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Viperin has species-specific roles in response to herpes simplex virus infection

Yeu-Yang Tseng[1], Anjali Gowripalan[1], Sarah N. Croft[1], Stewart A. Smith[1], Karla J. Helbig[2], Si Ming Man[1] and David C. Tscharke[1,*]

Abstract

Viperin is a gene with a broad spectrum of antiviral functions and various mechanisms of action. The role of viperin in herpes simplex virus type 1 (HSV-1) infection is unclear, with conflicting data in the literature that is derived from a single human cell type. We have addressed this gap by investigating viperin during HSV-1 infection in several cell types, spanning species and including immortalized, non-immortalized and primary cells. We demonstrate that viperin upregulation by HSV-1 infection is cell-type-specific, with mouse cells typically showing greater increases compared with those of human origin. Further, over-expression and knockout of mouse, but not human viperin significantly impedes and increases HSV-1 replication, respectively. In primary mouse fibroblasts, viperin upregulation by infection requires viral gene transcription and occurs in a predominantly IFN-independent manner. Further we identify the N-terminal domain of viperin as being required for the anti-HSV-1 activity. Interestingly, this is the region of viperin that differs most between mouse and human, which may explain the apparent species-specific activity against HSV-1. Finally, we show that HSV-1 virion host shutoff (vhs) protein is a key viral factor that antagonises viperin in mouse cells. We conclude that viperin can be upregulated by HSV-1 in mouse and human cells, and that mouse viperin has anti-HSV-1 activity.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a large, enveloped double-stranded DNA virus, belonging to the *Herpesviridae* family. Globally, it is wide-spread and can result in various pathologies, including oral lesions, herpetic keratitis of the eye, genital lesions and rare but serious infections in neonates. Typically, in response to viral infection, host cells elicit a series of defensive reactions, where innate immune responses are critical to the first-line attack against invading viral pathogens [1]. In particular, the importance of type I interferons (IFNs) to the innate immune response against herpesvirus infection has been well established [2, 3]. IFNs are secreted from infected cells and initiate signal transduction by binding to their cognate receptors on surrounding cells. This process results in expression of numerous interferon-stimulated genes (ISGs) [4]. Whilst IFNs themselves do not directly have antiviral properties, ISGs effectively limit virus replication and spread [5]. Apart from IFN induction, entry of pathogens into cells can also stimulate production of a subset of ISGs via interferon regulatory factors (IRFs), such as IRF1 and 3, in the absence of IFNs [6, 7].

Viperin is an ISG with broad spectrum antiviral activity against both DNA and RNA viruses [8–15]. It was initially identified as an ISG in human macrophages and a human cytomegalovirus (HCMV)-inducible gene in human fibroblasts [9, 15]. Viperin is also conserved across species and has been cloned from humans to fish [16]. The viperin protein comprises three distinct domains: an N-terminal region with varied length and sequence between species, a highly conserved central domain that contains cysteine residues arranged in a CxxxCxxC motif and a C-terminal domain that shows low variability across species. An amphipathic helix located from residue 9 to 42 in the N-terminus of viperin associates with the cytosolic face of the endoplasmic reticulum, and also affords localisation of viperin to the lipid droplet [17]. The N-terminal sequences of viperin are also critical in

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Author affiliations: [1]John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia; [2]Department of Physiology, Anatomy and Microbiology, La Trobe University, Bundoora, VIC, Australia.
***Correspondence:** David C. Tscharke, david.tscharke@anu.edu.au
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Abbreviations: HCMV, human cytomegalovirus; HSV-1, herpes simplex type 1; IE, immediate early; IRF, interferon regulatory factor; ISG, interferon stimulated gene; PFU, plaque forming units.
One supplementary figure is available with the online version of this article.

its inhibition of multiple viruses, including chikungunya virus (CHIKV) and Hepatitis C Virus [18, 19]. The central domain of the protein contains four sequence motifs associated with the radical SAM (S-adenosyl-L-methionine) superfamily, and together these confer enzymatic function [20]. The role of the conserved C-terminus is in binding the substrate CTP, as well as facilitating interaction with the cytosolic iron-sulphur protein assembly protein, CIA1 [21, 22], which co-operate to form the novel ddhCTP (3′-deoxy-3′, 4′-didehydro-CTP) which has recently been demonstrated to act as a chain terminator for the RNA-dependent RNA polymerase of multiple *Flaviviridae* family members [21]. Interestingly, viperin has also been shown to limit the replication of *Flaviviridae* family members independently of its radical SAM activity, suggesting multiple mechanisms of viral inhibition [18, 23, 24].

Most of the current literature is aimed at RNA viruses, and whether viperin functions efficiently against DNA viruses is not as well-defined [9, 25, 26]. In *Herpesviridae*, viperin has been shown to affect replication of HCMV. The envelope glycoprotein B from HCMV can directly induce expression of different ISGs, including viperin [9, 27]. Human fibroblasts expressing viperin show a reduction in HCMV production, indicating that viperin is a potential antiviral against HCMV [9]. In order to evade the antiviral activity of viperin in HCMV infection, the HCMV vMIA protein can interact with viperin, resulting in the redistribution of viperin from endoplasmic reticulum to mitochondria. The role of viperin in HSV-1 infection has been examined by two studies, but with contrasting findings. In the first, wild-type HSV-1 infection was not associated with an increase in viperin expression, nor was ectopic expression of viperin able to inhibit infection [26]. This study identified UL41 (virion host shutoff, vhs) as a key viral factor that interfered with viperin by reducing the accumulation of viperin mRNA and found that ectopic viperin reduced the replication of a UL41-null HSV-1 [26]. By contrast, Zheng and Su concluded that wild-type HSV-1 can trigger MAVS-dependent induction of viperin [28]. Both studies reported using HEK293 cells and a similar multiplicity of infection leading to further uncertainty as to the role of viperin in HSV-1 infection. To address these conflicting reports, we revisit the role of viperin in HSV-1 infection using a range of cell types and lines across species.

METHODS

Cells and viruses

HeLa, MRC5, A549 cells were purchased from the American Type Culture Collection (ATCC). HFF were a kind gift from Giel van Dooren (Australian National University) and Pritinder Kaur (Curtin University). NIH3T3 and MC57G cells were kind gifts from C. Goodnow (ANU, Australia) and G. Karupiah, (ANU, Australia) respectively. The 293A, 143B(TK-), BS-C-1, and DC2.4 were a kind gift from Dr J. Yewdell (NIH). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% foetal calf serum (FCS; Serana) and 2 mM L-glutamine (Life Technologies). Vero cells were a gift from G. Karupiah (ANU, Australia). Vero and V27 cells were maintained in Minimal Essential Medium (MEM; Life Technologies) with 10% FCS, 4 mM L-glutamine, 5 mM HEPES and 50 µM 2-mercaptoethanol (Life Technologies). V27 cells were Vero cells expressing ICP27 upon HSV-1 infection [29]. IMR-90 and WI-38 cells were purchased from the ATCC and cultured in MEM with 10% FCS, 2 mM L-glutamine, 5 mM HEPES and non-essential amino acids. Wild-type and IFNAR$^{-/-}$ mouse fibroblasts (MF) were harvested from skin of newborn female C57BL/6 mice and were grown in DMEM with 10% FCS, 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids solution and 1% penicillin-streptomycin (Life Technologies). To inhibit viral protein synthesis, cells were pre-treated with CHX (Sigma) at 100 µg ml^{-1} in supplemented DMEM for 1 h at 37 °C and 5% CO_2, as previously described [30]. CHX was added to all relevant virus dilutions, so remained on the cells until the experimental time point.

