The interferon-inducible protein TDRD7 inhibits AMP-activated protein kinase and thereby restricts autophagy-independent virus replication

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The interferon system is the first line of defense against virus infection. Recently, using a high-throughput genetic screen of a human interferon-stimulated gene short-hairpin RNA library, we identified a viral restriction factor, TDRD7 (Tudor domain–containing 7). TDRD7 inhibits the paramyxo-/pneumoviruses (*e.g.* Sendai virus and respiratory syncytial virus) by interfering with the virus-induced cellular autophagy pathway, which these viruses use for their replication. Here, we report that TDRD7 is a viral restriction factor against herpes simplex virus (HSV-1). Using knockdown, knockout, and ectopic expression systems, we demonstrate the anti–HSV-1 activity of TDRD7 in multiple human and mouse cell types. TDRD7 inhibited the virus-activated AMP-activated protein kinase (AMPK), which was essential for HSV-1 replication. Genetic ablation or chemical inhibition of AMPK activity suppressed HSV-1 replication in multiple human and mouse cells. Mechanistically, HSV-1 replication after viral entry depended on AMPK but not on its function in autophagy. The antiviral activity of TDRD7 depended on its ability to inhibit virus-activated AMPK. In summary, our results indicate that the newly identified viral restriction factor TDRD7 inhibits AMPK and thereby blocks HSV-1 replication independently of the autophagy pathway. These findings suggest that AMPK inhibition represents a potential strategy to manage HSV-1 infections.

The interferon (IFN)[2] system is a critical component of the antiviral innate immune responses in the vertebrates (1–4). Virus infection is rapidly detected by the pattern recognition receptors (PRRs), *e.g.* Toll-like receptors, RIG-I–like receptors, cGMP-AMP synthase, and stimulator of IFN genes (STING). The PRRs recognize viral components, such as viral nucleic acids, either the viral genome or the replication products, in specific subcellular compartments (5–10). The PRRs, upon binding their respective ligands, trigger the downstream signaling pathways, which via the adaptor proteins and kinases activate the transcription factors, *e.g.* interferon regulatory factors (IRFs) and NF-κB. These transcription factors co-operatively trigger the transcriptional induction of type I IFN, *e.g.* IFNβ, the antiviral cytokine. IFNs are secreted and act on the virus-infected as well as the uninfected cells via Janus kinase/signal transducer of transcription signaling pathways to trigger the synthesis of IFN-stimulated genes (ISGs). Some of the ISGs can be induced directly by the transcriptional action of IRF3 (11–13).

The antiviral functions of the IFNs are mediated by the ISG-encoded protein products, which, either alone or in combination with other ISGs, inhibit virus replication (3, 14, 15). The ISG-encoded proteins exhibit viral restriction activities by directly interfering with the viral life cycle, amplifying IFN responses or triggering cell death in the infected or as-yet uninfected neighboring cells (3, 15). Viperin, IFN-induced transmembrane proteins, and tetherin (BST2) restrict virus replication by inhibiting specific stages of the viral life cycle. Viperin inhibits hepatitis C virus replication by localizing to the cellular lipid droplets, the site of viral replication (16). Viperin also inhibits influenza A virus budding and release by disrupting the lipid rafts (17). IFN-induced transmembrane proteins inhibit viral attachment and uncoating of several enveloped viruses (18, 19). Tetherin inhibits the release of human immunodeficiency virus, type 1 (HIV-1) by tethering HIV-1 virion particles to the cell surface (20). Some ISGs, *e.g.* IFIT (IFN-induced protein with tetratricopeptide repeats), protein kinase R (PKR), 2′,5′-oligoadenylate synthetase (OAS), and Mx1, display broad antiviral activities *in vitro* and *in vivo* (21–28). The IFIT proteins recognize viral mRNAs to inhibit their translation or modulate cellular translation machinery by inhibiting eukary-

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This article contains Figs. S1–S3.

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[2] The abbreviations used are: IFN, interferon; ISG, interferon-stimulated gene; shRNA, short-hairpin RNA; HSV, herpes simplex virus; AMPK, AMP-activated protein kinase; PRR, pattern recognition receptor; STING, stimulator of IFN genes; IRF, interferon regulatory factor; HIV-1, human immunodeficiency virus, type 1; PKR, protein kinase R; OAS, 2′,5′-oligoadenylate synthetase; SeV, Sendai virus; MEF, mouse embryonic fibroblast; CDG, cyclic di-GMP; MOI, multiplicity of infection; CC, compound C; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NT, nontargeting; 3-MA, 3-methyladenine; DMEM, Dulbecco's modified Eagle's medium; qRT-PCR, quantitative RT-PCR; qPCR, quantitative PCR; HA, hemagglutinin; KO, knockout; mTOR, mammalian target of rapamycin.

otic initiation factor 3 activity (25, 27, 29). PKR, activated by viral dsRNA, phosphorylates eukaryotic initiation factor 2α to inhibit the translation of cellular or viral mRNAs (30). OAS produces 2′,5′-oligoadenylates upon dsRNA stimulation to activate the latent RNase L, which degrades both cellular and viral RNAs (22). Mx1 functions by sequestering the viral components from the desired destination within the cells (26). ISGs also exert their antiviral actions by amplifying cellular IFN responses (14). Some ISGs serve as PRRs or signaling intermediates, which are expressed at low levels and are transcriptionally induced by IFN signaling.

Recently, we uncovered a new antiviral mechanism of the IFN system (31). Using a high-throughput genetic screen of a human ISG shRNA library, we identified a novel antiviral ISG, Tudor domain– containing 7 (*TDRD7* in humans and *Tdrd7* in mice). TDRD7, expressed at low endogenous levels in various cell types, is transcriptionally up-regulated upon virus infection or IFN treatment. Using knockdown, knockout, and ectopic expression strategies, we established the antiviral function of TDRD7 in a variety of human and mouse cells. We examined the antiviral activity of TDRD7 against paramyxo-/pneumoviruses, *viz.* Sendai virus (SeV), respiratory syncytial virus, and human parainfluenza virus 3. TDRD7 exhibits its antiviral activity by inhibiting the cellular autophagy pathway, which is required for paramyxo-/pneumovirus replication. An in-depth investigation revealed that TDRD7 inhibits the autophagy-initiating kinase AMPK to block the autophagy pathway. Genetic or chemical inhibition of AMPK suppresses viral replication. The anti-autophagic activity is a new property of TDRD7 that has not earlier been studied as either an ISG or an antiviral protein.

Virus replication is an energy-dependent process that requires high cellular ATP levels, leading to increased AMP levels (32–34). Elevated levels of AMP activate the metabolic kinase AMPK, which some viruses utilize to promote their replication. Viruses use AMPK in both autophagy-dependent and independent cellular pathways. Dengue virus activates AMPK to promote lipophagy, which targets lipid droplets (35). Vaccinia virus activates AMPK-dependent macropinocytosis and actin dynamics for cell entry (36). Kaposi's sarcoma–associated *herpesvirus* viral protein K1 directly interacts with AMPK to promote viral replication (37). Human cytomegalovirus activates AMPK to facilitate viral replication by increasing glucose flux (38, 39). AMPK activation inhibits mTOR, which benefits some viruses by suppressing protein synthesis (34, 40). Therefore, AMPK is a critical cellular factor that many viruses utilize for replication. Herpesviruses differentially use autophagy for their replication. HSV-2 and varicella-zoster virus, but not HSV-1, depend on autophagy pathway for virus replication (41). HSV-1 triggers autophagy in the early stage of its replication cycle, and in the later phase, it inhibits autophagy by the viral protein ICP34.5 and ICP0 (42, 43). Here, our study revealed that HSV-1 replication depends on AMPK activity but not the autophagy pathway. The AMPK dependence of HSV-1 led us to examine whether the anti-AMPK ISG TDRD7 inhibits HSV-1 replication. Our results demonstrate that TDRD7 inhibits its HSV-1 replication by inhibiting virus-activated AMPK.

