Herpes simplex virus 1 expressing GFP-tagged virion host shutoff (vhs) protein uncouples the activities of degradation and nuclear retention of the infected cell transcriptome

Emma L Wise¹, Jerzy Samolej^{1#}, Gillian Elliott^{1*}

¹ Section of Virology, Department of Microbial Sciences, Faculty of Health and Medical Sciences, University of Surrey, Guildford, United Kingdom

* Corresponding author: Gillian Elliott g.elliott@surrey.ac.uk

[#] Present affiliation: Institute of Microbiology and Infection, University of Birmingham, Birmingham, United Kingdom

Running title: Separating the activities of HSV1 vhs endoribonuclease

Summary

Virion host shutoff (vhs) protein is an endoribonuclease encoded by herpes simplex virus 1 (HSV1). Vhs causes a number of changes to the infected cell environment that favour translation of late (L) virus proteins: cellular mRNAs are degraded, immediate-early (IE) and early (E) viral transcripts are sequestered in the nucleus with polyA binding protein (PABPC1), and dsRNA is degraded to help dampen the PKR-dependent stress response. To further our understanding of the cell biology of vhs, we constructed a virus expressing vhs tagged at its C-terminus with GFP. When first expressed, vhs-GFP localised to juxtanuclear clusters, and later it colocalised and interacted with its binding partner VP16, and was packaged into virions. Despite vhs-GFP maintaining activity when expressed in isolation, it failed to degrade mRNA or relocate PABPC1 during infection, while viral transcript levels were similar to those seen for a vhs knockout virus. PKR phosphorylation was also enhanced in vhs-GFP infected cells, in line with a failure to degrade dsRNA. Nonetheless, mRNA FISH revealed that as in Wt but not Δ vhs infection, IE and E, but not L transcripts were retained in the nucleus of vhs-GFP infected cells at late times. Moreover, a representative cellular transcript which is ordinarily highly susceptible to vhs degradation, was also retained in the nucleus. These results reveal that the vhs-induced nuclear retention of the infected cell transcriptome is dependent on vhs expression but not on its endoribonuclease activity, uncoupling these two functions of vhs.

42 **Importance**

43 Like many viruses, herpes simplex virus 1 (HSV1) expresses an endoribonuclease, the
 44 virion host shutoff (vhs) protein, which regulates the RNA environment of the infected cell
 45 and facilitates the classical cascade of virus protein translation. It does this by causing the
 46 degradation of some mRNA molecules and the nuclear retention of others. Here we describe
 47 a virus expressing vhs tagged at its C-terminus with green fluorescent protein (GFP) and
 48 show that the vhs-GFP fusion protein retains the physical properties of native vhs, but does
 49 not induce the degradation of mRNA. Nonetheless, vhs-GFP maintains the ability to trap the
 50 infected cell transcriptome in the nucleus, proving for the first time that mRNA degradation
 51 is not a prerequisite for vhs effects on the nuclear transcriptome. This virus has therefore
 52 uncoupled the nuclear retention and degradation activities of vhs, providing new
 53 understanding of vhs during infection.

54

55

56

57

58

Introduction

The UL41 gene of herpes simplex virus 1 (HSV-1) encodes the virion host shutoff (vhs) protein, an important virulence factor that is conserved across the *alphaherpesvirinae* subfamily (1). vhs is an endoribonuclease that induces the degradation of mRNA but not rRNA or tRNA (2-4), by binding to the cellular translation initiation machinery through the eIF4A and eIF4H components of the eIF4F cap-binding complex, resulting in mRNA cleavage (1, 5-7). This activity effectively shuts down cellular protein synthesis (8, 9), freeing up ribosomes to translate viral mRNA. One consequence of vhs activity is that cellular transcripts encoding interferon-stimulated genes (ISGs), generated in response to virus infection, are targeted for degradation, thereby blunting the host innate response (10, 11). More recently, vhs has also been shown to induce the degradation of double-stranded RNA contributing to the inhibition of the protein kinase R (PKR) response seen in virus infection and revealing an additional role for vhs in counteracting host defences (12).

In theory, vhs should target all translating transcripts equally, both host and viral. However, transcriptomic analyses from our group have demonstrated that cellular mRNAs exhibit a wide range of susceptibility to vhs activity, ranging from almost complete resistance to 1000-fold reduction, suggesting that specificity for vhs activity exists (10); this result was also confirmed in another recent study (13). In the case of virus transcripts, vhs has been shown to play a key role in regulating viral gene expression during the classical herpesvirus cascade, by reducing the levels of immediate-early (IE) and early (E) viral transcripts at the transition to late (L) gene expression (2, 14). In addition, previous work from our group has revealed a novel mechanism whereby vhs causes the nuclear retention of mRNAs, providing an alternative mechanism of translation inhibition (10, 15). In particular, using mRNA FISH, IE and E transcripts were seen to be retained in the nucleus in a vhs-dependant fashion from around 10 h onwards, whereas L transcripts were efficiently exported to the cytoplasm (15). In the absence of vhs, all viral transcripts were cytoplasmic.

85 Concomitant with this nuclear retention of mRNA, the steady state localisation of the polyA
 86 binding proteins PABPC1 is altered from cytoplasmic to nuclear in HSV1 infected cells (10,
 87 15, 16). PABPC1 binds the polyA tails of mRNA transcripts in the nucleus and shuttles
 88 between the nucleus and cytoplasm as mRNAs undergo nuclear export, translation and
 89 normal cellular degradation (17, 18). In uninfected cells, PABPC1 has a steady-state
 90 cytoplasmic localisation, however upon infection with HSV-1, or expression of vhs by
 91 transient transfection, PABPC1 accumulates in the nucleus in a vhs-dependant manner (10,
 92 15), meaning that relative PABPC1 localisation is a useful surrogate for vhs function. These
 93 observations follow those seen previously for the Kaposi's sarcoma herpesvirus (KSHV)
 94 SOX protein (19, 20), revealing the potential for a universal mechanism across the
 95 herpesviruses for regulating RNA metabolism.

96 Given its profound activity on the cellular transcriptome, vhs would be expected to be lethal
 97 to the virus if left unchecked, and in recent years it has become clear that the virus uses
 98 several mechanisms to lessen the consequences of vhs activity. First, the vhs transcript is
 99 inherently untranslatable, a feature which has been attributed to the presence of transferable
 100 inhibitory sequences within the mRNA (15). Second, the vhs transcript itself is predominantly
 101 localised in the nucleus when expressed during virus infection or in isolation, providing
 102 another layer of translation inhibition (15). A third process for regulating vhs activity in virus
 103 infection is through the formation of a trimeric complex between the vhs protein and the viral
 104 proteins VP16 and VP22 (15, 21), in which VP16 binds directly to vhs (22) and VP22 binds
 105 directly to VP16 (23). As such, both VP16 and VP22 deletion viruses exhibit profound shutoff
 106 of translation in the infected cell (10, 24-26), while $\Delta 22$ viruses are rescued by spontaneous
 107 mutations in vhs (10, 24, 27). This originally led to the hypothesis that vhs endoribonuclease
 108 activity is overactive in the absence of VP22, but our recent work has shown that the main
 109 outcome of deleting VP22 is not increased mRNA degradation *per se*, but increased nuclear

110 retention of the transcriptome (10). As such, the nuclear export of L transcripts and
111 subsequent late protein synthesis was dependent on the presence of VP22 (10).