Wild-type HSV-1 strain KOS was a gift from Francis Carbone (University of Melbourne). HSV-GFP (originally referred to as HSV-1 pICP47_eGC) is a recombinant virus with wild-type properties *in vitro* and *in vivo* expressing eGFP-Cre driven by the ICP47 promoter in the intergenic space between UL3 and UL4 [30]. All other recombinant HSV-1 viruses used in these studies were derived from wild-type HSV-1 strain KOS, including ΔSma with a functional deletion in the UL41 gene, 5dl1.2, an ICP27 deletion mutant and LJS1, a double deletion mutant generated from the two viruses described above [31, 32]. Both 5dl1.2 and LJS1 mutants were cultivated and titrated in V27 cells. Wild-type HSV-1, HSV-GFP and ΔSma were cultured and titrated in Vero cells. We sincerely thank Professor James Smiley (Department of Medical Microbiology and Immunology, University of Alberta) for kindly providing ΔSma, 5dl1.2, LJS1 mutants and V27 cells.

HSV-GFP was inactivated by dilution of virus in intercalating agent, 4′-aminomethyltrioxsalen hydrochloride (100 µg ml^{-1}, AMT; Sigma) and treatment with long-wave UV light (λ 365 nm; Vilber, VL-215.L) for 45 min on ice, with periodic mixing.

Plasmids

A series of plasmids having various lengths of viperin were constructed for transient expression. Briefly, cDNA was synthesised from an RNA template isolated from mouse skin fibroblasts. Full-length viperin was amplified from the resulting cDNA by using specific primer sets with sequences of *Kpn*I and *Xba*I on forward and reverse primers respectively. After digestion by corresponding restriction enzymes, the insert was ligated with the linearized p3xFLAG-CMV-14 plasmid (Sigma-Aldrich) to build the p3xFLAG-viperin vector. The rest of DNA fragments with deletions of different regions of viperin, including the deletion of residues 9 to 42 (Δ9–42), 71 to 182 (Δ71–182) and 218 to 361 (Δ218–361), were amplified from the resulting full-length mouse viperin plasmid (p3xFLAG-viperin) and the amplicons then were digested with corresponding restriction enzymes, followed

by ligation with p3xFLAG-CMV-14 vector linearized by *Kpn*I and *Xba*I enzymes. Human viperin-GFP-c1 or pGFP-c1 empty vector [33] was supplied by K. Helbig. Specific guide RNA targeting viperin (5′-CACCGTGTCATTAATCGCTT CAACG-3′ and 5′-AAACCGTTGAAGCGATTAATGACAC-3′) was inserted into a lentiCRISPR v2 vector containing mCherry coding sequence in the Th111I enzyme cutting site.

Transfection

Nucleofector transfection kit (Lonza) was applied to transfect plasmids or siRNA (human viperin: SASI_Hs02_00362416; mouse viperin: SASI_Mm01_00031984; Irf1: SASI_ Mm01_00151781; Irf3: SASI_Mm02_00323626; Irf7: SASI_ Mm01_00188289) (Sigma Aldrich) into MF cells, following the manufacturer's instruction. In brief, around 1×10^6 of MF cells were transfected with 2 µg or indicated amounts of plasmid vectors or 50 nM siRNA via the Nucleofector 2b Device (Lonza). The cells were ready for infection post-24 h transfection.

RNA isolation, cDNA synthesis and qPCR

The TRIZOL reagent (Invitrogen) or RNAqueous total RNA extraction kit (Invitrogen) and the SuperScript VILO kit (Invitrogen) was used to isolate total RNA and synthesize cDNA respectively. Specific primers target mouse (5′- CTCAAACAGGCTGGTTTGG-3′ and 5′-CTTGC-CCAAGTATTCACCC-3′) and human (5′-GTGGTTCC AGAATTATGGTGAG-3′ and 5′-ATAAGGACATTGACTT CCTCGT-3′) viperin, and ICP4 (5′- CGGTGATGAAGG AGCTGCTGTTGC-3′ and 5′-CTGATCACGCGGCTGC TGTACA-3′) [34] were used in qPCR with the Power SYBR Green PCR Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The qPCR conditions were set with 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The expression of viperin was normalized to mouse (5′-GCAATTATTCCCCATGAACG-3′ and 5′- GGCCTCACTAAACCATCCAA-3′) or human (5′-CTACCACATCCAAGGAAGCA-3′ and 5′- TTTTTCGT CACTACCTCCCCG-3′) 18S rRNA and fold changes were determined by the $2^{-\Delta\Delta Ct}$ method comparing expression at 4 h.p.i. or 8 h.p.i. to mock infection.

Western blotting

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane, followed by probing with a primary antibody specific for viperin (1:500; Abcam; ab107359), FLAG (1:5000; Sigma; F3165) or β-catenin (1:2000; Cell Signalling; #9582). A goat anti-mouse (1:10000; Abcam; ab6789) or anti-rabbit (1:10000; Abcam; ab97051) IgG-conjugated HRP antibody was applied as a secondary antibody and incubated with membranes for 1 h at room temperature. An enzyme-linked chemiluminescence system (ECL; GE Healthcare) was used to treat PVDF membranes and then the signal was detected by chemiluminescence using ImageQuant LAS 4000 (GE Healthcare). Washing procedures were conducted by PBST (PBS with 0.1% Tween 20) between each step.

Confocal microscopy

MF cells were seeded at a concentration of 1×10^6 cells prior to transfection with 2 µg p3xFLAG-viperin plasmid. A p3xFLAG vector was used as a negative control. Post-transfection, cells were seeded on coverslips and infected with HSV-GFP at an MOI of 1 for 8 h. Cells were then fixed in 4% PFA and stained with DAPI for 10 min prior to mounting and visualisation on a Leica SP5 confocal system.

Statistics

All analysis was conducted using GraphPad Prism software. One-way or two-way ANOVAs with Tukey's post-tests were used for this study. Error bars refer to standard error unless otherwise stated. All experiments shown represent three independent replicates.

