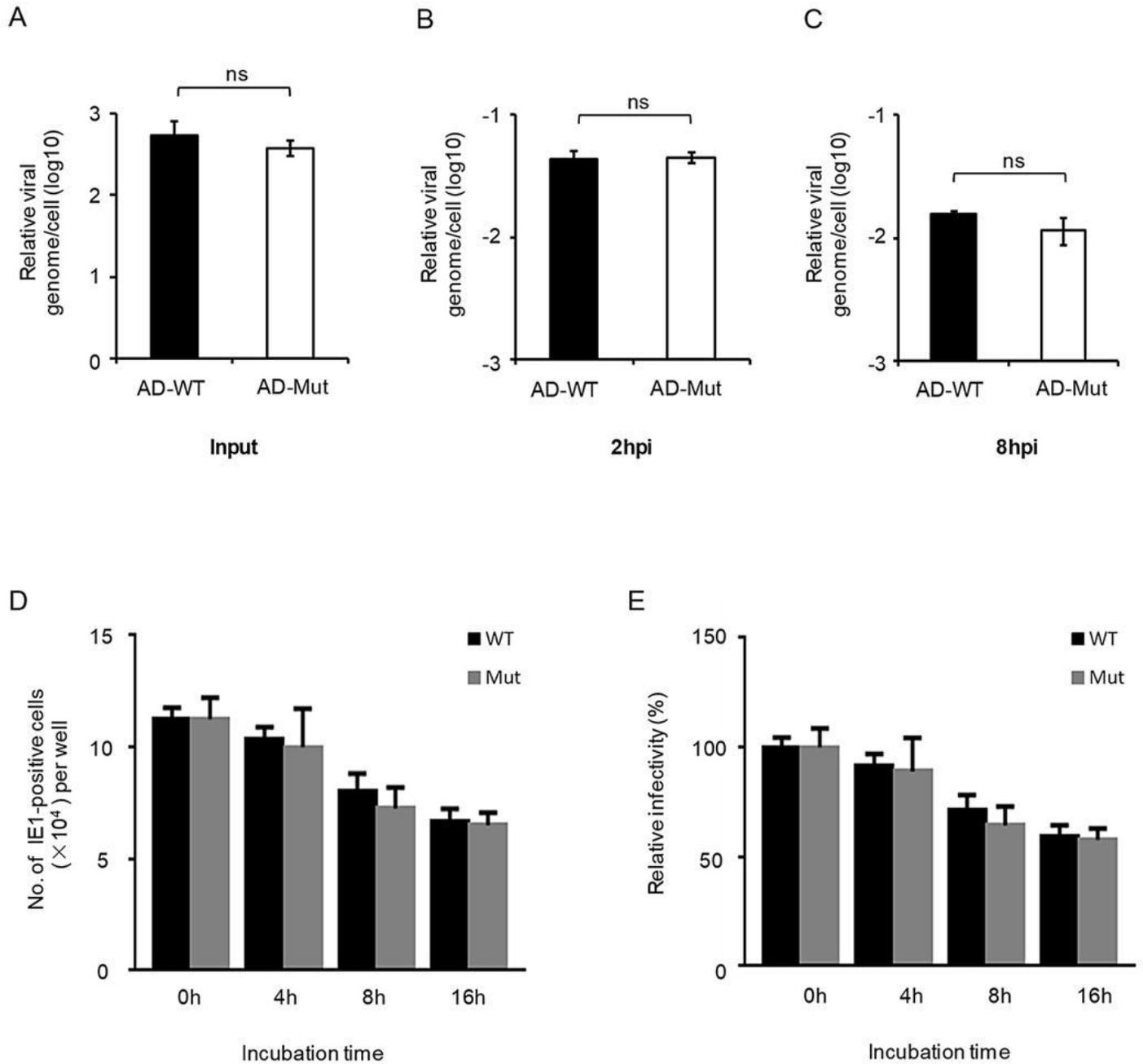


Figure 5

Double knockout of UL24 and UL43 did not affect viral entry and its stability. (A) Take some virus of AD-WT or AD-Mut at an MOI of 1, (B) cells were collected at 2hpi, and (C) at 8hpi. The viral genome was extracted and analyzed by quantitative PCR, ns= no significant. A representative result of two biological and two technical replicates for a single experiment is shown. (D) The viruses were treated at 37°C for certain times and then used to infect cells at 24hpi, and the number of infected cells (IE1-positive cells) was observed and counted under the fluorescent microscope. (E) The virions' stability is represented by the relative infectivity.