112 To date, our cell biological studies of vhs have been impeded by the lack of an effective anti-
113 vhs antibody for downstream studies. Here we report the generation and characterisation of
114 a recombinant HSV1 which expresses vhs tagged with GFP at its C- terminus (vhs-GFP).
115 We show that HSV1 vhs-GFP maintains many characteristics of Wt virus, including similar
116 plaque size, viral gene expression, the ability to form a complex with VP16 and VP22 and
117 virion packaging of vhs itself. We also show that when first expressed, vhs-GFP
118 concentrates in a juxtannuclear position in close proximity to but not associated with the Golgi
119 apparatus, while later in infection it co-localises with VP16. Despite vhs-GFP retaining
120 activity in transient transfection, it failed to induce mRNA degradation or PABPC1
121 relocalisation to the nucleus in the context of virus infection, whereas PKR phosphorylation
122 was enhanced, suggesting that vhs enzymatic activity is abrogated in this virus.
123 Nonetheless, mRNA FISH of virus transcripts revealed that nuclear retention of IE and E
124 mRNA was maintained in cells infected with HSV1 vhs-GFP, despite a lack of mRNA
125 degradation. This unexpected result uncouples the endoribonuclease and nuclear retention
126 activities of vhs, providing us with a unique tool to investigate these discrete molecular
127 aspects of vhs function.

128

129

Results

Vhs tagged with GFP at its C-terminus retains endoribonuclease activity. To determine if vhs retains activity when fused to GFP, we first tagged vhs at either its N- or C-terminus and expressed it by transient transfection in HeLa cells. Total protein analysed by SDS-PAGE and Western blotting for GFP revealed that as expected, GFP accumulated to high levels in transfected cells, but GFP-vhs was around 15-fold lower, while expression of vhs-GFP was not detectable (Fig 1A). These results are in line with previous experiments by us and others where it was shown that the vhs transcript is inherently untranslatable, and vhs protein fails to accumulate during transient transfection despite vhs mRNA levels being similar to those in infected cells (1, 15, 21). The ability of GFP-vhs or vhs-GFP to relocalise PABPC1 to the nucleus of HeLa cells, indicative of endoribonuclease activity, was measured using immunofluorescence of PABPC1. Following transfection with a plasmid expressing GFP alone, PABPC1 remained cytoplasmic (Fig 1B). However, while vhs-GFP caused PABPC1 relocalisation to the nucleus, GFP-vhs failed to do so, indicating that vhs functionality is retained only in vhs tagged at its C-terminus with GFP (Fig 1B). Furthermore, mRNA FISH using a probe for GFP indicated that while the GFP and GFP-vhs transcripts were localised in the cytoplasm, the vhs-GFP transcript was retained in the nucleus (Fig 2). This confirms that unlike GFP-vhs, vhs-GFP seems to retain the full characteristics of the native vhs transcript (15).

Characterisation of recombinant HSV1 expressing a vhs-GFP fusion protein. A recombinant HSV1 in strain Sc16 was subsequently generated using homologous recombination to express vhs-GFP in place of vhs (Fig 3A). Following three rounds of plaque purification on Vero cells, vhs-GFP expression was confirmed by analysing total infected cell protein by SDS-PAGE and Western blotting for GFP, vhs, and a loading control of α -tubulin (Fig 3B). Another recombinant virus expressing a different tagged protein – YFP-

156 UL47 - was analysed simultaneously to directly compare the relative expression of vhs with
 157 a second tagged virus protein (Fig 3B). In HSV1-vhs-GFP infected cells, a protein band of
 158 the correct size was detected with both the vhs and the GFP antibodies, confirming the
 159 expression of vhs-GFP in this virus (Fig 3B). Moreover, while the vhs antibody indicated that
 160 vhs was expressed at roughly the same level in cells infected with HSV1 expressing either
 161 the vhs-GFP or YFP-UL47, quantification of the respective vhs-GFP and YFP-UL47 bands
 162 using the GFP antibody (which detects YFP with equal efficiency), indicated that vhs is
 163 expressed at a level 15-fold lower than UL47, re-enforcing the relatively poor expression of
 164 vhs (Fig 3B). Plaque assays on Vero and HFFF cells further indicated that the plaque size
 165 of HSV1-vhs-GFP was similar to that of the Wt Sc16 virus suggesting that virus replication
 166 was not significantly impaired by the expression of vhs-GFP in place of vhs (Fig 3C). The
 167 growth characteristics of vhs-GFP were next compared to that of Wt strain Sc16 in a single-
 168 step growth curve in HFFF cells. Cells were infected at MOI 2 and total virus was harvested
 169 every 5 hours up to 30 h, and titrated on Vero cells, indicating that vhs-GFP replication was
 170 delayed by around 5 hours compared to Wt virus but ultimately reached approximately the
 171 same titre (Fig 3D). A similar infection time-course was analysed for protein expression by
 172 SDS-PAGE and Western blotting to assess the kinetics of expression of representative viral
 173 proteins (Fig 3E). Blotting for ICP27, TK and VP16, representative of the viral gene classes
 174 IE, E, and L respectively, showed that the kinetics of expression of all three classes was
 175 similar between Wt and HSV1-vhs-GFP infections, while vhs and GFP blots confirmed the
 176 expression of vhs-GFP with similar kinetics to vhs alone, although vhs-GFP accumulated to
 177 lower levels than vhs (Fig 3E).

178 To investigate where vhs localises in the infected cell, HFFF cells infected with HSV1 vhs-
 179 GFP were fixed at various times after infection and imaged for GFP fluorescence using
 180 confocal microscopy. In line with Western blotting, vhs-GFP was first detected at low levels
 181 at around 10 hours and increased in intensity to 15 hours, when it was particularly

concentrated close to the nucleus and at the cell periphery (Fig 4A). Closer examination of the 10-hour sample revealed that vhs-GFP was clustered in a juxtannuclear location when first expressed at detectable levels (Fig 4B), but staining of cells at this time with an antibody for giantin indicated that these clusters, while in proximity of the Golgi apparatus, were not associated with it (Fig 4C).

The vhs-GFP fusion protein maintains the ability to form the vhs-VP16-VP22 complex in infected cells. Viral protein VP16 binds directly to vhs, and together with VP22 regulates the activity of vhs (22, 26). Immunofluorescence of VP16 in HSV1 vhs-GFP infected cells indicated that these two proteins were extensively co-localised at later times in infection when virus assembly would be optimal (Fig 5A). To determine if vhs-GFP interacts with VP16 and VP22 during infection, a GFP-TRAP pulldown was performed on extracts of HaCat cells infected with either vhs-GFP or Wt viruses at MOI 5, harvested at 24 h. The resulting pulldown complex was analysed by Western blotting, indicating that both VP16 and VP22 were present in the vhs-GFP interactome but not the negative control (Fig 5B), suggesting that the fusion protein is capable of binding its usual viral protein partners.