RESULTS

Cell type-specific upregulation of viperin by HSV-1 infection

Previous work using HEK293 cells demonstrated either none, or a small capacity for HSV-1 to induce viperin expression in infected cells [26, 28]. To re-examine these variable reports, we tested viperin (*Rsad2*) mRNA regulation by HSV-1 infection in several cell types all of which are fully permissive for HSV replication. These cells included primary human foreskin fibroblasts (HFF), MRC5 a non-immortalised fibroblast line derived from human lung, the human immortalised cell lines 293A (a HEK293 derivative) and HeLa, and primary mouse skin fibroblasts (MF, made from female newborn mice). Cells were infected at 0.5 p.f.u. $cell^{-1}$ and reverse transcription of whole RNA followed by quantitative PCR (qPCR) was used to quantify viperin-encoding transcripts. We used 18S rRNA as a normalisation control because it is not degraded by virion host shutoff (vhs) protein expressed by HSV-1 [35]. In MF cells, viperin mRNA was increased approximately nine-fold and 40-fold at 4 and at 8 h post-infection (h.p.i), respectively (Fig. 1a). However, of the four human cell types, viperin was only upregulated by the HeLa cells and this was seen at 8 h.p.i. This upregulation of viperin in HeLa cells was clear, but still substantially less than what was observed in MF cells. These data indicate that viperin induction by HSV-1 occurs in a cell- and possibly species-specific manner.

Next, Western blotting (WB) was used to verify that HSV-1 infection leads to increased viperin protein (Fig. 1b, c). We chose three cell types for this experiment, including 293A, HeLa and MF cells and examined viperin expression at 4, 8, 12 and 24 h after infection. The 293A cells were utilized for comparison with previous reports [26, 28]. HeLa cells and MF cells were used, because HSV-1 induced more viperin in HeLa compared with other human cell types and MF cells displayed the most upregulation of viperin. In 293A cells, there was no significant increase of viperin protein during HSV-1 infection, which is consistent with previous findings [26]. In HeLa

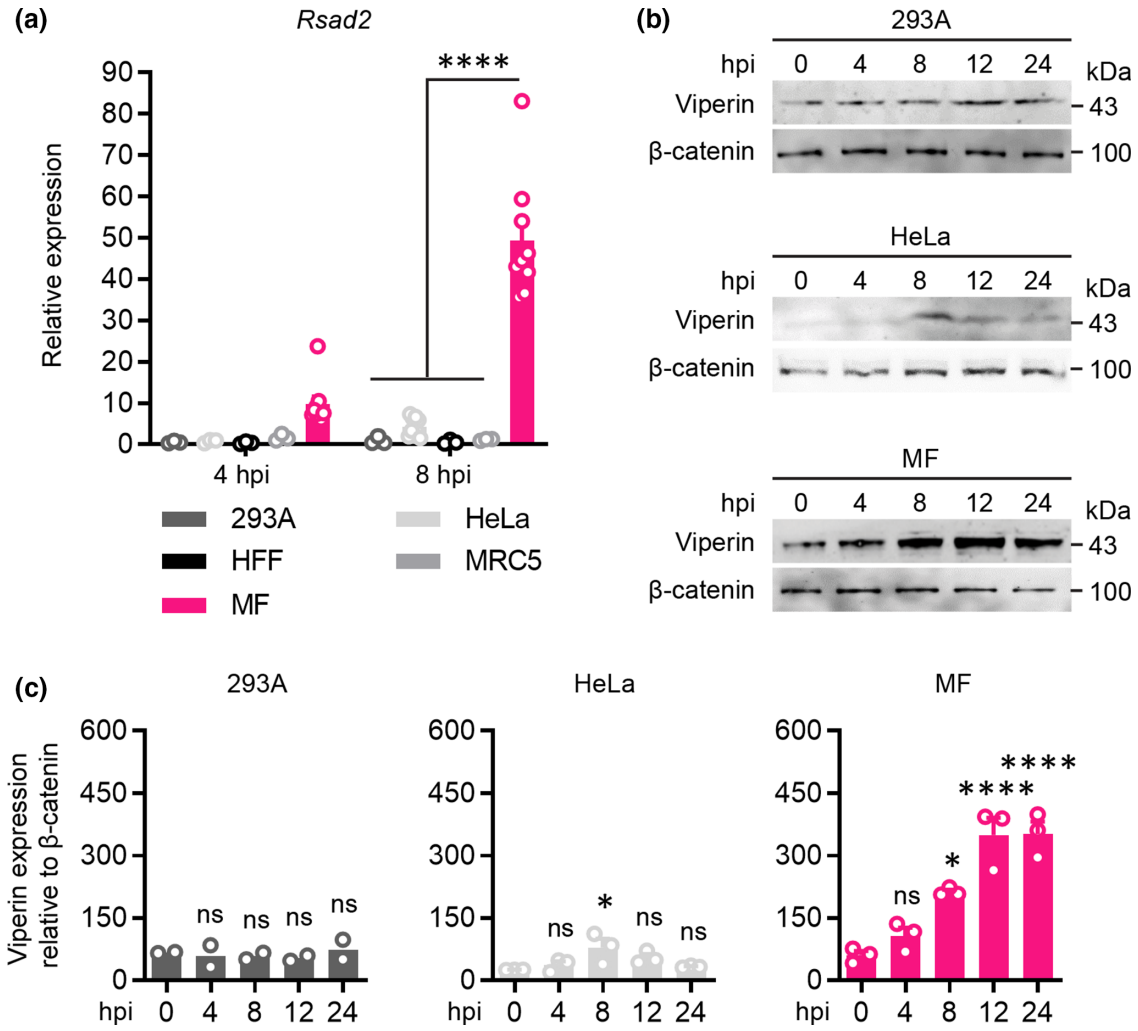

Fig. 1. Regulation of viperin by HSV-1 infection. (a) MF cells from four individual mice and three replicates of 293A, HFF, MRC5 and HeLa cells were infected with HSV-GFP at an MOI of 0.5 for 4 and 8 h. Relative mRNA levels compared with mock-infected cells were assessed by qPCR. (b,c) Here 293A, HeLa and MF cells were infected with HSV-GFP at an MOI of 2 and viperin protein detected by WB at the times shown. (b) Representative blots are shown. (c) Quantification of blots by densitometry with viperin normalized to *β*-catenin. The results are shown as mean±SEM. One-way ANOVA was used with Tukey's tests done post-hoc to compare differences between means. **P* <0.05; *****P* <0.0001; ns, not significant.

cells, a transient increase in viperin protein was seen with a low peak at 8 h.p.i. By contrast, in MF cells the protein level of viperin showed a strong increase, starting at 4 h.p.i. and reaching a plateau at 12 h.p.i. that was maintained until at least 24 h.p.i. The comparatively large increase in viperin after HSV-1 infection in MF cells, marginal increase in HeLa and lack of increase in 293A cells can be seen in individual blots and after quantification of replicate experiments (Fig. 1b, c). Taken together we found that increased viperin expression can be detected at mRNA and protein level after HSV-1 infection, depending on cell type.

Having shown that viperin expression after HSV-1 infection varies between cell types from mouse or human origin, we wanted to extend these findings to an extended array of human, primate and mouse cell lines. Again we found striking differences in relative fold-change of viperin expression across different cell types (Fig. 2a). For example, viperin upregulation was significantly greater in WI-38 cells (a non-immortalised human lung fibroblast line) compared with the other human cell lines, except for HeLa. Likewise, a difference was noted between the mouse MC57G fibrosarcoma cell line and the other murine cell lines. Analysing these data in a different way and comparing baseline as well as post-infection viperin mRNA levels provided another way to test the significance of any apparent upregulation of viperin mRNA. This revealed that viperin up-regulation in response to HSV-1 infection was only low and infrequent across human and primate cells, with only two of nine cell lines having significant increases (Fig. 2b). By contrast, viperin mRNA was strongly, and significantly increased in three of four mouse cell lines.