Results

The IFN-inducible TDRD7 is a novel restriction factor against HSV-1 replication

To explore whether the newly identified anti-autophagic ISG TDRD7 inhibits, in addition to the paramyxo-/pneumoviruses, the DNA viruses, we used HSV-1, a human pathogen, as a model because of its broad host range. Human and mouse cells constitutively express endogenous TDRD7 at various levels (31). HSV-1 infection or cytoplasmic dsDNA poly(dA·dT) induced up-regulation of TDRD7 protein in HeLa cells (Fig. S1*A*). As expected, IFNβ treatment also induced TDRD7 protein expression in these cells (Fig. S1*A*). Importantly, Tdrd7 mRNA was up-regulated in HSV-1–infected mouse brains, a primary site for *herpesvirus* pathogenesis (Fig. 1*A*). Mouse embryonic fibroblasts (MEFs), upon infection with HSV-1 or administration of cyclic di-GMP (CDG), an agonist of STING, a cellular sensor for HSV-1, triggered transcriptional induction of Tdrd7 mRNA (Fig. 1*B*). In the next series of experiments, we determined whether TDRD7 inhibits HSV-1 replication, using knockdown or knockout of endogenous TDRD7 in multiple human cells. We generated TDRD7 knockdown human ARPE19 cells, confirmed by reduced TDRD7 mRNA expression upon IFNβ treatment (Fig. 1*C*). In TDRD7 knockdown ARPE19 cells, HSV-1 replication was enhanced, which was analyzed by the increased expression of a viral immediate early gene product, ICP0 (Fig. 1*D*). We used ICP0 protein expression as a primary readout of HSV-1 replication in our subsequent studies. To further investigate the role of TDRD7 in protection against HSV-1, we generated TDRD7 knockout (TDRD7$^{-/-}$) human cells using the CRISPR/Cas9 system (Fig. 1*E*, *lower panel*). When infected with two multiplicities of infection (MOI, 0.1 and 1), the TDRD7$^{-/-}$ cells exhibited a robust increase in the protein expression of ICP0, ICP4, and ICP27, the immediate early gene products, as well as ICP8, an early gene product of HSV-1 (Fig. 1*E* and Fig. S1*B*). To investigate whether the anti–HSV-1 function of TDRD7 is at the viral protein level, we measured viral DNA in WT and TDRD7$^{-/-}$ cells. Viral DNA, measured by qPCR, was strongly enhanced in TDRD7$^{-/-}$ cells, compared with the WT control (Fig. S1*C*). The increased viral gene expression led to enhanced production of infectious virion by TDRD7$^{-/-}$ cells, compared with the WT control cells (Fig. 1*F*). We validated these results using another strain of HSV-1 (F strain), which also showed a similar increase in ICP0 expression in the TDRD7$^{-/-}$ cells (Fig. S1*D*). Together, these results demonstrate that the IFN-inducible TDRD7 inhibits HSV-1 replication.

AMPK is required for HSV-1 replication

Recently, we reported that TDRD7 exhibits its antiviral activity by inhibiting virus-activated AMPK (31). To test whether HSV-1 replication depends on AMPK, we used AMPK knockdown HeLa cells (Fig. 2*A*, *lower panel*), in which HSV-1 replication was inhibited, analyzed by viral protein (ICP0) expression when infected with two MOIs (Fig. 2*A*). The AMPK knockdown cells also displayed reduced infectious virion production (Fig. S2*A*). Similar results were obtained from the AMPK knockout mouse cells; the AMPKα1$^{-/-}$ MEFs pro-

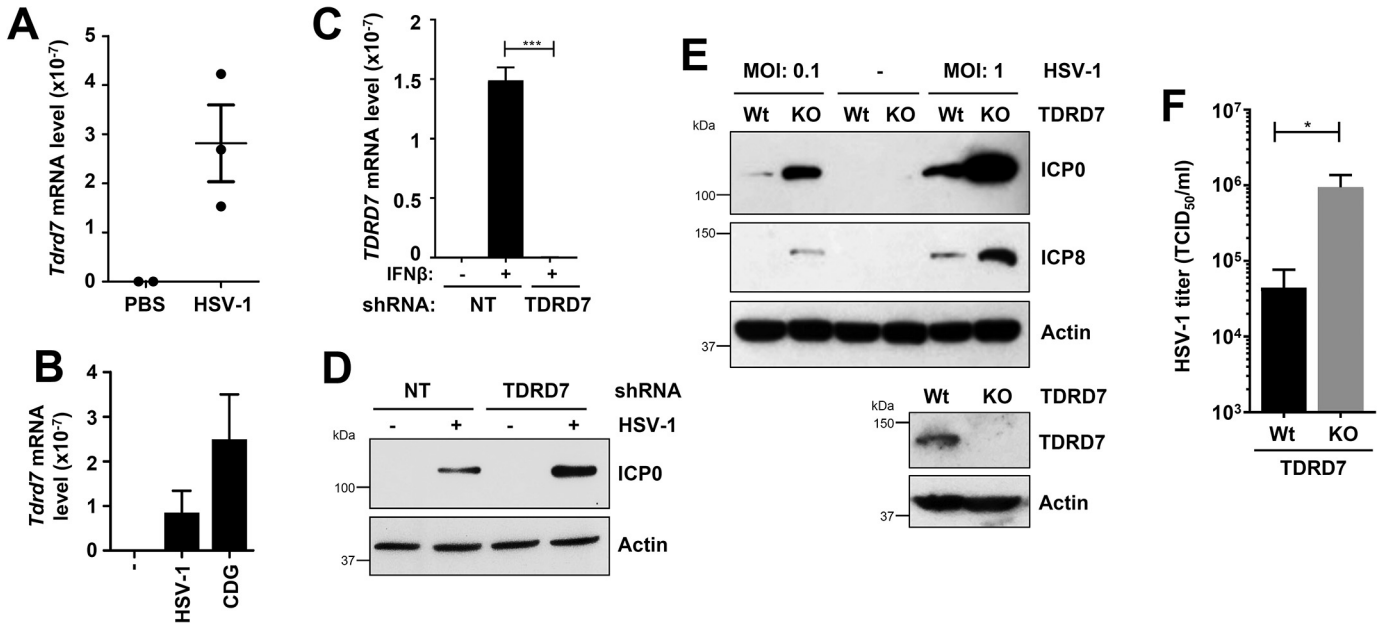

Figure 1. The IFN-inducible TDRD7 is an anti–HSV-1 restriction factor. *A*, mouse brains either mock-infected (PBS) or infected with HSV-1 (KOS strain) were analyzed for Tdrd7 mRNA expression using qRT-PCR. *B*, WT MEFs infected with HSV-1 (MOI: 1) or transfected with CDG were analyzed for TDRD7 mRNA induction by qRT-PCR. *C*, ARPE19 cells expressing NT or TDRD7-specific shRNA were treated with hIFNβ, and TDRD7 mRNA expression was analyzed by qRT-PCR. *D*, ARPE19 cells expressing NT or TDRD7-specific shRNA were infected with HSV-1, and the viral protein (ICP0) expression was analyzed by immunoblot. *E*, WT or TDRD7$^{-/-}$ (KO) HT1080 cells were infected with HSV-1 (at the indicated MOIs), and the expression of viral proteins (ICP0 and ICP8) was analyzed by immunoblot (*upper panel*). TDRD7 protein expression in WT and KO cells was analyzed by immunoblot (*lower panel*). *F*, WT or TDRD7$^{-/-}$ (KO) HT1080 cells were infected with HSV-1 (MOI:1), and the infectious virion release in culture media was analyzed by $TCID_{50}$/ml. *, $p < 0.05$.