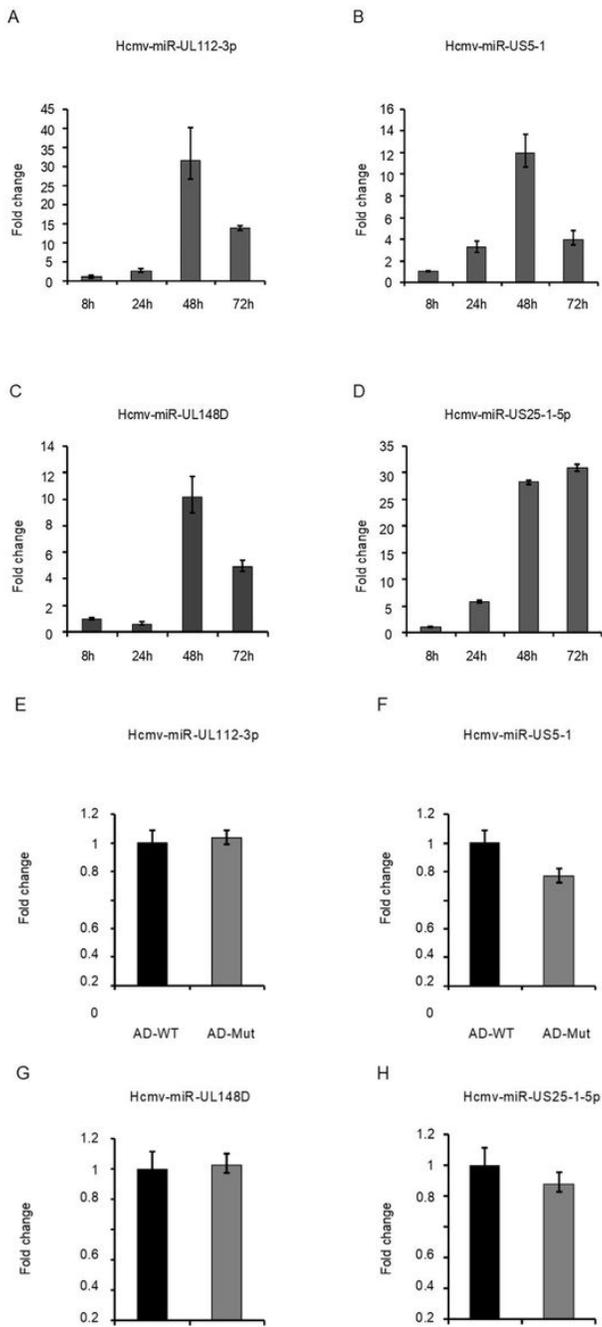


Figure 6

Double knockout of UL24 and UL43 didn't affect the expression of HCMV miRNAs. (A-D) MRC5 cells were infected with AD-WT at an MOI of 1, the cells were collected at indicated time points post-infection. Following the RNA extraction, the expression of miR-UL112-3p, miR-US5-1, miR-UL148D, miR-US25-1-5p were analyzed by quantitative qPCR. (E-H) MRC5 cells were infected with AD-WT or AD-Mut at an MOI of 1, and cells were collected at 48 hpi, and finally, the RNA extracted and the expression of miR-UL112-3p, miR-US5-1, miR-UL148D, miR-US25-1-5p were measured as previously by quantitative qPCR. A representative result of two biological and two technical replicates for a single experiment is shown.