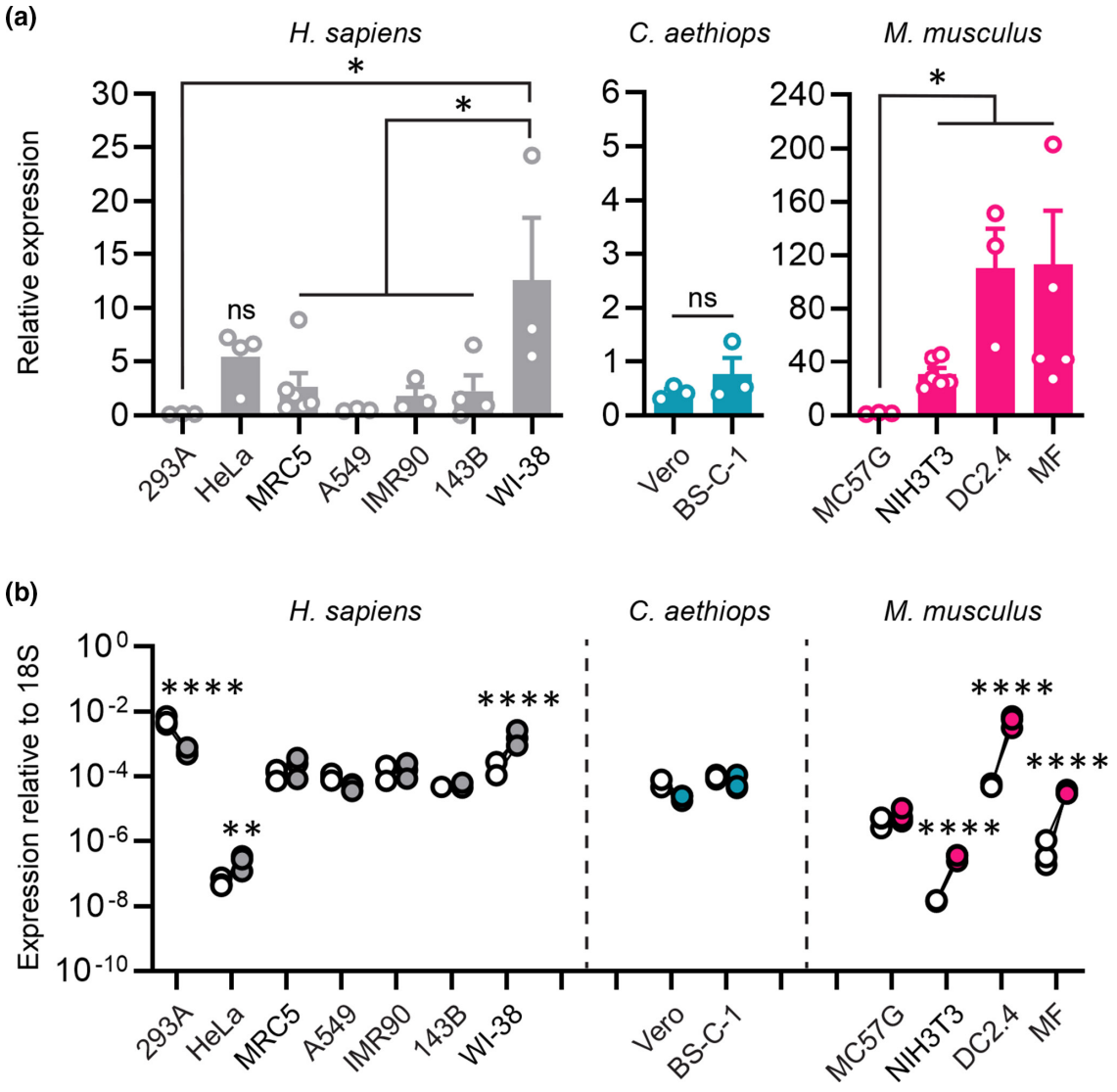

Fig. 2. Upregulation of viperin by HSV-1 is cell type-specific. (a, b) Cell lines / types as shown were infected with HSV-GFP at 0.5 p.f.u. cell^{-1} and analysed for viperin mRNA expression at 8 h.p.i. (a) Expression relative to mock infection is shown as mean±SEM with individual replicates. One-way ANOVA was used with Tukey's test done post-hoc to determine pair-wise difference between means. (b) Expression of viperin in mock (empty circles) and HSV-GFP-infected cells (filled circles) was determined relative to 18S rRNA. Two-way ANOVA was used followed by Sidak's test to determine the significance between pairs for each cell line. $^{**}P$ <0.01; $^{***}P$ <0.001; ns, not significant.

Interestingly, as a general finding the human and primate cells tended to have higher levels of viperin mRNA before infection, but this may be an artefact of the different qPCR assays used. Together these data show that viperin expression varies markedly across cell lines, both in homeostatic levels and in response to HSV infection.

Mouse viperin has anti-viral activity against HSV-1

The upregulation of viperin by HSV infection was most marked in MF cells and so we looked for an anti-viral function for the protein in these cells first. MF cells were transfected with 0, 0.5, 1, 2, or 4 µg of a plasmid expressing viperin and then infected with HSV-1 for 24 h. Viperin levels were confirmed by WB (Fig. 3a, top) and virus titres in cell lysates quantified by plaque assay, which revealed a dose-dependent inhibition of HSV-1 infection that was significant where 1 µg or more of plasmid was transfected (Fig. 3a, bottom). The result was confirmed in a similar experiment in which transfection of the viperin plasmid reduced GFP-expression in cells infected with HSV-GFP (Fig. 3b).

Next, the role of viperin at natural levels during infection was assessed by transient transfection of a lentiCRISPR plasmid expressing Cas9 and a viperin-specific gRNA to knockout the viperin gene [36] (Fig. 3c). HSV-1 infection in the viperin knockout cells resulted in a significant, approximately ten-fold enhancement of HSV-1 production at 24 h.p.i. compared with control in MF cells (Fig. 3c). This result was supported