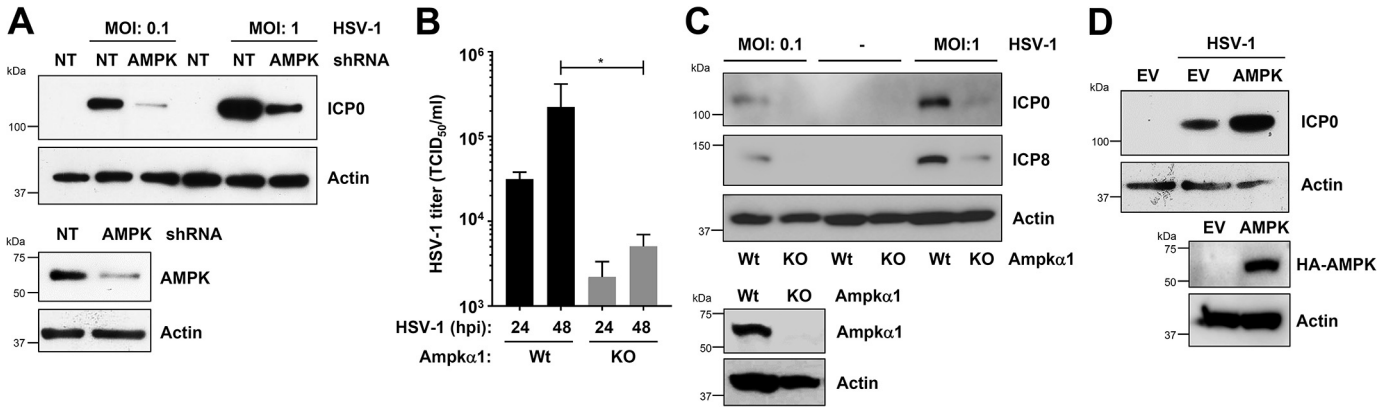

Figure 2. Pro-viral role of AMPK in HSV-1 replication. *A*, HeLa cells expressing NT or AMPK-specific shRNA were infected with HSV-1 (KOS strain, at the indicated MOIs), and viral protein (ICP0) expression was analyzed by immunoblot (*upper panel*). AMPK protein expression was analyzed in these cells by immunoblot (*lower panel*). *B*, WT or AMPK$\alpha1^{-/-}$ MEFs were infected with HSV-1, and the infectious virion release was analyzed by $TCID_{50}$/ml at the indicated time post-infection. *C*, WT or AMPK$\alpha1^{-/-}$ (KO) MEFs were infected with HSV-1 (F strain), and the expression of viral proteins (ICP0 and ICP8) was analyzed by immunoblot (*upper panel*). AMPK protein expression was analyzed in these cells by immunoblot (*lower panel*). *D*, L929 cells ectopically expressing HA-AMPK (*lower panel*) were infected with HSV-1, and the viral protein (ICP0) expression (*upper panel*) was analyzed by immunoblot. *EV*, empty vector. *, $p < 0.05$.

duced significantly reduced HSV-1 infectious virions when compared with the WT control (Fig. 2*B*). We validated these results using the HSV-1 (F strain), which showed a reduction in the expression of viral proteins (ICP0 and ICP8) in the AMPK$\alpha1^{-/-}$ MEFs (Fig. 2*C*). In a reciprocal approach, we ectopically expressed AMPK in mouse cells (L929), which exhibited increased expression of viral ICP0 protein compared with the control cells (Fig. 2*D*). These results demonstrate that HSV-1 depends on AMPK for its replication.

To determine whether only the physical presence or the kinase activity of AMPK is required for HSV-1 replication, we used a chemical inhibitor of AMPK, compound C (CC) (31, 44, 45). Pretreatment of cells with CC strongly inhibited HSV-1 replication in a dose-dependent manner, as observed by the reduced expression of ICP0 protein (Fig. 3*A*). HSV-1 F strain also showed a strong reduction of ICP0 expression at the two doses of CC (Fig. 3*B*). We validated these results using a derivative of CC, dorsomorphin dihydrochloride, which also strongly inhibited HSV-1 replication at the lower doses (Fig. S2*B*). To further confirm our results, we took a microscopic approach to quantify the ICP0-expressing cells. Similar to the mouse cells (Fig. 2*D*), the ectopic expression of AMPK in human cells significantly enhanced the number of ICP0-expressing cells (Fig. 3, *C* and *D*). The AMPK-dependent increase

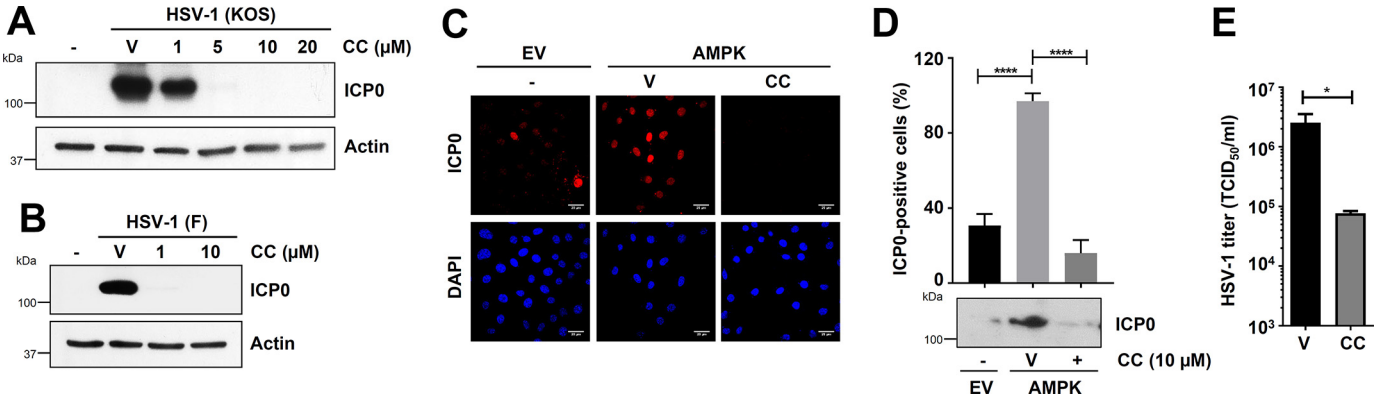

Figure 3. Pharmacological inhibition of AMPK suppresses HSV-1 replication. *A*, primary human fibroblasts (NuFF) were pretreated with the AMPK inhibitor (CC) at the indicated concentrations and infected with HSV-1 (KOS strain), and the viral protein (ICP0) expression was analyzed by immunoblot. *B*, NuFF cells were pretreated with CC at the indicated concentrations and infected with HSV-1 (F strain); the viral protein (ICP0) expression was analyzed by immunoblot. *C*, HeLa cells ectopically expressing HA-AMPK were infected with HSV-1 in the absence or the presence of CC (10 μM). ICP0-expressing cells were analyzed by confocal microscopy (60× magnification). *D*, HeLa cells ectopically expressing HA-AMPK were infected with HSV-1 in the absence or the presence of CC, and the ICP0-expressing cells were quantified using confocal microscopy (*bar graph* in the *upper panel* from multiple fields). ICP0 expression in these cells was analyzed by immunoblot (*lower panel*). *E*, NuFF cells were pretreated with CC and infected with HSV-1 (F strain); the infectious virion release was analyzed by $TCID_{50}$/ml. *EV*, empty vector; *V*, vehicle (DMSO). *, $p < 0.05$.

in the number of ICP0-expressing cells was significantly inhibited by CC (Fig. 3, *C* and *D*). The inhibition of viral protein expression led to a significant reduction in infectious virion production upon CC treatment (Fig. 3*E*). We confirmed that the inhibitors, tested at their antiviral doses, did not cause significant cytotoxicity, measured by MTT assay in multiple cell types (>80% cell viability relative to the vehicle control; Fig. S2*C*), as well as the number of cells (microscopy, Fig. 3*C*).

To gain further insight into the CC-mediated suppression of HSV-1 replication, we compared virus replication in cells treated with CC, before (pre) or after (post) virus adsorption to the cells, to distinguish the effect of CC pre- or post-entry of the virus particles. Both pre- and post-treatment with CC similarly suppressed the expression of ICP0 expression in primary human cells (Fig. 4*A*) and mouse (Fig. 4*B*) cells. We further used the microscopic approach to confirm these results in primary human fibroblasts. Both pre- and post-treatment with CC significantly inhibited the ICP0-expressing cells (Fig. 4, *C* and *D*). Finally, we investigated whether the reduction of viral protein by both treatment strategies led to the suppression of infectious virion production. Indeed, the pre- and post-treatment with CC led to a significant reduction of HSV-1 infectious virion production (Fig. 4*E*). These results demonstrate that the chemical inhibitor of AMPK, treated either pre- or post-viral adsorption, strongly inhibited the HSV-1 replication in both human and mouse cells.