The interaction between VP16 and vhs has also been implicated in the packaging of vhs into the virion (22). To test the assembly of vhs-GFP, extracellular HSV1 vhs-GFP and Wt virions were purified from infected HaCat cells by centrifuging on a 5 to 15% Ficoll gradient. Solubilised virions were analysed by SDS-PAGE followed by Coomassie blue staining and Western blotting (Fig 5C and 5D). Using the VP5 major capsid protein band to equalise virion loading, the total protein profiles for each virus was determined to be comparable (Fig 5C). Western blotting for viral proteins vhs, GFP, VP16 and VP22, using VP5 as a loading control confirmed the presence of the ~55 kDa vhs protein in Wt virions and the ~82 kDa fusion protein in vhs-GFP virions (Fig 5D). Although VP16 was equally packaged in both

207 virion samples, vhs-GFP was present around five-fold lower than Wt vhs (Fig 5D) suggesting
208 that packaging of vhs-GFP into virions may be less efficient than Wt virus.

209

210 **vhs-GFP lacks endoribonuclease activity in infected cells.** We previously showed that
211 PABPC1 was relocalised from the cytoplasm to the nucleus in a vhs-dependent manner in
212 both HSV1 infected cells and in cells transfected with a plasmid expressing vhs (22). During
213 the initial transfection experiments we also showed that when transfected into Hela cells,
214 vhs-GFP was also capable of relocalising PABPC1 to the nucleus (Fig 1B). To confirm that
215 this activity was maintained in HSV1 vhs-GFP, HFFF cells infected with either Wt or HSV1
216 vhs-GFP were fixed at 16 h and stained for PABPC1. As shown previously, PABPC1 was
217 concentrated in nuclear accumulations in Wt infected cells (Fig 6). By contrast, PABPC1
218 remained cytoplasmic in HSV1 vhs-GFP infected cells even as late as 20 h after infection
219 (Fig 6).

220 The lack of PABPC1 relocalisation to the nucleus raised the possibility that vhs may not be
221 active as an endoribonuclease when expressed in the context of virus infection. To test the
222 ability of the vhs-GFP fusion protein to degrade cellular mRNA, relative levels of two ISG
223 transcripts (IFIT1 and IFIT2) and two transcripts previously shown to be hypersensitive to
224 vhs activity (MMP1 and MMP3) were measured by RT-qPCR at 16 h. IFIT1 and IFIT2
225 transcripts were reduced in Wt infection compared to mock-infected control (Fig 7A) as
226 shown in our previous work (10). By contrast these ISG transcripts were both upregulated
227 in HSV1 vhs-GFP infection by around Log₂ fold change of 3, suggesting that these
228 transcripts were not degraded by vhs-GFP and were being activated in response to virus
229 infection, as shown previously in cells infected. With a vhs knockout virus (10). In the case
230 of MMP1 and MMP3, these transcripts were reduced by Log₂ fold change of 8 in Wt
231 infection, again as shown previously (10), but these transcripts were unchanged in the HSV1

vhs-GFP infection compared to the mock-infected control at 16 h (Fig 7A). For all four transcripts, very similar results were observed at 24 h to those seen at 16 h, suggesting that the ability of vhs-GFP to degrade cellular transcripts is impaired, rather than delayed.

Viral transcripts representing IE (ICP22), E (TK) and L (gC) genes were measured in the same way as the cellular transcripts, but in this case the log₂ FC was compared to Wt infected cells. ICP22 and TK transcripts were significantly more abundant in HSV1 vhs-GFP infection compared to Wt (Fig 7B), but the level of the late gC transcript was not significantly different between the two viruses, in agreement with our previous results on Δ vhs infected cells (10). Hence, the significant differences in IE and E mRNA transcript levels between HSV1 vhs-GFP and Wt infection further suggests that the vhs-GFP fusion protein is severely impaired or non-functional with respect to its mRNA degradation capabilities.

One other consequence of vhs endoribonuclease activity is the degradation of double-stranded RNA, formed by the annealing of virus mRNA transcribed from both strands of the virus genome, thereby contributing to the suppression of PKR phosphorylation and subsequent eIF2 α phosphorylation in infected cells to subsequently counteract translational shutoff (12). This suggests that vhs acts in tandem with two other virus factors: Us11, which blocks PKR phosphorylation (28) and ICP34.5, which controls eIF2 α phosphorylation (29). Western blotting of Wt and HSV1 vhs-GFP infected cell extracts indicated that PKR phosphorylation was indeed enhanced in cells expressing vhs-GFP compared to Wt virus infection (Fig 7C). Increased PKR phosphorylation was also reflected in an increase in eIF2 α phosphorylation in HSV1 vhs-GFP infection.

Nuclear retention of the infected cell transcriptome in HSV1 vhs-GFP infected cells.

The failure of vhs-GFP to degrade infected cell mRNA led us to hypothesize that this virus would be equivalent to a Δ vhs virus in that all vhs activities would be abrogated by the fusion

257 of GFP at its C-terminus in the context of virus infection. As we have not yet formally shown
 258 that vhs endoribonuclease activity is required for the nuclear retention of the infected cell
 259 transcriptome (10, 15), mRNA FISH was carried out on infected HFFF cells for
 260 representative IE (ICP27) E (TK) and L (gD) transcripts. Wt infected cells recapitulated our
 261 previous results that IE and E transcripts but not L transcripts were retained in the nucleus
 262 at later times of infection (Fig 8A, Wt), but in Δ vhs infected cells, all classes of transcript
 263 were entirely cytoplasmic, thereby confirming that vhs is required for nuclear retention of
 264 these transcripts (Fig 8A, Δ vhs). However, in the HSV1 vhs-GFP infected cells, IE and E
 265 transcripts were also seen to be retained in the nucleus (Fig 8A, vhs-GFP), despite vhs-GFP
 266 failing to induce the degradation of mRNA or the nuclear retention of PABPC1. Likewise,
 267 the cellular transcript for MMP1, which is efficiently degraded by vhs but not vhs-GFP (Fig
 268 7A), was also retained in the nucleus despite not being degraded in HSV1 vhs-GFP infected
 269 cells, in contrast to Δ vhs infected cells (Fig 8B). Finally, the localisation of the vhs-GFP
 270 transcript itself was examined in infected cells using a probe for GFP or vhs. This revealed
 271 that as for Wt infection, where the vhs transcript was predominantly nuclear, the vhs-GFP
 272 transcript was also retained in the nucleus (Fig 8C), confirming the results obtained above
 273 in cells expressing vhs-GFP by transfection.