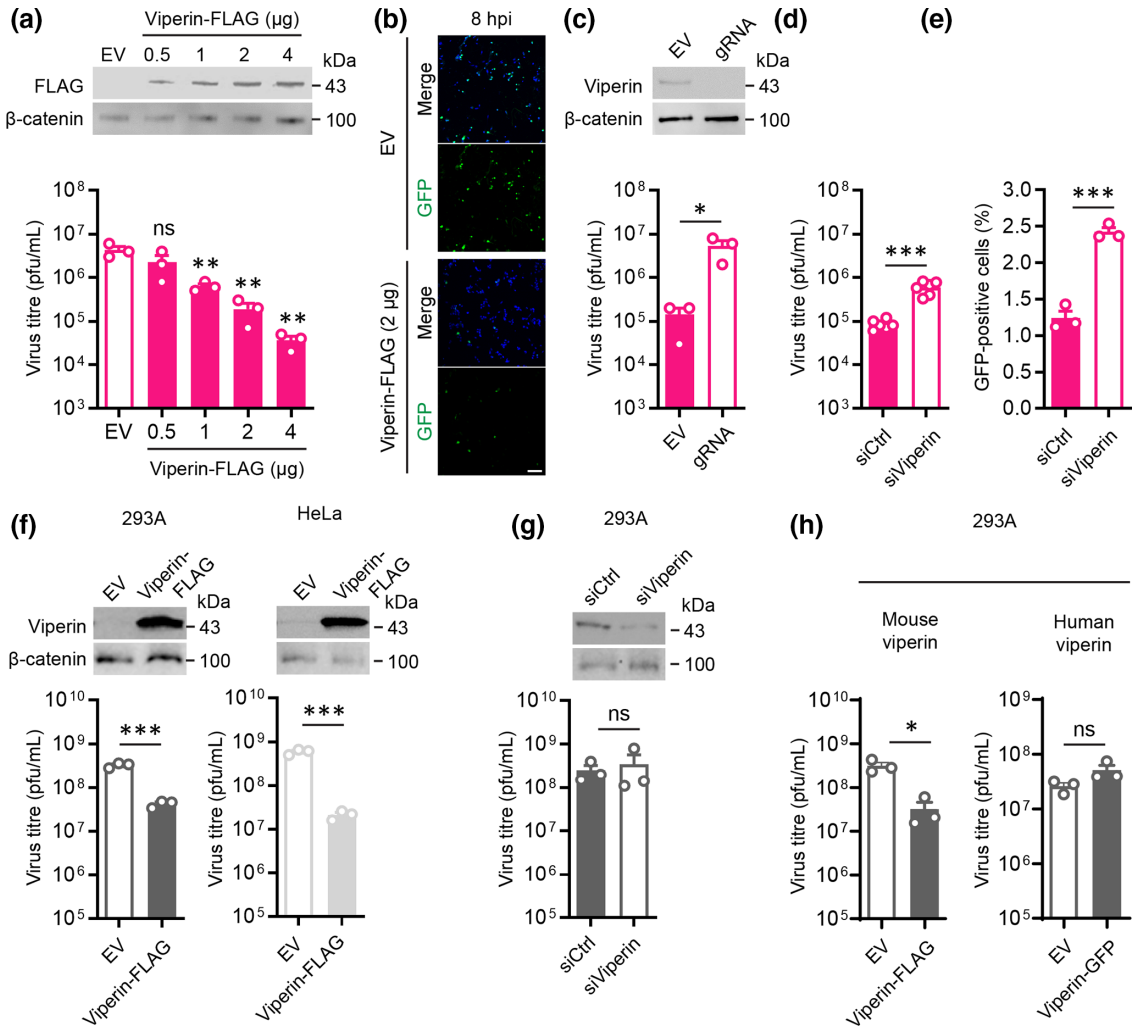

Fig. 3. Mouse viperin inhibits HSV-1 replication. (a) MF cells from three individual mice were transfected with p3xFLAG-viperin at indicated concentrations and infected with HSV-1 KOS at an MOI of 1 for 24 h. Viperin expression from the construct was detected using an anti-FLAG and WB (top) and virus titres were determined by plaque assay (bottom). (b) MF cells were transfected with 2 μg p3xFLAG-viperin (or vector control) and infected with HSV-GFP at 1 p.f.u. cell^{-1} and GFP expression visualised by confocal microscopy at 8 h.p.i. (Scale bar=100 μm). (c) LentiCRISPR vectors (2 μg) with a gRNA targeting viperin (gRNA) or without (EV) were transfected into MF cells for 24 h and then infected with HSV-1 KOS at 0.1 p.f.u. cell^{-1} for 24 h. Knockout of viperin in uninfected cells (top) and virus titres (bottom) are shown. (d, e) MF cells were transfected with 50 nM of control siRNA (siCtrl) or siRNA specific for viperin (siViperin) for 24 h and were then infected with HSV-GFP at an MOI of 0.0003 in MF cells for 72 h. Virus titres (d) and percent of cells positive for GFP by flow cytometry at 72 h.p.i. are shown (e). (f) 293A and HeLa cells were transfected with 1 μg p3xFLAG-viperin for 24 h before infection with 1 p.f.u. cell^{-1} of HSV-1 and virus titres were measured at 24 h.p.i. Overexpression of viperin in uninfected cells is shown by Western (above each graph). (g) 293A cells were transfected with siControl or siViperin prior to infection with HSV-1 as in (f). Virus titres were measured by plaque assay and Viperin knockdown was confirmed by WB (above graph). (h) 293A cells were transfected with 1 μg of plasmids expressing mouse (left) or human (right) viperin, which were FLAG- and GFP-tagged respectively, for 48 h and then infected with HSV-1 with 0.5 p.f.u. cell^{-1} of HSV-1 and virus titres were measured at 24 h.p.i. In the case of human viperin-GFP, transfection efficiency of >80% was verified by flow cytometry. All data are shown as mean±SEM, with individual replicates also shown. One-way ANOVA was used with Tukey's post-tests to evaluate pair-wise differences between means. *P <0.05; **P <0.01; ***P <0.001; ns, not significant.

by a similar experiment in which viperin was knocked down with siRNA and a low inoculum of HSV-GFP was used to measure spread over many rounds of viral replication in a single culture (Fig. 3d, e). This found that virus replication (Fig. 3d) and number of infected cells (Fig. 3e) were enhanced by viperin knockdown. We then used over-expression to examine the anti-viral potential of viperin in 293A and HeLa cells. This found that for both human cell lines, virus replication was reduced in cells transfected with a viperin expression plasmid (Fig. 3f). By contrast, siRNA knock down of viperin did not increase virus replication in 293A cells (Fig. 3g). All our over-expression experiments to this point used mouse viperin, irrespective of the species of host cell. Further, a comparison of mouse and human viperin shows there is a

marked lack of conservation between these orthologues for the first 70 aa, suggesting that these proteins might not be functionally interchangeable (Fig. S1, available in the online version of this article). For this reason we wondered whether the lack of impact in knocking down viperin in human 293A cells might be due to differences in effectiveness of mouse and human viperin against HSV-1. To test this, we overexpressed either mouse or human viperin, or corresponding empty-vectors, in 293A cells before infecting with HSV-1 and measuring viral growth by plaque assay. Corroborating our previous results, we found that HSV-1 replication was restricted with overexpression of mouse viperin, but human viperin had no anti-viral activity (Fig. 3h).

Taken together, ectopically expressed murine viperin was found to inhibit HSV-1 infection in all cell types tested, however viperin was not required in the physiological response to HSV of human cells.