HSV-1 requires AMPK in the absence of autophagy pathway

Activated AMPK triggers cellular autophagy pathway (Fig. 5*A*) (46, 47), which we examined in HSV-1–infected cells, using both genetic and pharmacological approaches. We used human cells, stably expressing a nontargeting (NT) or ATG5-specific shRNA (ATG5-KD; Fig. 5*B*, *lower panel*), to investigate whether HSV-1 infection activates the canonical (ATG5-dependent) autophagy pathway. Consistent with the current literature (43, 48, 49), our results indicate that HSV-1 infection activated an early induction of autophagy, analyzed by LC3-II accumulation (LC3-II/Actin), which was significantly inhibited in the later phase of virus replication (Fig. 5*B*, *upper panel*, *lanes 3*, *5*, and *7*). Both early and late autophagic responses, as expected, were inhibited in the ATG5-KD cells, which showed reduced accumulation of LC3-II (LC3-II/Actin; Fig. 5*B*, *upper panel*, *lanes 4*, *6*, and *8*). To investigate whether HSV-1 replication depends on the virus-induced canonical autophagy, we used the ATG5-KD human cells. In ATG5-KD cells, viral protein (ICP0) expression was unchanged when compared with the NT control cells, examined at two different MOIs (Fig. 5*C*). In line with these results, infectious virion production was unchanged in ATG5-KD cells when compared with the NT control cells (Fig. 5*D*). Similar to the results obtained from the genetic experiments, a known chemical inhibitor of autophagy, 3-methyladenine (3-MA) (Fig. 5*A*), inhibited the early autophagic response by HSV-1, analyzed by the reduced LC3-II accumulation (LC3-II/Actin; Fig. 5*E*). As expected, 3-MA treatment did not alter the expression of ICP0 in these cells (Fig. 5*F*). These results indicate that HSV-1 replication does not depend on the virus-induced cellular autophagy pathway.

To strengthen that HSV-1 requires an autophagy-independent activity of AMPK, we used the autophagy-deficient human cell line ATG5-KD (Fig. 5*B*). This approach is physiologically relevant because HSV-1 infection naturally blocks autophagy by interfering with steps downstream of AMPK (42, 43). In these cells, HSV-1 infection activated the AMPK signaling pathway, the initiation stage of the autophagy pathway. Previous studies have indicated that HSV-1 infection activates AMPK signaling (50). HSV-1 infection triggered robust phosphorylation of AMPK on Thr^{172} (Fig. 6*A*) and dephosphorylation of mTOR of Ser^{2448} (Fig. 6*B*) in the ATG5-KD cells. These results led us to examine the role of AMPK in the absence of its autophagy branch. Pharmacological inhibition of AMPK activity by CC, as expected, inhibited HSV-1 replication in ATG5-KD cells (Fig. 6*C*). To confirm that HSV-1 replication depends on AMPK but not its autophagy branch, we took a genetic approach and generated AMPK knockout ATG5-KD (ATG5-KD $AMPK^{-/-}$) human cells (Fig. 6*D*, *lower panel*). In

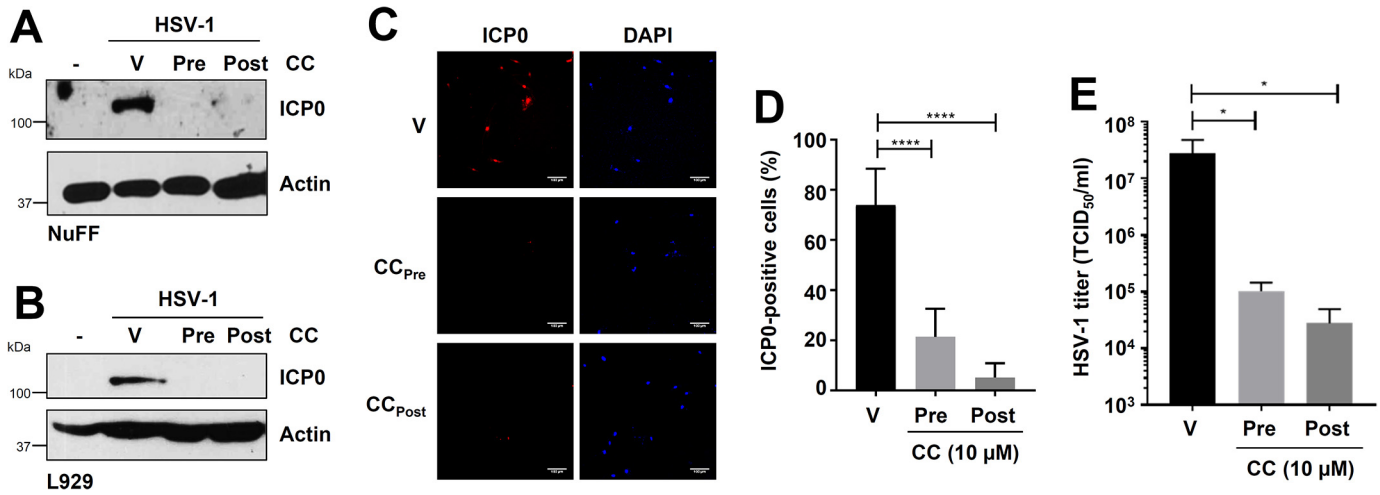

Figure 4. AMPK inhibition, either pre- or post-viral adsorption, blocks HSV-1 replication. *A* and *B*, NuFF (*A*) or L929 (*B*) cells either pre- or post-treated (viral adsorption) with 10 μM of CC were infected with HSV-1, and the viral protein (ICP0) expression was analyzed by immunoblot. *C*, NuFF cells were either pre- or post-treated with CC (10 μM, as in *A*), and HSV-1–infected cells were analyzed by immunostaining for ICP0-expressing cells using confocal microscopy. *D*, quantification of ICP0-expressing cells using confocal microscopy (20× magnification) from multiple fields. *E*, NuFF cells were pre- or post-treated with 10 μM CC and infected with HSV-1; the infectious virion release was analyzed by $TCID_{50}$/ml. *V*, vehicle (DMSO). *, $p < 0.05$.