274 These unexpected results indicate that although vhs-GFP lacks endoribonuclease activity
 275 in HSV1 infected cells, it retains the ability to alter the compartmentalisation of the infected
 276 cell transcriptome, both viral and cellular. Hence, these two properties of vhs have been
 277 uncoupled in the HSV1 vhs-GFP virus, providing scope for further molecular understanding
 278 of vhs activity.

279

280

Discussion

The vhs protein of HSV1 is involved in regulating the RNA environment of the infected cell. Not only does it induce the degradation of mRNA and dsRNA (3, 4, 12), but it also causes the nuclear retention of both the viral and the cellular transcriptome (10). The overall effect of this activity is to enhance translation of the late virus transcriptome not only by sequestration of the early virus transcriptome in the nucleus, but also by reducing the PKR stress response (12, 30). As a virulence factor, vhs therefore helps to optimise the virus infection process and block the establishment of an antiviral environment in the cell. In this study we aimed to develop a cell biology tool for studying vhs, using a vhs-GFP fusion protein which had been demonstrated to function when expressed in isolation. Having constructed recombinant HSV1 expressing vhs-GFP in place of native vhs, we characterised its localisation, binding partners, virion packaging, and endoribonuclease activity during infection. Surprisingly, despite functioning in isolation and maintaining its physical characteristics during infection, vhs-GFP exhibited little if any endoribonuclease activity, a result that was backed up by the failure of PABPC1 to relocate to the nucleus. This suggests that the addition of GFP to the C-terminus of vhs differentially affects its activity depending on whether it is expressed in isolation or in virus infection.

The vhs-GFP protein was expressed at a relatively low level during transfection or infection, with a 20-fold or over reduction in GFP expression by fusing it to the vhs open reading frame at either the N- or C- terminus, (Fig 1A). This is not unexpected as we have previously demonstrated that vhs autoregulates its own expression by various means, including the nuclear retention of its own transcript, and inefficient translation of its transcript (15). Despite the low level of vhs expression during infection, the HSV1 vhs-GFP virus nonetheless afforded us with the opportunity to investigate vhs localisation throughout the virus infection cycle. The vhs-GFP fusion protein was only detectable at 8 to 10 hours after infection, at which time it localised to juxtanuclear clusters that were close to but not associated with the

307 Golgi apparatus. As infection progressed, fluorescence levels increased and vhs-GFP
308 appeared throughout the cytoplasm and at the cell periphery, where it colocalised with its
309 known binding partner, VP16. As both vhs and VP16 are assembled in to the HSV1 virion,
310 these sites of vhs positivity are likely to represent different stages of the HSV1
311 morphogenesis pathway including the trafficking of structural proteins on the path to virus
312 envelopment and the egress of enveloped virions (31).

313 Herpesvirus encoded endoribonucleases act by enhancing the normal rate of turnover of
314 cellular mRNA; mRNA is first cleaved by the endoribonuclease before the cellular mRNA
315 decay machinery acts on it (32, 33). This accelerated cleavage alters the steady-state
316 localisation of PABPC1 from cytoplasmic to nuclear as it shuttles between the nucleus and
317 the cytoplasm (17). The most striking result from this study is that despite the lack of
318 endoribonuclease activity and PABPC1 retention in the nucleus, vhs-GFP maintains the
319 ability to cause the vhs-dependent nuclear retention of IE and E transcripts seen in Wt
320 infection. This reveals for the first time that vhs cleavage and degradation of mRNA is not a
321 prerequisite for enhanced nuclear compartmentalisation of the infected cell transcriptome.
322 It also emphasizes that the relative steady-state localisation of PABPC1 is a direct
323 consequence of vhs endoribonuclease activity and mRNA degradation in the cytoplasm,
324 rather than a correlate for the relative compartmentalisation of the transcriptome: PABPC1
325 bound to the polyA tails of cytoplasmic transcripts can only return to the nucleus after mRNA
326 degradation has released it for nuclear import (34). Much of what is known about PABPC1
327 compartmentalisation has been elucidated from work on virus endoribonucleases, and in
328 particular the SOX protein from Kaposi's sarcoma herpesvirus (KSHV). As for vhs, the
329 activity of SOX in the cytoplasm causes the nuclear retention of both PABPC1 and mRNA
330 (19), but in the case of SOX, previous work has revealed that the inhibition of mRNA export
331 is a direct consequence of the nuclear accumulation of PABPC1 (35). However, our results
332 shown here demonstrate that vhs induces the nuclear accumulation of mRNA without

333 concomitant relocalisation of PABPC1, showing not only that these two properties of vhs are
334 separable, but also that PABPC1 accumulation in the nucleus is not a direct consequence
335 of increased nuclear concentration of mRNA (36).

336 The molecular explanation for the vhs-induced sequestration of the transcriptome in the
337 nucleus will require further studies, but one potential explanation is that vhs causes the
338 cytoplasmic retention of a nuclear export factor, preventing it from returning to the nucleus
339 and thereby blocking the subsequent export of IE and E transcripts. If this is the case then
340 it raises the question of how L transcripts are successfully exported for translation of
341 structural proteins. The activities of several other virus proteins may provide clues to this.
342 First, the IE protein ICP27 is known to be essential for late protein expression (37, 38).
343 ICP27 is a nucleocytoplasmic shuttling protein that binds mRNA transcripts in the nucleus
344 and helps export them in co-ordination with the TREX (transcription-coupled export)
345 complex, specifically through binding to the Aly/REF factor within the complex (39, 40).
346 ICP27 may therefore counteract the nuclear retention activity of vhs by bypassing the need
347 for any specific factors retained by vhs in the cytoplasm. Interestingly, ICP27 has been
348 shown to stimulate the translation of a subset of late HSV1 mRNAs (41), a property likely to
349 be explained by its ability to directly bind PABPC1 and recruit it to these mRNAs (42).
350 Additionally, deletion of either of the VP16 and VP22 components of the vhs-VP16-VP22
351 complex causes translational shut-off, and in the case of VP22 at least, this shutoff
352 correlates with the entrapment of late transcripts in the nucleus (10). Hence, VP22 and VP16
353 quench the ability of vhs to retain the nuclear transcriptome, potentially by competing for vhs
354 binding to specific factors in the cytoplasm, to function in co-ordination with ICP27 to activate
355 late protein expression. GFP-Trap analysis of vhs-GFP expressed during infection will now
356 provide us with the means of investigating the binding partners of vhs during infection,
357 allowing us to investigate the full vhs interactome and identify potential candidates required
358 for export of viral mRNAs late in infection.