Viperin expression is predominantly IFN-independent and requires HSV immediate-early gene expression

Viperin expression has been shown to occur via IFN-dependent signalling, where activation of DNA sensors drives type I IFN production, which signals via its receptor (IFNAR) to upregulate ISGs, including viperin [8, 10–12, 14]. However, in the absence of TLRs and IFNAR, viruses can stimulate expression of viperin directly through MDA5, RIG-I and downstream IRFs [6, 7, 23], (Fig. 4a). We wanted to explore which of these potential mechanisms was in play in MF cells, which showed the most marked upregulation of viperin expression after HSV-1 infection. To do this, wild-type and IFNAR$^{-/-}$ MF cells were infected for 24 h with HSV-1 KOS before being analysed for viperin mRNA expression. Viperin upregulation was similar in magnitude as seen in previous data, but there was no significant difference in these levels between IFNAR$^{-/-}$ and wild-type cells after infection with HSV-1 (Fig. 4b). These data suggest that IFN-independent signalling is sufficient for upregulation of viperin transcription during HSV-1 infection, at least in MF cells. These results were corroborated by siRNA knock down experiments that identified IRF1, which functions solely in the IFN-independent viperin pathway, as being an absolute requirement for viperin upregulation (Fig. 4c). Interestingly, we found that IRF3 and IRF7 were also required for full viperin upregulation, implying that perhaps IFN-dependent signalling played a minor or secondary role. However, knockdowns of these IRFs had the same impact on viperin mRNA upregulation after infection in IFNAR$^{-/-}$ and wild-type cells (Fig. 4d). Taken together, the data indicate that HSV-1 drives viperin expression in an IRF-dependent, but predominantly IFNAR-independent mechanism.

Having insight into the cellular processes that result in viperin upregulation, we wanted to understand what virological process is potentially triggering the expression. To do this, we used UV-inactivated HSV-GFP, to abolish all gene expression, and infected MF cells with the same virus in the presence of the translation inhibitor cycloheximide (CHX), to restrict viral activity to transcription of immediate early (IE) genes, and examined viperin mRNA. After 8 h of infection, viperin upregulation was equivalent in CHX-treated and untreated cells, but UV-inactivated HSV was not able to upregulate viperin transcription (Fig. 4e). To confirm that all viral gene expression was abolished by UV-inactivation but that IE transcription was intact in the presence of CHX, we also measured transcripts for ICP4, a key HSV-1 IE gene. As expected, ICP4 mRNA was detected in CHX-treated samples, but not when HSV-1 was UV-inactivated (Fig. 4f). Further, microscopy for GFP verified the absence of viral protein production both when UV-inactivated virus was used and in the presence of CHX (not shown). Together these results suggest that viperin upregulation seen in HSV-1 infection, requires IE transcription but not viral protein production.

HSV-1 vhs but not ICP27 is important for the inhibition of viperin activity

Published data indicate that HSV-1 vhs is sufficient to limit anti-HSV-1 activity of viperin in human cells [26]. However, ICP27 collaborates with vhs to reduce the abundance of host mRNA through the inhibition of host mRNA biogenesis during infection [37, 38]. In addition, vhs and ICP27 have been found to occlude IFN signalling and induction of ISGs more generally.

To explore the roles of these proteins we used three HSV-1 mutant viruses: a vhs functional deletion (ΔSma), an ICP27 null mutant (5dl1.2) and an ICP27/vhs double deletion (LJS1). MF cells were transfected with plasmids expressing full-length viperin for 24 h and then infected with HSV-1 strain KOS, ΔSma, 5dl1.2 or LJS1 at an MOI of 2 for 24 h. In cells transfected with the control vector, wild-type HSV-1 and ΔSma replicated to similar titres, but virus yields of 5dl1.2 and LJS1 were reduced significantly, by approximately 5–10-fold compared with the wild-type virus (Fig. 5a). These results are consistent with the work of others with ICP27-null mutants of HSV-1 [29, 31]. Of more interest here, overexpression of viperin significantly suppressed HSV-1 replication of all of these viruses, but to varying levels (Fig. 5a). Notably, ectopic expression of viperin resulted in significantly greater inhibition of ΔSma and LJS1, compared with wild-type and 5dl1.2 (Fig. 5b). The similarly strong effect of viperin on LJS1 and ΔSma, which both lack vhs, suggests that this viral factor is an antagonist of viperin function in MF cells, consistent with published results obtained using a human cell line [26].

The N-terminal amphipathic helix domain of viperin is required for inhibition of HSV-1

Viperin has several functional domains that have differing roles across a range of viral infections [13, 23]. Therefore, we set out to determine which of the domains were required for the antiviral ability of viperin against HSV-1 (Fig. 6a). To do this, a panel of viperin mutants, including