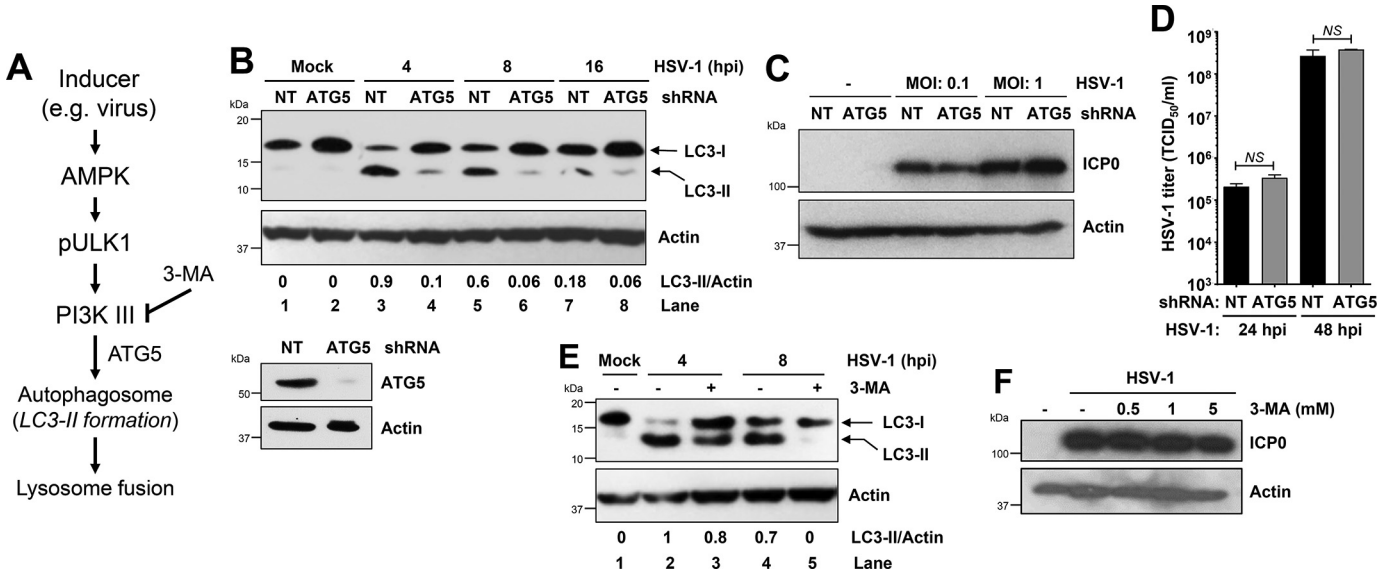

Figure 5. HSV-1 activates an early autophagy but does not require it for viral replication. *A*, cellular canonical autophagy pathway and its critical protein components are shown. *B*, HT1080 cells expressing NT or ATG5-specific shRNA were infected with HSV-1 (KOS) at the indicated time when LC3-II accumulation was analyzed by immunoblot (*upper panel*). LC3-II/Actin ratios were analyzed by ImageJ and are shown *below* each lane. ATG5 protein expression was analyzed by immunoblot (*lower panel*). *C*, HT1080 cells expressing NT or ATG5-specific shRNA were infected with HSV-1 at the indicated MOIs, and viral protein (ICP0) expression was analyzed by immunoblot. *D*, HT1080 cells expressing NT or ATG5-specific shRNA were infected with HSV-1 for the indicated time when the infectious virion release was analyzed by $TCID_{50}$/ml. *E*, HT1080 cells were infected with HSV-1 in the absence or the presence of 3-MA for the indicated time when LC3-II accumulation was analyzed by immunoblot. LC3-II/Actin ratios were analyzed by ImageJ and are shown *below* each lane. *F*, HT1080 cells were infected with HSV-1 in the absence or the presence of 3-MA, and the expression of viral protein (ICP0) was analyzed by immunoblot. *NS*, nonsignificant.

these cells, HSV-1 replication was strongly inhibited, as analyzed by the reduction in viral proteins (ICP0 and ICP8; Fig. 6*D*). Together, our results demonstrate that HSV-1 activates but does not require autophagy; moreover, an autophagy-independent AMPK activity is required for HSV-1 replication.

TDRD7 inhibits AMPK activation to suppress HSV-1 replication

In the next series of experiments, we investigated whether the anti–HSV-1 activity of TDRD7 is related to its anti-AMPK functions. TDRD7 inhibits paramyxovirus-activated AMPK (31); here, we examined whether TDRD7 inhibits AMPK activation in HSV-1–infected cells. In L929, HSV-1 infection triggered robust phosphorylation of AMPK (pAMPK), which was inhibited by the ectopic expression of Tdrd7 (Fig. 7*A*). Similarly, restoration of TDRD7 expression in $TDRD7^{-/-}$ cells strongly inhibited the HSV-1–induced pAMPK (Fig. 7*B*). AMPK is also activated by nonviral inducers, *e.g.* serum starvation, which caused pAMPK in $TDRD7^{-/-}$ cells (Fig. S3*A*). Restoration of TDRD7 inhibited pAMPK (Fig. S3*A*), indicating that the anti-AMPK activity of TDRD7 is not limited to virus-infected cells. We examined whether the Tdrd7-mediated inhibition of AMPK activation led to the inhibition of viral replica-

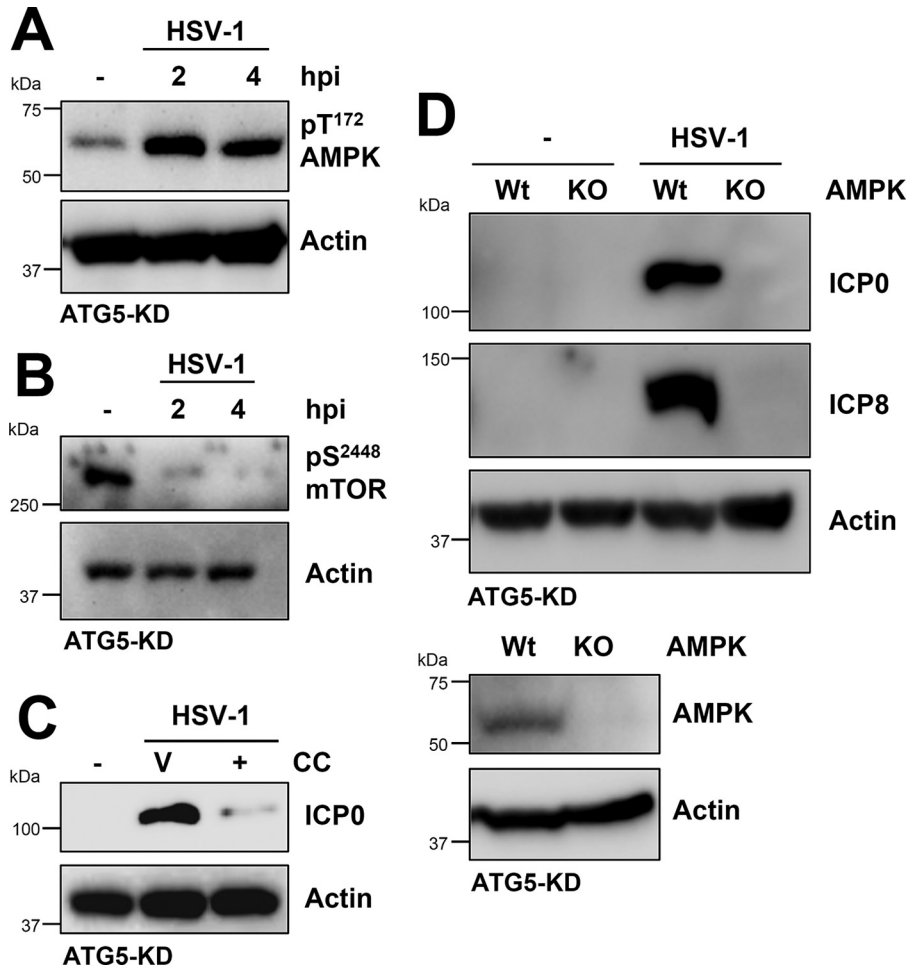

Figure 6. HSV-1 infection activates and requires AMPK in the absence of autophagy. HT1080 cells stably expressing ATG5-specific shRNA (shATG5) were used for the following experiments. *A* and *B*, the cells were infected with HSV-1, and the phosphorylation of AMPK (pAMPK on Thr^{172}, *A*) and mTOR (p-mTOR on Ser^{2448}, *B*) was analyzed at the indicated time postinfection by immunoblot. *C*, the cells were pretreated with CC (10 μM), and the viral protein (ICP0) expression was analyzed by immunoblot. *D*, WT or the $AMPK^{-/-}$ (KO) HT1080/shATG5 cells were infected with HSV-1 and analyzed for viral proteins (ICP0 and ICP8) by immunoblot (*upper panel*). AMPK protein expression was analyzed by immunoblot (*lower panel*). *V*, vehicle (DMSO); *hpi*, hours post infection.

tion. Indeed, ectopic expression of Tdrd7 suppressed ICP0 expression in mouse (L929; Fig. S3*B*) and human (HeLa; Fig. S3*C*) cells. We further validated these results in primary human fibroblasts (NuFF), which upon ectopic expression of TDRD7, strongly inhibited viral protein (ICP0) expression (Fig. 7*C*). To connect the anti–HSV-1 and anti-AMPK activities of TDRD7 genetically, we examined whether TDRD7 inhibits AMPK-dependent HSV-1 replication. As shown before (Figs. 2*D* and 3, *C* and *D*), ectopic expression of AMPK strongly enhanced the levels of viral protein (ICP0; Fig. 7*D*, *lanes 2* and *3*). The AMPK-mediated increase in viral protein (ICP0) expression was significantly suppressed by TDRD7 (Fig. 7*D*, *lanes 3–5*, and ICP0/actin levels in *top panel*). Next, we determined whether the anti–HSV-1 function of TDRD7 depends on cellular AMPK activity. TDRD7, as expected, significantly inhibited viral protein (ICP0) expression, analyzed by confocal microscopy (Fig. 7*E* and Fig. S3*D*). However, the antiviral activity of TDRD7 was diminished in CC-treated cells (Fig. 7*E* and Fig. S3*D*). We further validated these results using immunoblot analyses, which showed that TDRD7-mediated inhibition of viral proteins (ICP0 and ICP27) depended on cellular AMPK activity (Fig. 7*F*). Inhibition of AMPK activity dampened the antiviral effect of TDRD7. Together, these results demonstrate that HSV-1 replication depends on cellular AMPK activity, the inhibition of which by TDRD7 is a novel viral restriction mechanism.