359 These results further emphasize the role that vhs plays in regulating the antiviral response
 360 to HSV1 infection. As we have shown before, ISG transcripts are initially upregulated in
 361 HSV1 infected HFFF, before dropping again as infection progresses, with this decrease in
 362 ISG level being dependent on vhs activity (10). Here we have shown that vhs-GFP also fails
 363 to degrade IFIT1 and IFIT2 transcripts, confirming the dependence of this process on vhs
 364 endoribonuclease activity. The PKR stress response is also upregulated in HSV1 vhs-GFP
 365 infection with a seven-fold increase in PKR phosphorylation and a 3.5-fold increase in eIF2 α
 366 phosphorylation compared to Wt infection, suggesting that the level of dsRNA may be
 367 increased in these infected cells. Nonetheless, there was no evidence of translational shutoff
 368 in the HSV1 vhs-GFP infected cells, indicating that the neurovirulence factor ICP34.5, which
 369 induces the dephosphorylation of eIF2 α (29), is likely to counteract the increase in eIF2 α
 370 phosphorylation induced by the increased PKR phosphorylation seen in cells infected with
 371 virus expressing vhs-GFP. This lack of stress-induced translational shutoff is further
 372 emphasized by the fact that, despite remaining cytoplasmic in HSV1 vhs-GFP infected cells,
 373 PABPC1 did not localise to cytoplasmic foci, suggesting that recruitment of mRNA into
 374 stress granules was not a feature of this infection (43).

375 In combination, these results serve to further emphasize the interplay between a range of
 376 virus factors, including the dsRNA binding protein Us11 which inhibits PKR, the
 377 neurovirulence factor ICP34.5, which reverses the phosphorylation of eIF2 α , and vhs, which
 378 degrades ISG transcripts and dsRNA in the antagonism of host responses to HSV infection
 379 (44). Our new virus tool that separates out vhs-induced mRNA degradation from its effects
 380 on the localisation of the virus transcriptome affords us a unique opportunity to investigate
 381 the relative contributions of these vhs activities to the success of an HSV1 infection.

382 **Materials and Methods**

383 **Cells and viruses.** Vero, HFFF, HaCat and HeLa cells were cultured in Dulbecco modified
 384 Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Viruses were
 385 routinely propagated and titrated in Vero cells using DMEM with 2% FBS and supplemented
 386 with 5% human serum for titrations. The parental virus strains used in this study were HSV-
 387 1 strain Sc16 and s17. The s17-derived vhs knockout virus (Δ vhs) has been described
 388 before (28). Extracellular virions were gradient purified as described previously (16). Briefly,
 389 ten 175-cm² flasks of confluent HaCaT cells were infected at a multiplicity of 0.05. Once
 390 cytopathic effect was advanced (3-4 days post-infection), the extracellular medium was
 391 collected and centrifuged at 3,000 rpm for 30 min at 4°C in a fixed-angle rotor to remove cell
 392 debris. Virus particles were pelleted from the supernatant at 9,000 rpm for 90 min at 4°C.
 393 The particle pellet was resuspended in 0.5 mL phosphate-buffered saline (PBS) and layered
 394 onto a preformed 11 mL 5% to 15% (wt/vol) Ficoll gradient in a 13.2 mL thin-wall polyallomer
 395 ultracentrifuge tube (Beckman Coulter). Gradients were centrifuged at 12,000 rpm for 2 h at
 396 4°C in an SW41 Ti swinging-bucket rotor in a Sorvall Discovery SE ultracentrifuge. Virions
 397 were harvested by needle puncture through the side of the tube with a 23-gauge hypodermic
 398 needle in a volume of <1 mL, diluted in 10 mL PBS, and pelleted at 25,000 rpm for 1 h at
 399 4°C using the same rotor and ultracentrifuge. The pellets were resuspended in 100 μ L PBS
 400 and stored at -80°C. Virions were solubilised in SDS-PAGE lysis buffer prior to analysis by
 401 SDS-PAGE and Western blotting.

402 **Plasmids.** To construct pGFP-vhs, the vhs open reading frame (UL41) was transferred from
 403 plasmid V5-vhs to pEGFPC1. To construct pvhs-GFP, UL41 was amplified by PCR from
 404 plasmid pGE204 (15) using primers F- ggagatctacatgggttgttcgggatga and R-
 405 gcgaattctcgtcccagaattt, and inserted into pEGFPN1. Plasmids were transfected into HeLa
 406 cells with Lipofectamine 2000.

407 A transfer vector to produce recombinant HSV1 vhs-GFP was constructed by amplifying the
408 800 bp downstream flanking region of the vhs-encoding gene UL41 from the Sc16 genome
409 as a Not1 fragment using primers F-5'cggcggccgccgctcagacgagcgcgcttg3' and R-
410 5'cggcggccgccggtggccggtaccatcaac3', and inserting the amplified fragment in the Not1 site
411 downstream of GFP in pvhs-GFP.

412 **Construction of vhs-GFP virus.** Equal amounts of plasmid vhs-GFP and infectious HSV1
413 strain Sc16 DNA were transfected into 2x10⁵ Vero cells grown in a well of a 12-well culture
414 plate using lipofectamine 2000. Five days later when cytopathic effect was extensive, the
415 infected cells were scraped into the cell medium, subjected three times to freeze-thawing
416 and titrated on Vero cells. Fluorescent plaques were picked and plaque-purified a further
417 two times before further analysis.

418 **Antibodies.** The VP22 (AGV031) antibody has been described elsewhere (29). Other
419 antibodies used in this study were kindly provided by the following individuals: VP16 (LP1),
420 Colin Crump (University of Cambridge); vhs, Duncan Wilson (Albert Einstein College of
421 Medicine); TK, Frazer Rixon (Centre for Virus Research, Glasgow). Other antibodies were
422 purchased commercially: ICP27 (AbCam); α -tubulin (Sigma), ICP5 (Virusys), PABPC1
423 (Santa Cruz), GFP (Clontech), PKR, eIF2 α (Cell Signalling Technology), Phospho-PKR and
424 giantin (both AbCam). Fluorescent IRDye secondary antibodies were from LI-COR.

425 **SDS-PAGE and Western blotting.** Total protein samples were analysed by SDS-
426 polyacrylamide gel electrophoresis (PAGE) and were either stained with Coomassie blue or
427 transferred to a nitrocellulose membrane for Western blot analysis. Western blots were
428 visualised on the Odyssey CLx system (LI-COR). Phosphorylated eIF2 α was distinguished
429 from eIF2 α using Phos-tag SDS-PAGE (Alpha Laboratories).

430 **GFP-TRAP pulldown assay.** A GFP-TRAP agarose kit (Chromotek) was used to purify
431 GFP-tagged protein from infected HaCat cells according to the manufacturer's instructions.

432 Briefly, infected cells were harvested at 24 hpi, washed in D-PBS then lysed in RIPA buffer.
 433 Cell lysates were centrifuged to clear cellular debris, before mixing with dilution buffer and
 434 adding GFP-TRAP beads. At this point a modification was made to the protocol: bead
 435 binding was performed overnight rather than for 1 h. Finally, the beads were washed three
 436 times before being resuspended in 2x SDS-PAGE lysis buffer and boiled for 5 minutes prior
 437 to analysis by SDS-PAGE.