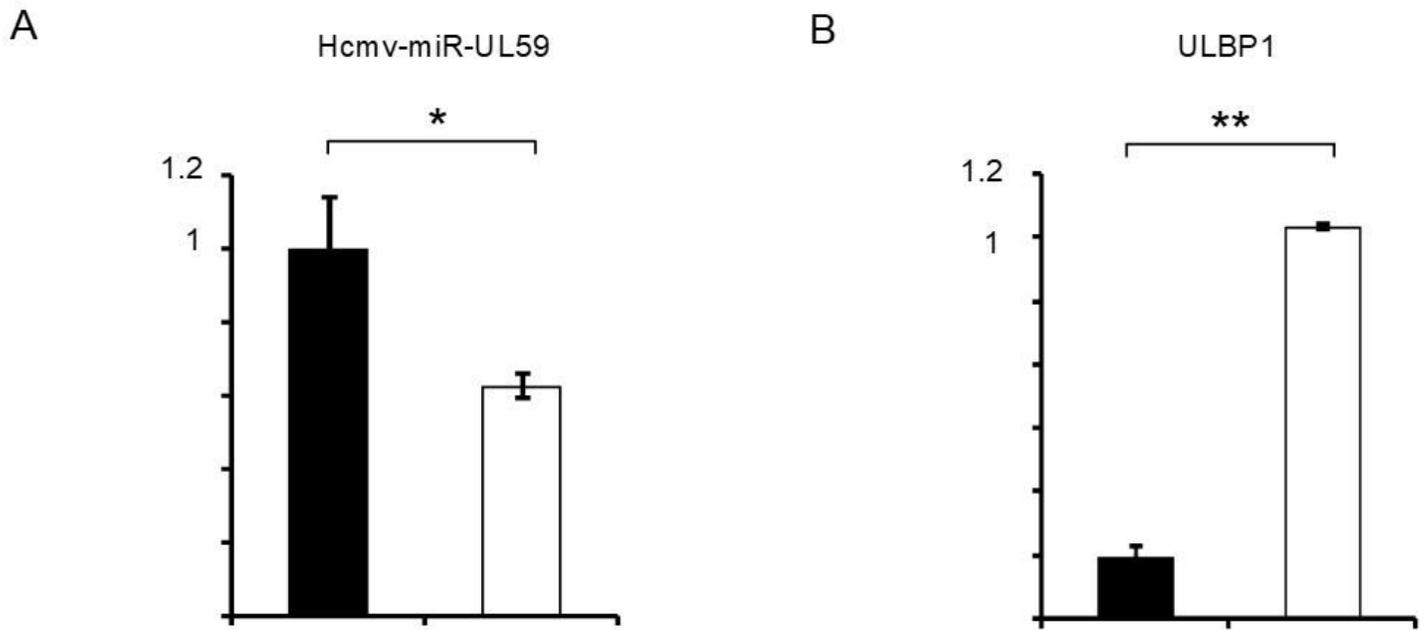


Figure 7

Double knockout of UL24 and UL43 down-regulated miR-UL59. (A-B) MRC5 cells were infected with AD-WT or AD-Mut at an MOI of 1. Then the cells were collected at 48 hpi, RNA was extracted and RT-qPCR analysis was applied to detect (A) miR-UL59 and (B) ULBP1 expression levels. *, P<0.05; **, P<0.01.