Discussion

We report a novel antiviral mechanism of the host via inhibiting cellular AMPK activity by the newly identified viral restriction factor TDRD7 (Fig. 8). Because TDRD7 inhibits virus-induced autophagy to block paramyxo-/pneumovirus replication, we explored this antiviral mechanism against other viruses that also activate autophagy. TDRD7-deficient cells were more susceptible to HSV-1 replication, indicating the antiviral function of TDRD7 against HSV-1. However, HSV-1 replication did not depend on cellular autophagy pathway, which we established by using autophagy-deficient human cells. These results led to the conclusion that HSV-1 replication requires the autophagy-initiating kinase AMPK but not its autophagy activity. Pharmacological or genetic inhibition of AMPK, in the absence or the presence of autophagy, blocked HSV-1 replication in human and mouse cells. Importantly, the antiviral activity of TDRD7 depended on its ability to

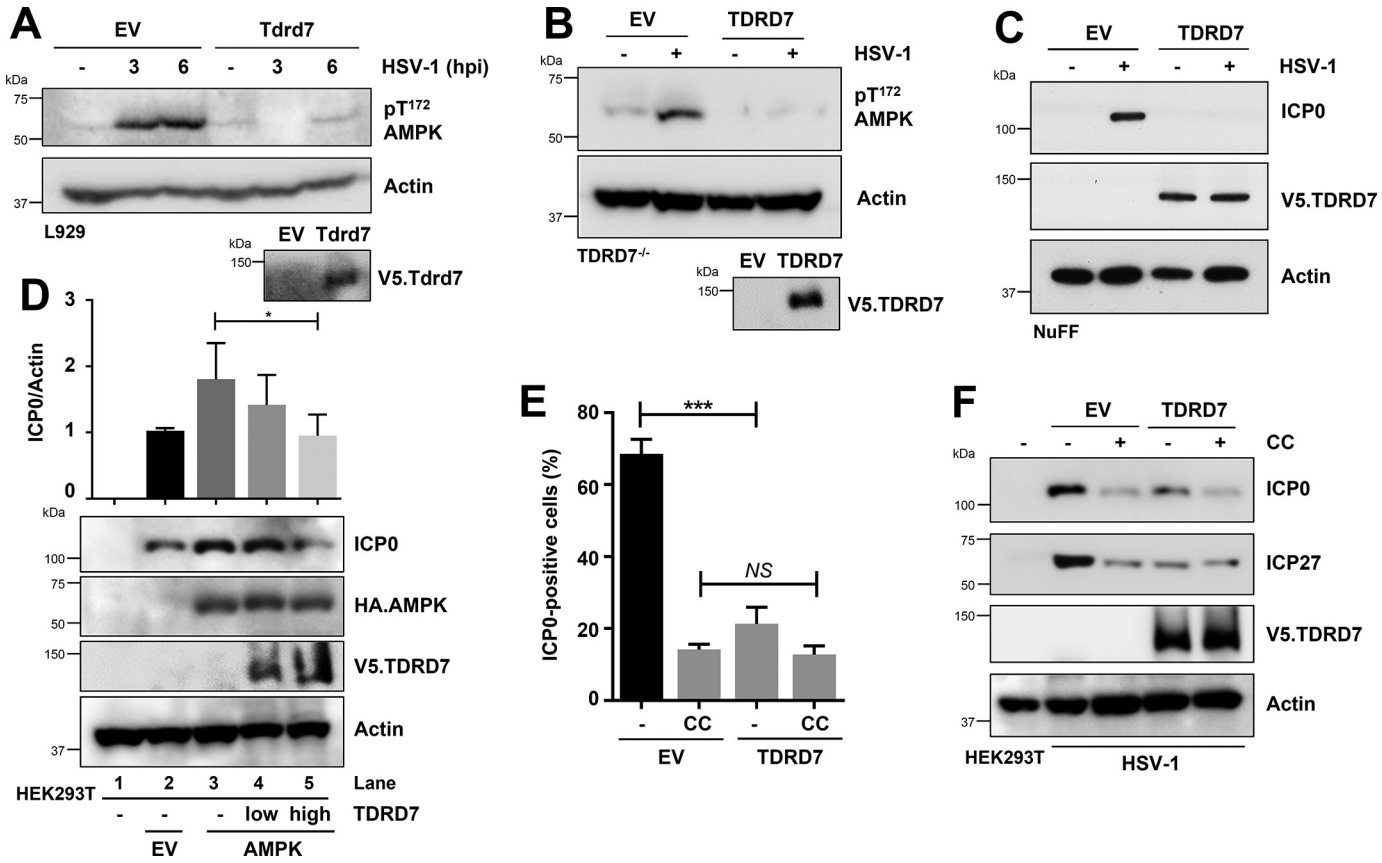

Figure 7. TDRD7 inhibits virus-activated AMPK to block HSV-1 replication. *A*, L929 cells stably expressing V5-Tdrd7 (*lower panel*) were infected with HSV-1, and the phosphorylation of AMPK (pAMPK on Thr^{172}) was analyzed by immunoblot. *B*, TDRD7$^{-/-}$ (KO) cells stably expressing V5-TDRD7 (*lower panel*) were infected with HSV-1, and the phosphorylation of AMPK (pAMPK on Thr^{172}) was analyzed by immunoblot. *C*, NuFF cells ectopically expressing V5-TDRD7 were infected with HSV-1, and the viral protein (ICP0) expression was analyzed by immunoblot. *D*, HEK293T cells were co-transfected with HA-AMPK and V5-TDRD7 as indicated and infected with HSV-1, and the viral protein (ICP0) expression was analyzed by immunoblot. The *bar graph* indicates ICP0 levels (ICP0/Actin) quantified from multiple experiments (*upper panel*). *E*, HEK293T cells transfected with V5-TDRD7 were infected with HSV-1 in the absence or the presence of CC as indicated, and ICP0-expressing cells were analyzed by confocal microscopy and quantified from multiple fields. *F*, HEK293T cells transfected with V5-TDRD7 were infected with HSV-1 in the absence or the presence of CC as indicated, and the expression of viral proteins (ICP0 and ICP27) was analyzed by immunoblot. *EV*, empty vector. *, $p < 0.05$.

inhibit AMPK. In contrast to HSV-1, the replication of SeV depends on the autophagy pathway of AMPK (31). Therefore, AMPK inhibition may be a potential strategy to inhibit both autophagy-dependent and independent viruses. Our study, therefore, reveals a metabolic control of virus replication by the IFN system.

Autophagy, benefits paramyxo-/pneumoviruses, contributes differentially to HSV-1 replication (41, 51–54). The exact role of autophagy in HSV-1 replication is not completely clear (41). HSV-1 infection triggers autophagy in the early stage of virus infection; however, at the later stage, the viral protein ICP34.5 binds to Beclin-1 to inhibit autophagy (42, 55, 56). Viral ICP0 protein also inhibits p62 and optineurin to block the HSV-1–induced early autophagy (43). HSV-1 replication does not depend on autophagy, established using ATG5$^{-/-}$ MEFs, which are deficient in autophagy pathway but support normal viral replication (41, 54, 55). We provide additional insight that HSV-1 replication remains unaltered in autophagy-deficient human cells. *In vivo*, autophagy in dendritic cells contributes to HSV-1–induced keratitis (57) and enhances MHC class I presentation (58). PKR-induced autophagy protects against HSV-1 infection in the neuron (59, 60). These studies indicate that HSV-1–induced autophagy may have cell type–specific role in viral replication and pathogenesis. In contrast to HSV-1, other herpesviruses, HSV-2 and varicella-zoster virus, rely on autophagy pathway for virus replication (41).