438 **Quantitative RT-PCR (qRT-PCR)** Total RNA was extracted from cells using the RNeasy
 439 plus mini kit (Qiagen). Excess DNA was removed by incubation with DNase I (Invitrogen)
 440 for 15 min at room temperature, followed by inactivation for 10 min at 65°C in 25 nM of
 441 EDTA. Superscript III (Invitrogen) was used to synthesise cDNA using random primers
 442 according to the manufacturer's instructions. All qRT-PCR assays were carried out in 96-
 443 well plates using Takyon™ No ROX SYBR 2X MasterMix blue dTTP (Eurogentec). Primers
 444 for cellular and viral genes are shown in S1 table. Cycling was carried out in a Lightcycler
 445 (Roche), and relative expression was determined using the $\Delta\Delta CT$ method (30), using 18s
 446 RNA as reference. Statistical analyses were carried out using unpaired t tests in GraphPad
 447 Prism 8.2.1.

448 **Immunofluorescence.** Cells for immunofluorescence were grown on coverslips and fixed
 449 with 4% paraformaldehyde in PBS for 20 min at room temperature, followed by
 450 permeabilisation with 0.5% Triton-X100 for 10 min. Fixed cells were blocked by incubation
 451 in PBS with 10% newborn calf serum (block buffer) for 20 min, before the addition of primary
 452 antibody in block buffer, and a further 30-min incubation. After extensive washing with PBS,
 453 the appropriate Alexafluor conjugated secondary antibody was added in block buffer and
 454 incubated for a further 30 min. The coverslips were washed extensively in PBS and mounted
 455 in Mowiol containing DAPI. Images were acquired using a Nikon A1 confocal microscope
 456 and processed using ImageJ software (45).

457 **Fluorescent *in situ* hybridisation (FISH) of mRNA.** Cells were grown in 2-well slide
 458 chambers (Fisher Scientific) and infected with virus. At the appropriate time, cells were fixed
 459 for 20 min in 4% PFA, then dehydrated by sequential 5 min incubations in 50%, 70% and
 460 100% ethanol. FISH was then carried out using Applied Cell Diagnostics (ACD) RNAscope
 461 reagents according to manufacturer's instructions. Briefly, cells were rehydrated by
 462 sequential 2 min incubations in 70%, 50% ethanol and PBS, and treated for 30 min at 37 °C
 463 with DNase, followed by 15 min at room temperature with protease. Cells were then
 464 incubated for 2 h at 40 °C with RNAscope probes for virus transcripts ICP27, TK and
 465 glycoprotein D, or cellular transcripts MMP1 and serpinE1, as designed by Advanced Cell
 466 Diagnostics, ACD, followed by washes and amplification stages according to instructions.
 467 After incubation with the final fluorescent probe, the cells were mounted in Mowiol containing
 468 DAPI to stain nuclei, and images acquired with a Nikon A2 inverted confocal microscope
 469 and processed using Adobe Photoshop software.

470

471

472 **Acknowledgements**

473 The authors would like to thank Colin Crump, Duncan Wilson and Frazer Rixon for
 474 antibodies used in this study. This work was funded by a grant from Medical Research
 475 Council, UK (MR/T0001038/1).

476

477

Table 1. Primer pairs used for qRT-PCR (sequences shown 5'-3')

Gene	Forward	Reverse
ICP22	tgtgcaagcttccttggttg	ggcatcggagatttcatcat
TK	tacccgagccgatgacttac	gttatctgggcgcttgcat
gC	gggtccgtccccccaat	cgtaggttgggggcgct
18s	ccagtaagtgcgggtcataagc	gcctcactaaaccatccaatcgg
IFIT1	cctgaaaggccagaatgagg	tccacctgtccaggtaagt
MMP1	HP100549 (SinoBiological)	
MMP3	HP100493 (SinoBiological)	
IFIT2	PPH02853A (Qiagen)	

Figure Legends

FIG 1. Expression of GFP-tagged vhs proteins. **(A)** HeLa cells were mock transfected or transfected with plasmids expressing GFP-tagged proteins as shown, and after 20 h total cell lysates were analysed by Western blotting for GFP. Relative expression of GFP was quantitated using LICOR ImageStudio and is represented as percentage of untagged GFP level. **(B)** As for (A), but transfected cells grown on coverslips were fixed and stained for PABPC1 (red) and nuclei stained with DAPI (blue). Scale bar = 20 μ m.

FIG 2. Nuclear localisation of the vhs-GFP transcript in transfected cells. HeLa cells grown in two-well chamber slides were transfected with plasmids expressing GFP-tagged proteins as shown, and after 20 h were fixed in 4% paraformaldehyde. Cells were then processed for mRNA FISH using a GFP-specific probe (red) and nuclei stained with DAPI (blue). GFP fluorescence is in green. Scale bar = 20 μ m.

FIG 3. Construction and characterisation of HSV1 expressing vhs-GFP. **(A)** HSV1 expressing vhs-GFP in place of GFP was constructed by co-transfecting Vero cells with infectious Sc16 genomic DNA and plasmid containing the UL41GFP fusion gene surrounded by the flanking sequences from UL40 and UL42. **(B)** HFFF cells were mock infected or infected with Sc16, plaque purified vhs-GFP or YFP-UL47 viruses at MOI 2, and total protein harvested at 16 hpi prior to separation by SDS-PAGE and Western blotting with antibodies for GFP, vhs and α -tubulin. **(C)** Vero and HFFF cells were infected with around 40 pfu of Sc16 or vhs-GFP and incubated for three days prior to fixation and staining with crystal violet. **(D)** One-step growth curves for Wt (Sc16) and HSV1 vhs-GFP viruses were carried out by harvesting total virus from HFFF cells infected at MOI 2 every 5 hours up to 30 hours. Samples were titrated onto Vero cells. The mean titre of three replicates and associated

506 SEM are plotted. **(E)** Total extracts of HFFF cells infected as in (D) were analysed by SDS-
507 PAGE and Western blotting for the indicated virus proteins and α -tubulin as a loading
508 control.

509
510 **FIG 4.** Localisation of vhs-GFP during HSV1 infection. **(A)** HFFF cells infected with HSV1-
511 vhs-GFP MOI 2 were fixed at the indicated times, stained with DAPI (blue) and imaged for
512 GFP fluorescence (white) using confocal microscopy. Scale bar = 50 μ m. **(B)** Examples of
513 the 10 h sample from (A) imaged at higher magnification. Scale bar = 20 μ m. **(C)** HFFF cells
514 infected as in (A) were fixed and stained for the Golgi marker giantin. Scale bar = 10 μ m.

515
516 **FIG 5.** Interaction of vhs-GFP with VP16 and assembly into virions. **(A)** HFFF cells infected
517 with HSV1 vhs-GFP MOI 2 were fixed at 16 h, stained for VP16 (red) and imaged for GFP
518 (green). Nuclei were stained with DAPI (blue). Scale bar = 20 μ m. **(B)** GFP-TRAP pulldown
519 was carried out on HaCat cells infected with Wt (Sc16) or vhs-GFP at moi 5 and harvested
520 24 hpi. The pulldowns were analysed by SDS-PAGE and Western blotting with antibodies
521 to GFP, VP16 and VP22. In = input, B = pulldown **(C) & (D)** Extracellular virions were purified
522 on 5 – 15% Ficoll gradients and analysed by SDS-PAGE followed by Coomassie blue
523 staining (C) or Western blotting with antibodies as indicated (D).