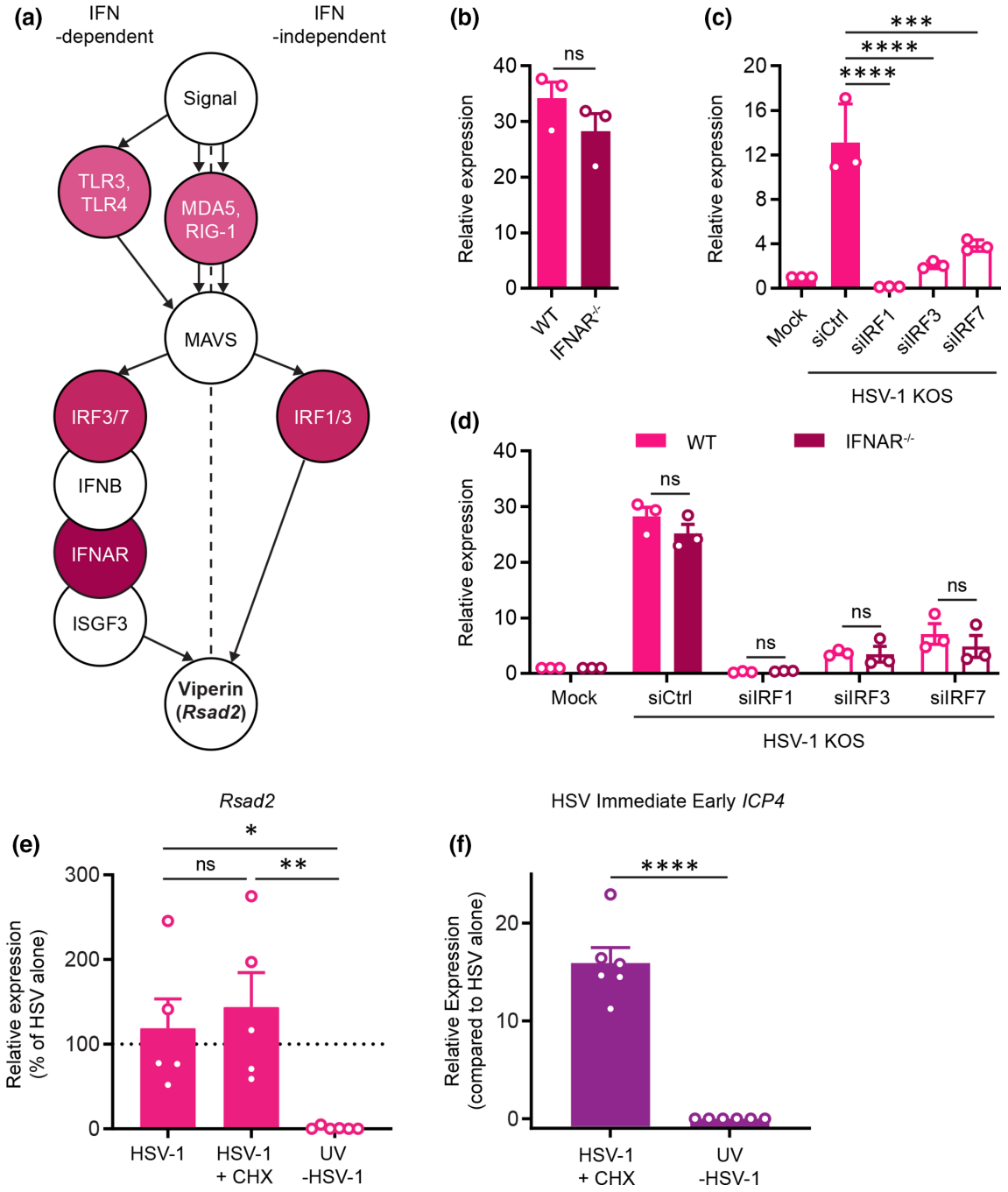

Fig. 4. HSV-1 induced viperin expression is IFN-independent. (a) Schematic depicting two pathways of viperin expression and key players. (b) WT and IFNAR$^{-/-}$ MF cells infected with HSV-1 KOS at 0.5 p.f.u. cell^{-1} were analysed by qPCR for viperin mRNA at 8 h.p.i. (c, d) Knockdown of IRF1, IRF3 and IRF7 by siRNA in WT (c) or WT and IFNAR$^{-/-}$ (d) MF cells prior to HSV-1 infection as in (a) with viperin mRNA shown at 8 h.p.i., relative to mock. (e, f) MF, with or without CHX were infected with HSV-GFP, or were infected with UV-inactivated HSV-GFP (UV-HSV-1) at 0.5 p.f.u. cell^{-1} for 8 h. Viperin (e) and ICP4 (f) transcripts were measured by qPCR. Viperin mRNA amounts are displayed as a percentage of the mean level in HSV-GFP-infected MF (e) and ICP4 mRNA as fold-change compared with that in HSV-GFP-infected MF (f). Data are mean±SEM with individual replicates also shown. One-way ANOVA was used with Tukey's tests done post-hoc to determine pair-wise differences between means. ****P <0.0001; ns, not significant.

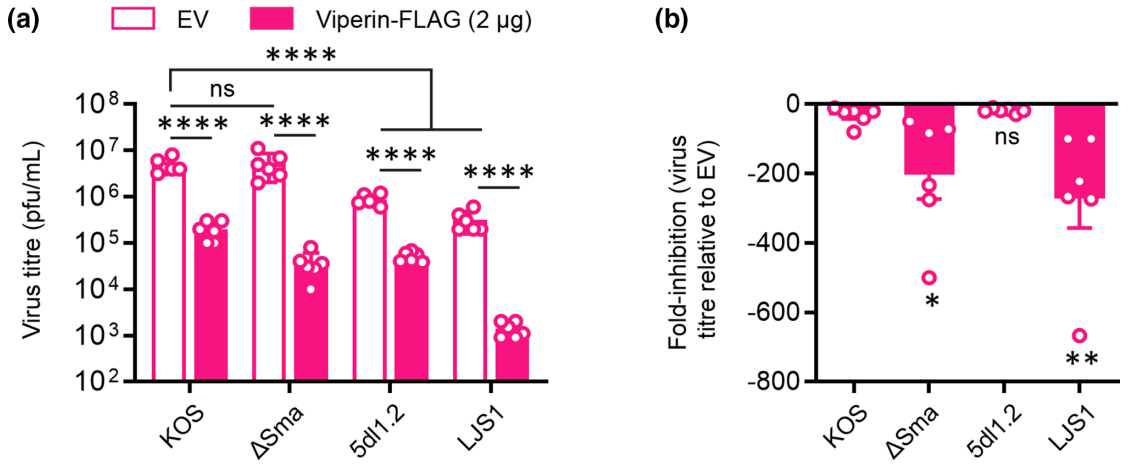

Fig. 5. HSV-1 vhs inhibits viperin function. (a, b) MF cells from three individual mice were transfected with 2 µg of p3xFLAG-viperin (pink) or EV (white) for 24 h before infection with the HSV-1 strains as shown (WT, KOS; vhs mutant, ΔSma; ICP27 mutant, 5dl1.2; ICP27/vhs double mutant, LJS1) at an MOI of 2. Virus titres were measured at 24 h.p.i. (a) and fold-inhibition due to viperin over-expression was calculated as titre in viperin-FLAG / EV transfected cells for each MF line (b). Data are mean±SEM with individual replicates also shown. Two-way ANOVA or One-way ANOVA were used with Tukey's test to evaluate pair-wise differences between means. **P* <0.05; ***P* <0.01; *****P* <0.0001; ns, not significant.

deletions of amphipathic helix domain (N-terminal domain; Δ9–42), radical SAM domain (central domain; Δ71–182) and C-terminal domain (Δ218–361), were constructed and transfected into MF cells, followed by HSV-1 infection. Transfection stability and efficiency of plasmids expressing full-length viperin, as well as Δ9–42, Δ71–182 and Δ218–361 mutants of viperin in MF cells, was demonstrated by WB (Fig. 6b). Transient overexpression of full-length, Δ71–182 and Δ218–361 viperin constructs significantly reduced HSV-1 replication by around ten-fold (Fig. 6c). By contrast, removing only the 34 aa of the N-terminal domain abolished the anti-HSV-1 activity of viperin. These results indicate that the N-terminal domain, but not the radical SAM