We have uncovered an unexpected role of the HSV-1–induced autophagy pathway in virus replication. Although HSV-1 does not require autophagy, it depends on the autophagy-initiating kinase AMPK. AMPK is a multifunctional metabolic kinase that controls many cellular activities, including autophagy (61). SeV infection activates AMPK, which subsequently phosphorylates ULK1 to initiate cellular autophagy pathway. The inhibition of AMPK or a downstream protein (ATG5) that triggers autophagy blocks SeV replication (31). However, HSV-1 infection activates AMPK but does not depend on its autophagy function for virus replication. The pro-viral activity of AMPK is required likely for a step post–viral entry. Among other cellular pathways, AMPK inhibits mTOR signaling pathway by directly phosphorylating an intermediate protein TSC2 (62). Because mTOR signaling regulates cellular protein synthesis, viruses manipulate this pathway to promote replication. HSV-1 proteins, US3, and VP11/12 inhibit TSC2, a downstream target of AMPK, or activate mTOR signaling under cellular stress conditions (50). However, these mechanisms are independent of AMPK activity. Our results

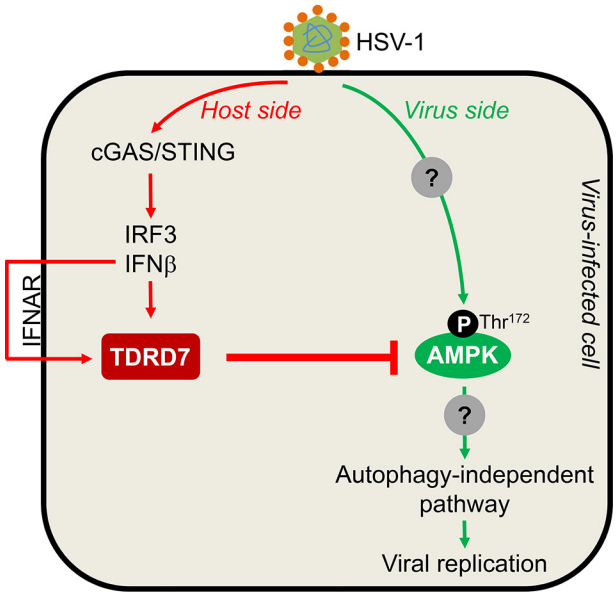

Figure 8. The IFN-inducible protein TDRD7 inhibits AMPK to suppress HSV-1 replication in an autophagy-independent pathway. HSV-1 infection activates AMPK in an autophagy-independent cellular pathway to facilitate virus replication. The virus-infected cells trigger the induction of ISGs via IRF3/IFNβ, and the antiviral ISG TDRD7 inhibits the virus-activated AMPK to suppress virus replication; *IFNAR*, IFN-α/β receptor.

indicate that mTOR signaling pathway is inhibited in HSV-1–infected cells, presumably as a consequence of AMPK activation, in the autophagy-deficient cells (Fig. 7*B*). Among other AMPK-dependent pathways, p53, SiRT1, and GLUT4 are potential regulators of HSV-1 replication. HSV-1 replication and neuropathogenesis are dependent on p53; p53 knockout mice display inhibited viral replication in the brain and subsequently reduced viral pathogenesis (63). Sirtuins (SiRT) are cellular regulators of viral replication, and SiRT1 regulates numerous RNA and DNA virus replication in relevant cell types (64). Viral infection triggers the transcriptional induction of glucose transporter GLUT4. Human cytomegalovirus replication, which is also dependent on AMPK activity, requires GLUT4 expression (38, 65, 66). Additional AMPK-dependent but autophagy-independent pathways, such as lipophagy and micropinocytosis, may also contribute to virus replication. Dengue virus activates AMPK to promote lipophagy, the selective autophagy that targets lipid droplets (35). Vaccinia virus utilizes AMPK to trigger macropinocytosis and actin dynamics for the entry of viral particles into the cells (36). Therefore, it is conceivable that the viruses, which are inhibited by the autophagy pathway likely via the autolysosome-dependent degradation, may still activate AMPK to facilitate virus replication. Genetic dissociation of AMPK from other parts of the autophagy, such as in the ATG5-KD AMPK$^{-/-}$ cells, will further clarify this. Future studies will further reveal whether the autophagy-dependent viruses also utilize some of the additional autophagy-independent AMPK pathways to facilitate virus replication.

Virus replication is an energy-consuming event that leads to the reduction of cellular ATP levels. Low cellular ATP leads to increased levels of AMP, which activates AMPK by direct interaction (34). Many viruses take advantage of the activated AMPK for their replication cycle. Activated AMPK also regulates the host defense mechanisms. Therefore, AMPK activity benefits both the host and the pathogen in a context-dependent manner. AMPK is also involved in regulating host immune response by cytoplasmic DNA signaling (67, 68). Therefore, the use of AMPK inhibitors to block virus replication *in vivo* would require caution because of their interference with host immune response. Replication of many bacteria depends on AMPK, and therefore, the inhibitors may also prevent bacterial pathogenesis *in vivo*. AMPK inhibitor reduces the intracellular replication of *Staphylococcus aureus* (69). The protozoan parasite *Trypanosoma brucei* encodes a parasitic AMPK gene and is susceptible to AMPK inhibitor (70). AMPK activators, metformin, and 5-aminoimidazole-4-carboxamide ribonucleotide are used therapeutically to treat metabolic disorders, *e.g.* diabetes (71). It will be interesting to investigate whether the patients receiving these drugs are susceptible to AMPK-dependent viruses. Such issues will first be addressed in a mouse model using these therapeutics. Many pathogens, including hepatitis C virus, inhibit AMPK activity; it is speculative that TDRD7 may have beneficial effects on these pathogens. We have shown that TDRD7 facilitates EMCV replication; however, the mechanism is currently not known (31). The increased EMCV replication by TDRD7 may be due to an inhibitory role of AMPK on the virus replication cycle.

How viruses activate AMPK is a critical aspect that requires in-depth investigation. Viruses activate AMPK either by the direct interaction of viral proteins with AMPK or by triggering the upstream cellular signaling cascades. The exact mechanism of TDRD7-mediated inhibition of AMPK activity will depend on these results. AMPK has multiple cellular activities; TDRD7 may specifically target some or all of them to control virus replication. Whether the effect of TDRD7 on AMPK is direct or intermediate cellular proteins are involved will require further investigation. It is speculated that the anti-AMPK function of TDRD7 may be associated with its ability to regulate stress granules (72), which are also related to virus replication (73). Future studies will be required to generate TDRD7 mutants, which do not localize to the stress granules, to examine anti-AMPK and antiviral activity. IFN signaling inhibits AMPK activation (74), and our results revealed that the TDRD7 is an IFN-inducible protein that inhibits AMPK. TDRD7 is expressed variably in multiple cell types, with ocular cells expressing relatively higher levels of TDRD7 (31). The endogenous TDRD7 protein level may contribute to the reduced AMPK activities in these cells. Because multiple pathogens, including viruses, bacteria, and parasites, rely on AMPK activity, TDRD7 may also inhibit the nonviral pathogens.

In summary, we demonstrated that TDRD7, in addition to the paramyxo-/pneumoviruses, is a new restriction factor against HSV-1. Because many anti–HSV-1 restriction factors, *e.g.* PKR and OAS, are efficiently antagonized by HSV-1, TDRD7 provides a novel strategy to study viral restriction. Furthermore, the idea that HSV-1 replication depends on AMPK activity but not the autophagy pathway is a novel concept that may be applied to other autophagy-independent viruses.