524
525 **FIG 6.** PABPC1 is not relocalised in HSV1 vhs-GFP infection. HFFF cells infected with Wt
526 (Sc16) or HSV1 vhs-GFP viruses at MOI 2 were fixed at 16 hours, stained with an antibody
527 for PABPC1 (red) and nuclei stained with DAPI (blue). Scale bar = 20 μ m.

528

529 **FIG 7.** vhs-GFP expressed in virus infection is deficient in endoribonuclease activity. **(A)**
 530 HFFF cells were infected with Wt or HSV1 vhs-GFP viruses at MOI of 5. Total RNA was
 531 purified at 16 hpi and subjected to RT-qPCR for cellular transcripts previously identified as
 532 being susceptible to vhs activity. **(B)** RNA samples from (A) were subjected to RT-qPCR of
 533 viral transcripts ICP22 (IE), TK (E) and gC (L). The mean and $\pm$ standard error for $n = 3$ is
 534 shown. Statistical analysis was carried out using an unpaired t test. ***, $p < 0.001$. ****, $p <$
 535 0.0001 . ns, not significant. **(C)** HFFF cells infected with Wt or HSV1 vhs-GFP viruses at MOI
 536 of 2 were harvested at 16 h and analysed by SDS-PAGE and Western blotting with
 537 antibodies as indicated. Western blotting for eIF2 α was carried out using 10 μ m Phos-Tag
 538 PAGE. Relative level of phospho-PKR and phospho-eIF2 α was quantitated using LICOR
 539 Image Studio and is represented relative to the level in uninfected cells.

540

541 **FIG 8.** vhs-GFP maintains the ability to cause the nuclear retention of viral and cellular
 542 transcripts. HFFF cells grown in two-well slide chambers were infected with Wt, Δ vhs or
 543 HSV1 vhs-GFP MOI 2 and fixed after 16 hours in 4% paraformaldehyde. Cells were then
 544 processed for mRNA FISH using probes specific for **(A)** IE (ICP27), E (TK) and L (gD)
 545 transcripts or **(B)** MMP1 (all in white). NB. The same field in Wt and Δ vhs infection is shown
 546 for ICP27 and gD mRNA FISH. **(C)** As above but Wt or HSV1 vhs-GFP infected cells were
 547 stained with a probe for UL41 or GFP (in white). Nuclei were stained with DAPI (blue). Scale
 548 bar = 50 μ m.

549

550

FIG 1. Expression of GFP-tagged vhs proteins. **(A)** HeLa cells were mock transfected or transfected with plasmids expressing GFP-tagged proteins as shown, and after 20 h total cell lysates were analysed by Western blotting for GFP. Relative expression of GFP was quantitated using LICOR ImageStudio and is represented as percentage of untagged GFP level. **(B)** As for (A), but transfected cells grown on coverslips were fixed and stained for PABPC1 (red) and nuclei stained with DAPI (blue). Scale bar = 20 μ m.

FIG 2. Nuclear retention of the vhs-GFP transcript in transfected cells. HeLa cells grown in two-well chamber slides were transfected with plasmids expressing GFP-tagged proteins as shown, and after 20 h were fixed in 4% paraformaldehyde. Cells were then processed for mRNA FISH using a GFP-specific probe (red) and nuclei stained with DAPI (blue). GFP fluorescence is in green. Scale bar = 20 μ m.

579

FIG 3. Construction and characterisation of HSV1 expressing vhs-GFP. **(A)** HSV1 expressing vhs-GFP in place of GFP was constructed by co-transfecting Vero cells with infectious Sc16 genomic DNA and plasmid containing the UL41GFP fusion gene surrounded by the flanking sequences from UL40 and UL42. **(B)** HFF cells were mock infected or infected with Sc16, plaque purified vhs-GFP or YFP-UL47 viruses at MOI 2, and total protein harvested at 16 hpi prior to separation by SDS-PAGE and Western blotting with antibodies for GFP, vhs and α -tubulin. **(C)** Vero and HFF cells were infected with around 40 pfu of Sc16 or vhs-GFP and incubated for three days prior to fixation and staining with crystal violet. **(D)** One-step growth curves for Wt (Sc16) and HSV1 vhs-GFP viruses were carried out by harvesting total virus from HFF cells infected at MOI 2 every 5 hours up to 30 hours. Samples were titrated onto Vero cells. The mean titre of three replicates and associated SEM are plotted. **(E)** Total extracts of HFF cells infected as in (D) were analysed by SDS-PAGE and Western blotting for the indicated virus proteins and α -tubulin as a loading control.

594

FIG 4. Localisation of vhs-GFP during HSV1 infection. **(A)** HFFF cells infected with HSV1-vhs-GFP MOI 2 were fixed at the indicated times, stained with DAPI (blue) and imaged for GFP fluorescence (white) using confocal microscopy. Scale bar = 50 μ m. **(B)** Examples of the 10 h sample from (A) imaged at higher magnification. Scale bar = 20 μ m. **(C)** HFFF cells infected as in (A) were fixed and stained for the Golgi marker giantin. Scale bar = 10 μ m.

FIG 5. Interaction of vhs-GFP with VP16 and assembly into virions. **(A)** HFFF cells infected with HSV1 vhs-GFP MOI 2 were fixed at 16 h, stained for VP16 (red) and imaged for GFP (green). Nuclei were stained with DAPI (blue). Scale bar = 20 μ m. **(B)** GFP-TRAP pulldown was carried out on HaCat cells infected with Wt (Sc16) or vhs-GFP at moi 5 and harvested 24 hpi. The pulldowns were analysed by SDS-PAGE and Western blotting with antibodies to GFP, VP16 and VP22. In = input, B = pulldown **(C) & (D)** Extracellular virions were purified on 5 – 15% Ficoll gradients and analysed by SDS-PAGE followed by Coomassie blue staining (C) or Western blotting with antibodies as indicated (D).

FIG 6. PABPC1 is not relocalised in HSV1 vhs-GFP infection. HFFF cells infected with Wt (Sc16) or HSV1 vhs-GFP viruses at MOI 2 were fixed at 16 hours, stained with an antibody for PABPC1 (red) and nuclei stained with DAPI (blue). Scale bar = 20 μ m.

FIG 7. vhs-GFP expressed in virus infection is deficient in endoribonuclease activity. **(A)** HFFF cells were infected with Wt or HSV1 vhs-GFP viruses at MOI of 5. Total RNA was purified at 16 hpi and subjected to RT-qPCR for cellular transcripts previously identified as being susceptible to vhs activity. **(B)** RNA samples from (A) were subjected to RT-qPCR of viral transcripts ICP22 (IE), TK (E) and gC (L). The mean and $\pm$ standard error for $n = 3$ is shown. Statistical analysis was carried out using an unpaired t test. ***, $p < 0.001$. ****, $p < 0.0001$. ns, not significant. **(C)** HFFF cells infected with Wt or HSV1 vhs-GFP viruses at MOI of 2 were harvested at 16 h and analysed by SDS-PAGE and Western blotting with antibodies as indicated. Western blotting for eIF2 α was carried out using 10 μ m Phos-Tag PAGE. Relative level of phospho-PKR and phospho-eIF2 α was quantitated using LICOR Image Studio and is represented relative to the level in uninfected cells.