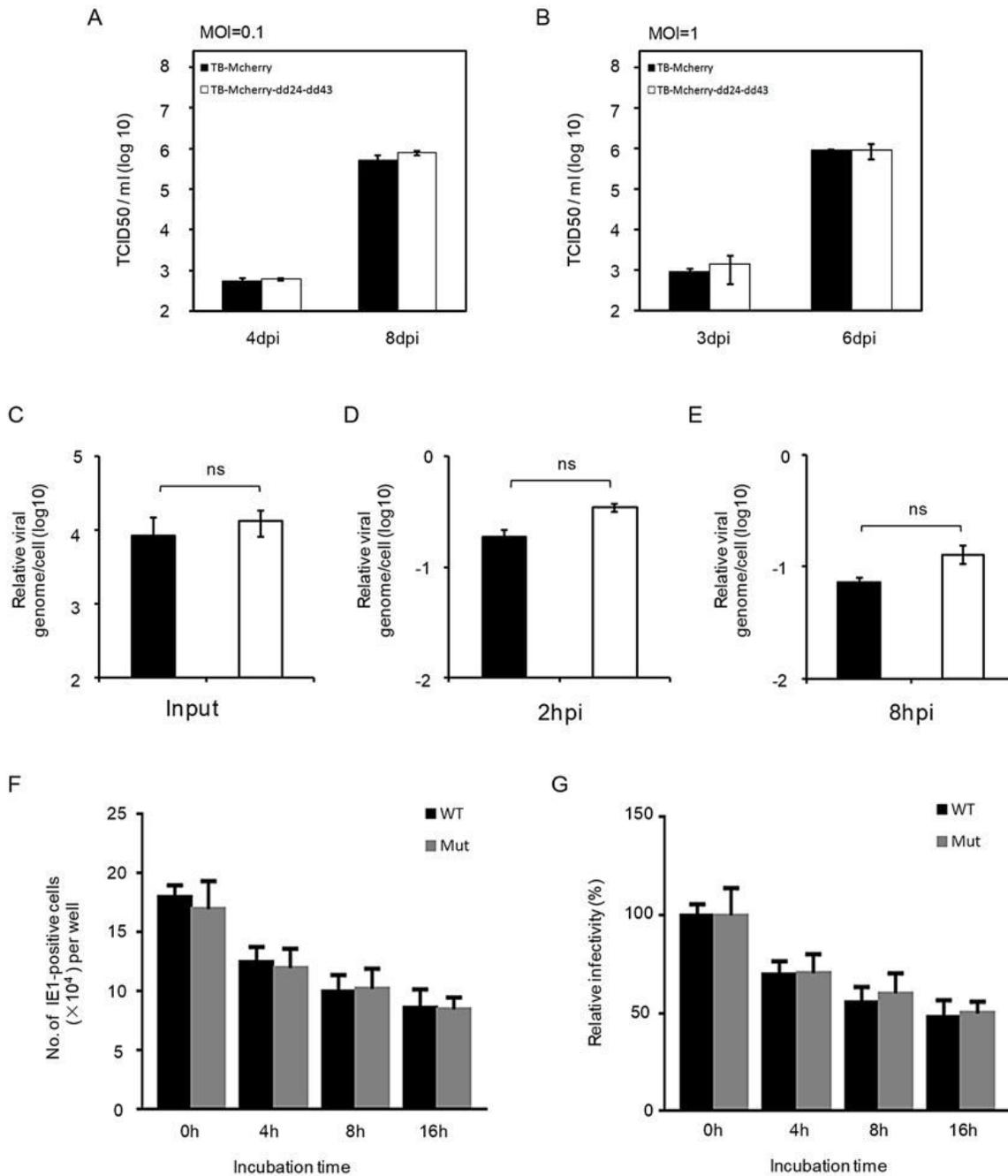


Figure 8

Double knockout of UL24 and UL43 with the clinical virus also does not affect virus replication, entry, and stability. (A-B) MRC5 cells were infected with TB-WT or TB-Mut at an MOI of 0.1 or 1. The supernatant was collected at indicated time points post-infection and ultimately, the virus titer was measured by tissue culture infectious dose 50% (TCID₅₀) assay. (C-E) Take TB-WT or TB-Mut virus at an MOI of 1, or collect cells at 2hpi and 8hpi, then extract viral genome and conduct quantitative PCR, ns, no significant. A representative result of two biological and two technical replicates for a single experiment is shown. (F) The viruses were treated at 37 °C for a certain time and then used to infect cells, at 24hpi, observed and counted the number of infected cells. (G) The virus stability is represented by the relative infectivity.

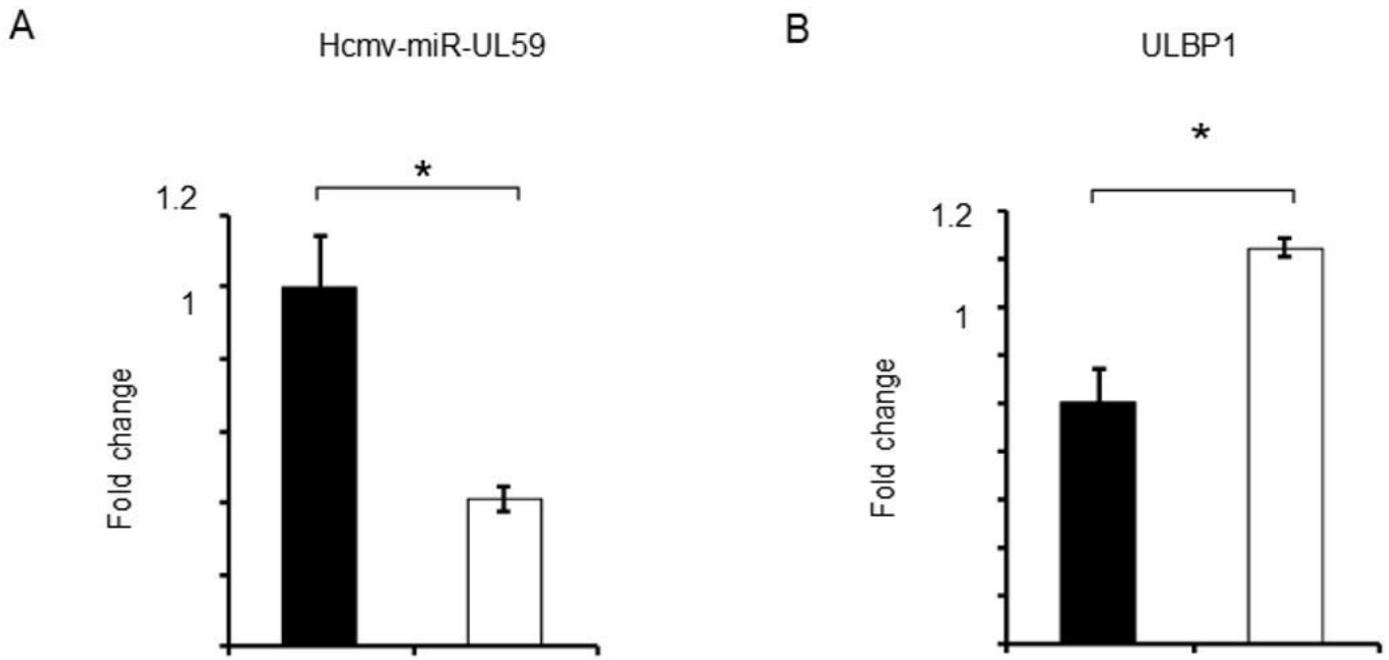


Figure 9

Double knockout of UL24 and UL43 down-regulated miR-UL59. (A) MRC5 cells were infected with TB-WT or TB-Mut at an MOI of 1. Cells were collected at 48hpi and extracted RNA. Detect miR-UL59 and (B) ULBP1 by quantitative qPCR, *, $P < 0.05$. A representative result of two biological and two technical replicates for a single experiment is shown.