Experimental procedures

Cells, plasmids, and reagents

HT1080, ARPE19, HeLa, HEK293T, NuFF, L929, and Vero cells were purchased from ATCC, and MEFs were described previously (75). All cell lines used in this study were maintained in the authors' laboratory. The cells were maintained in DMEM containing 10% fetal bovine serum, penicillin, and streptomycin. Expression vectors of TDRD7 and AMPK have been described previously (31). CC and 3-MA were obtained from Sigma–Aldrich, dorsomorphin dihydrochloride was obtained from Selleckchem, and Lipofectamine 2000 was obtained from Thermo Fisher Scientific. IFNβ was obtained from R&D, poly-(dA:dT) was obtained from Sigma, and CDG was obtained from Invivogen. The antibodies against the specific proteins were obtained as indicated below: anti-ICP0: (Abcam catalog no. ab6513, and Santa Cruz catalog no. sc-53070), anti-ICP27: (Santa Cruz catalog no. sc-17544), anti-ICP4 (Santa Cruz catalog no. sc-69809), anti-ICP8 (Santa Cruz catalog no. 53329):, anti-TDRD7 (Sigma–Aldrich catalog no. SAB1303547), anti-LC3 (CST catalog no. 2775), anti-pAMPK (Thr^{172}) (CST catalog no. 2535), anti-AMPK (CST catalog no. 2532), anti-ATG5 (CST catalog no. 2630), anti-pmTOR (Ser^{2448}) (CST catalog no. 2971), anti-Actin (Sigma–Aldrich catalog no. A5441), anti-V5 (Thermo Fisher Scientific catalog no. R960-25), and anti-HA (Abcam catalog no. ab18181). The goat anti-mouse and goat anti-rabbit secondary antibodies were obtained from Rockland.

Knockdown and ectopic expression

For generating stable knockdown of TDRD7 in human cells, the shRNA (GATCGCACATGTTTATTTA) was lentivirally expressed, and the transduced cells were selected in puromycin-containing medium, as described before (31). The stable knockdown cells were evaluated for the levels of TDRD7 by qRT-PCR in the absence or the presence of IFN treatment. ATG5 knockdown cells were generated by lentivirally expressing the respective shRNAs (Sigma catalog no. SHCLNG-NM_004849), and the transduced cells were selected in puromycin-containing medium. AMPK knockdown cells were generated as described previously (31), by lentiviral expression of shRNA plasmids (Sigma catalog no. SHCLNG-NM_006251) and selection of the transduced cells in puromycin-containing medium. Stable human and mouse cell lines ectopically expressing epitope (V5)–tagged TDRD7/Tdrd7 genes were generated by lentiviral transduction followed by selection in puromycin-containing medium. The stable cells were used for viral infection and other biochemical analyses. The cells ectopically expressing AMPK were generated by lentiviral transduction of HA-AMPK (HA-tagged AMPKα1) using pLVX-IRES-puro and selection in puromycin-containing medium.

Generation of CRISPR/Cas9-mediated TDRD7 and AMPK knockout cells

TDRD7 knockout (KO) human cells (HT1080) were generated as described before (31). Briefly, HT1080 cells were transfected with TDRD7-specific CRISPR/Cas9 plasmid (sc-407210). The transfected cells were sorted for high GFP expressers using flow cytometry and were expanded to isolate individual clones. Individual clones were screened for TDRD7 protein levels using immunoblot. ATG5 knockdown HT1080 cells were transfected with AMPK-specific CRISPR/Cas9 plasmid (sc-400104), and individual clones were isolated in a similar manner as described above.

Virus infections

HSV-1 KOS and F strains were described previously (76). For virus infection, the cells were adsorbed with the viruses (at the multiplicity of infection of 1.0 or as indicated in the figure legends) in serum-free DMEM for 2 h, after which the cells were washed and replaced with normal growth medium. The virus-infected cells were analyzed for viral protein expression at 24 h after infection or as described in the figure legends by immunoblot or confocal microscopy. Infectious virion release in the culture supernatants was determined by $TCID_{50}$ in Vero cells, as described before (76).

Treatment of cells for analyses of cellular pathways and cell viability

The cells were pretreated with the chemical inhibitors for 2 h prior to virus infection, as indicated in the figure legends. For post-treatment, the inhibitor was added to the culture medium after virus adsorption. For the analyses of activation of AMPK, mTOR, and the autophagy pathway, the control cells were mock-infected and harvested at the end of virus adsorption. For activation of AMPK by serum starvation, the cells were washed with, and incubated in, serum-free DMEM for 4 h, and pAMPK was analyzed by immunoblot. To measure cell viability, the cells were seeded in a 96-well plate and treated with vehicle or the inhibitors, as indicated, and MTT assay was performed using a previously described procedure (31). The absorbance in the vehicle (DMSO) treated cells was considered as 100, and the other values were normalized to this.

Cell lysis and immunoblot

Immunoblot was performed using previously described procedures (77, 78). Briefly, the cells were lysed in 50 mM Tris buffer, pH 7.4, containing 150 mM of NaCl, 0.1% Triton X-100, 1 mM sodium orthovanadate, 10 mM of sodium fluoride, 10 mM of β-glycerophosphate, 5 mM sodium pyrophosphate, protease, and phosphatase inhibitors (Roche). Total protein extracts were analyzed by SDS-PAGE followed by immunoblot. The immunoblots were developed using Syngene imaging system and processed using Adobe Photoshop for further analyses. Wherever indicated, the density of protein bands on the immunoblots was quantified using ImageJ program.

RNA isolation, qRT-PCR, and qPCR analyses

Total RNA was isolated using TRIzol extraction, and the cDNAs were prepared using the ImProm-II reverse transcription kit (Promega). For qRT-PCR, 0.5 ng of cDNA was analyzed using Radiant^{TM} SYBR Green reagent (Alkali Scientific Inc.) on a Roche LightCycler. The expression levels of the mRNAs were normalized to the 18S rRNA. For qPCR analyses of HSV-1 genomic DNA, total cellular DNA was isolated from the infected cells and was subjected to qPCR using primers targeting viral ICP27, and the copy number was relative to the DNA copies of cellular 18S rRNA. The

following primers were used for the qPCR analyses: TDRD7-fwd, CGAGCTGTTCTGCAGTCTCA; TDRD7-rev, GCCATGGCATAGCAGGTAAT; Tdrd7-fwd, CTAAGGGCTGTCCTGCAGTC; Tdrd7-rev, AGAGTTGCCTTTGGCTTT; ICP27-fwd, GCATCCTTCGTGTTTGTCATT; ICP27-rev, GCATCTTCTCTCCGACCCCG; 18S-fwd, ATTGACGGAAGGGCACCACCAG; and 18S-rev, CAAATCGCTCCACCAACTAAGAACG.

Confocal microscopy

To analyze ICP0-expressing cells, the infected cells were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and subjected to immunostaining by anti-ICP0 antibody followed by Alexa Fluor–conjugated secondary antibody (Invitrogen). The objects were mounted on slides using VectaShield/4′,6′-diamino-2-phenylindole and analyzed by confocal microscopy. The images were further processed and analyzed using Adobe Photoshop software. Multiple culture fields containing at least 100 cells were analyzed to select representative images and for quantification.

Virus infection in mice

The C57BL/6 mice (Taconic) were intraperitoneally injected with HSV-1 (10^8 plaque-forming units/mouse of HSV-1 KOS), using a previously described procedure (76). The brains of the HSV-1–infected mice were harvested for Tdrd7 mRNA expression analyses by qRT-PCR. All animal procedures are approved by the University of Toledo institutional animal care committee.

Statistical analyses

The statistical analyses were performed using GraphPad Prism 5.03 software. The p values were calculated using two-tailed, unpaired Student's t tests and are shown in the relevant figures. The results presented here are the representatives of at least three independent experiments.

Author contributions—G. S., S. P., and S. Chakravarty data curation; G. S. and S. Chattopadhyay formal analysis; G. S. and S. Chakravarty investigation; G. S., S. P., S. Chakravarty, R. T. T., and R. C. methodology; G. S., R. T. T., R. C., and S. Chattopadhyay writing-original draft; S. P. visualization; R. T. T. and R. C. resources; R. C. and S. Chattopadhyay conceptualization; R. C. and S. Chattopadhyay supervision; R. C. and S. Chattopadhyay funding acquisition; S. Chattopadhyay project administration.

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