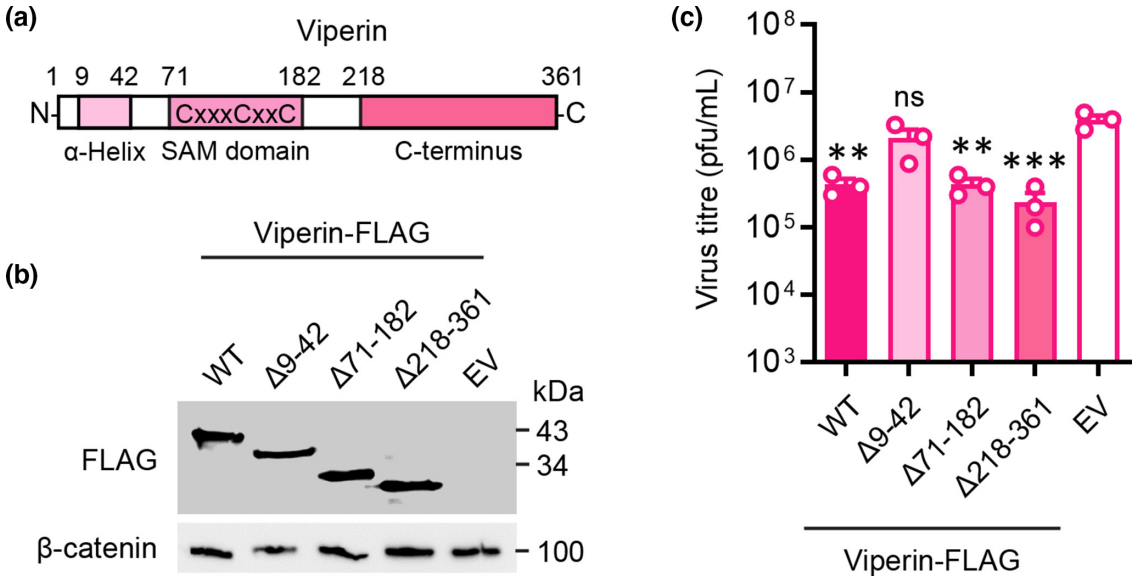

Fig. 6. The N-terminal domain of viperin is important for the anti-HSV-1 activity of viperin. (a–c) Plasmids expressing FLAG-tagged full-length viperin (WT), the viperin domain deletions Δ9–42, Δ71–182 and Δ218–361, or the empty vector (EV) were transfected into MF cells from three individual mice. After 24 h, viperin expression from the vectors were detected by WB (b) or infected with HSV-1 at 1 p.f.u. cell^{-1} and virus titre measured at 24 h.p.i. (c). Data in (c) are mean±SEM, with individual replicates shown. One-way ANOVA was used with Tukey's post-tests done for pair-wise comparisons between means. ***P* <0.01; ****P* <0.001; ns, not significant.

or C-terminal domains of viperin, is required for anti-HSV-1 activity.

DISCUSSION

Here we found HSV-1 infection leads to viperin upregulation in a host species and cell type-specific manner. Further, murine viperin is anti-viral against HSV as shown in over-expression and knockdown experiments. Viperin upregulation and anti-viral effect are most robust in mouse cells, suggesting that HSV-1 and human host co-evolution may have impacted the potency of viperin expression and antiviral function. Further, most of the cells examined are fibroblastoid cell types and where examined, the upregulation of viperin was independent of type I IFN, suggesting that viperin is part of a cell-intrinsic innate immune mechanism.

Cell-type-specific regulation of viperin has been seen previously with other viruses. For example, during rabies virus infection, viperin was upregulated significantly in mouse macrophage cells (RAW264.7), but much less so in neuroblastoma cells and baby hamster kidney cells (BHK-21) [39]. In another cell-type-specific case, Szretter *et al*. [40] demonstrated an increase in the viral load of West Nile virus (WNV) in primary macrophages and dendritic cells from viperin$^{-/-}$ compared with wild-type mice. However, there was no effect of viperin knockout on WNV infection of embryonic cortical neurons and fibroblasts [40]. Within our data, initial evidence indicated MF cells were uniquely capable of inducing strong viperin expression, but further investigations indicated this to be a common feature across mouse, but not human or primate cell lines. This is the first line of evidence that there is a human/mouse host species difference in the role of viperin in HSV-1 infection. Across the human cells we examined there was no obvious pattern by tissue of origin or level of immortalisation, with two non-immortalised lines tested, namely MRC-5 and WI-38, having different levels of viperin prior to infection and different response to infection. We did note that the two lines that upregulated viperin in response to HSV-1 were both of female origin, but sex of cells was not predictive because 293A and IMR-90, which are also of female origin, did not significantly upregulate viperin after infection.

Whilst upregulation of viperin can occur via IFN-dependent pathways, IFNAR was not required for an increase in viperin mRNA level after HSV-1 infection, suggesting a largely IFN-independent mechanism is at play. This IFNAR-independent viperin upregulation is akin to what is observed during HCMV and VSV infections [8, 9, 27, 41]. For instance, HCMV glycoprotein B directly drives activation and translocation of IRF3 to the nucleus, which correlates with the establishment of an antiviral state [27]. Our results indicate IRF3, as well as IRF1 and IRF7 are critical to the onset of viperin transcription after HSV-1 infection. We also were able to show the HSV-1 IE gene transcription was necessary and sufficient to induce viperin expression. The precise mechanism by which these IE mRNAs trigger IRF3, IRF1 and IRF7 mediated viperin upregulation remains unanswered.

We have confirmed the role of HSV-1 vhs in combating the impact of viperin on virus replication. This was done in MF cells here, which, when compared to published data for human HEK293 cells, suggests a similar role for vhs across these species [26]. Having said this, it could be that the impact of vhs on host mRNA degradation is greater in the HEK293 cells such that no upregulation of viperin mRNA was detected, either here or by others. This may be a general result across human cells, as opposed to those of a non-natural host like mice, but we note that complete ablation of viperin upregulation did not occur for all human cells in our study. Our data showing mouse, but not human viperin restricts HSV-1 in human cells raises the possibility that human viperin mRNA is specifically targeted by vhs. However, we consider this a less likely explanation of the species difference than divergent viperin protein functions between mouse and human, especially given the very broad endoribonuclease activity of vhs. Unlike the role of vhs in blunting the anti-viral potential of viperin, ICP27 appears to have little or no role either by itself or in combination with vhs. It remains possible that there are other mechanisms deployed by HSV-1 to reduce the response of viperin and other ISGs. For example, this may be done by interference with IRF1, IRF3 and/or IRF7, which we found here to be required for viperin upregulation, but others have demonstrated can be targeted by HSV-1 immune modulators [42–46].

The N-terminal domain of viperin has been found to be crucial for the association between the endoplasmic reticulum and lipid droplets. This is key to inhibition of HCV replication, whereby viperin interacts with HCV non-structural protein 5A in lipid droplets [17, 18, 47]. Likewise, the amphipathic helix in the N-terminus of viperin is necessary for its antiviral activity against the release of CHIKV and equine infectious anaemia virus [48, 19]. In the context of our data here with HSV it is notable that it is the N-terminus that is the least conserved region of viperin across species. Mouse and human viperin share 88.5% identity, with most of the variability lying in the N-terminal domain. Together with our finding that mouse, but not human viperin has anti-viral activity against HSV-1 it seems likely that it is these sequences differences at the N-terminus that drive this aspect of viperin species-specificity. Having noted this, viperin has multiple roles and these are contributed by different parts of the protein. For example the C-terminus of viperin can drive accumulation of molecules that specifically antagonise viral polymerases and forces chain termination during RNA virus replication [21]. Viperin is also capable of interfering with viral budding and release of influenza through disruption of lipid rafts, however the domain of viperin involved is yet to be determined [49]. However, given the ineffectual nature of regions other than the N-terminus and the conservation of these between mouse and human viperin it seems less likely that these mechanisms are in play in combatting HSV-1.

In conclusion, our data shows that HSV-1 induces viperin expression in a cell-specific manner and mouse, but not human viperin has an antiviral function via its N-terminus to restrict HSV-1 replication. Moreover, HSV-1 vhs is a key viral

factor to counteract viperin activity during infection. Cell species generally aligns with the antiviral response mounted, but other features such as cell sex may be a secondary determinant of viperin induction across species. These findings extend our understanding of the role of viperin against HSV-1 infection and add viperin function to the list of differences seen in host responses to this virus between humans and mice.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The generation of primary mouse fibroblasts from newborn mouse pups was done under ethics protocol A2016/045 from the ANU Animal Ethics and Experimentation Committee.

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