640 **FIG 8.** vhs-GFP maintains the ability to cause the nuclear retention of viral and cellular
641 transcripts. HFF cells grown in two-well slide chambers were infected with Wt, Δ vhs or
642 HSV1 vhs-GFP MOI 2 and fixed after 16 hours in 4% paraformaldehyde. Cells were then
643 processed for mRNA FISH using probes specific for (A) IE (ICP27), E (TK) and L (gD)
644 transcripts or (B) MMP1 (all in white). NB The same field in Wt and Δ vhs infection is shown
645 for ICP27 and gD mRNA FISH. (C) As above but Wt or HSV1 vhs-GFP infected cells were
646 stained with a probe for UL41 or GFP (in white). Nuclei were stained with DAPI (blue). Scale
647 bar = 50 μ m.

References

1. Everly DN, Jr., Feng P, Mian IS, Read GS. 2002. mRNA degradation by the virion host shutoff (Vhs) protein of herpes simplex virus: genetic and biochemical evidence that Vhs is a nuclease. *J Virol* 76:8560-71.
2. Oroskar AA, Read GS. 1989. Control of mRNA stability by the virion host shutoff function of herpes simplex virus. *J Virol* 63:1897-906.
3. Elgadi MM, Hayes CE, Smiley JR. 1999. The herpes simplex virus vhs protein induces endoribonucleolytic cleavage of target RNAs in cell extracts. *J Virol* 73:7153-64.
4. Taddeo B, Roizman B. 2006. The virion host shutoff protein (UL41) of herpes simplex virus 1 is an endoribonuclease with a substrate specificity similar to that of RNase A. *J Virol* 80:9341-5.
5. Feng P, Everly DN, Jr., Read GS. 2005. mRNA decay during herpes simplex virus (HSV) infections: protein-protein interactions involving the HSV virion host shutoff protein and translation factors eIF4H and eIF4A. *J Virol* 79:9651-64.
6. Page HG, Read GS. 2010. The virion host shutoff endonuclease (UL41) of herpes simplex virus interacts with the cellular cap-binding complex eIF4F. *J Virol* 84:6886-90.
7. Doepker RC, Hsu WL, Saffran HA, Smiley JR. 2004. Herpes simplex virus virion host shutoff protein is stimulated by translation initiation factors eIF4B and eIF4H. *J Virol* 78:4684-99.
8. Smibert CA, Johnson DC, Smiley JR. 1992. Identification and characterization of the virion-induced host shutoff product of herpes simplex virus gene UL41. *J Gen Virol* 73 467-70.

- 672 9. Read GS, Frenkel N. 1983. Herpes simplex virus mutants defective in the virion-
673 associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis
674 of alpha (immediate early) viral polypeptides. J Virol 46:498-512.
- 675 10. Pheasant K, Moller-Levet CS, Jones J, Depledge D, Breuer J, Elliott G. 2018.
676 Nuclear-cytoplasmic compartmentalization of the herpes simplex virus 1 infected
677 cell transcriptome is co-ordinated by the viral endoribonuclease vhs and cofactors
678 to facilitate the translation of late proteins. PLoS Pathog 14:e1007331.
- 679 11. Pasiaka TJ, Lu B, Crosby SD, Wylie KM, Morrison LA, Alexander DE, Menachery
680 VD, Leib DA. 2008. Herpes simplex virus virion host shutoff attenuates
681 establishment of the antiviral state. J Virol 82:5527-35.
- 682 12. Dauber B, Saffran HA, Smiley JR. 2019. The herpes simplex virus host shutoff (vhs)
683 RNase limits accumulation of double stranded RNA in infected cells: Evidence for
684 accelerated decay of duplex RNA. PLoS Pathog 15:e1008111.
- 685 13. Friedel CC, Whisnant AW, Djakovic L, Rutkowski AJ, Friedl MS, Kluge M,
686 Williamson JC, Sai S, Vidal RO, Sauer S, Hennig T, Grothey A, Milic A, Prusty BK,
687 Lehner PJ, Matheson NJ, Erhard F, Dolken L. 2021. Dissecting Herpes Simplex
688 Virus 1-Induced Host Shutoff at the RNA Level. J Virol 95.
- 689 14. Oroskar AA, Read GS. 1987. A mutant of herpes simplex virus type 1 exhibits
690 increased stability of immediate-early (alpha) mRNAs. J Virol 61:604-6.
- 691 15. Elliott G, Pheasant K, Ebert-Keel K, Stylianou J, Franklyn A, Jones J. 2018. Multiple
692 post-transcriptional strategies to regulate the herpes simplex virus type 1 vhs
693 endoribonuclease. J Virol doi:10.1128/JVI.00818-18.
- 694 16. Salaun C, MacDonald AI, Larralde O, Howard L, Lochtie K, Burgess HM, Brook M,
695 Malik P, Gray NK, Graham SV. 2010. Poly(A)-binding protein 1 partially relocalizes

to the nucleus during herpes simplex virus type 1 infection in an ICP27-independent manner and does not inhibit virus replication. J Virol 84:8539-48.

17. Afonina E, Stauber R, Pavlakis GN. 1998. The human poly(A)-binding protein 1 shuttles between the nucleus and the cytoplasm. J Biol Chem 273:13015-21.

18. Burgess HM, Richardson WA, Anderson RC, Salaun C, Graham SV, Gray NK. 2011. Nuclear relocalisation of cytoplasmic poly(A)-binding proteins PABP1 and PABP4 in response to UV irradiation reveals mRNA-dependent export of metazoan PABPs. J Cell Sci 124:3344-55.

19. Lee YJ, Glaunsinger BA. 2009. Aberrant herpesvirus-induced polyadenylation correlates with cellular messenger RNA destruction. PLoS Biol 7:e1000107.

20. Gilbertson S, Federspiel JD, Hartenian E, Cristea IM, Glaunsinger B. 2018. Changes in mRNA abundance drive shuttling of RNA binding proteins, linking cytoplasmic RNA degradation to transcription. Elife 7.

21. Taddeo B, Sciortino MT, Zhang W, Roizman B. 2007. Interaction of herpes simplex virus RNase with VP16 and VP22 is required for the accumulation of the protein but not for accumulation of mRNA. Proc Natl Acad Sci U S A 104:12163-8.

22. Smibert CA, Popova B, Xiao P, Capone JP, Smiley JR. 1994. Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs. J Virol 68:2339-46.

23. Elliott G, Mouzakis G, O'Hare P. 1995. VP16 interacts via its activation domain with VP22, a tegument protein of herpes simplex virus, and is relocated to a novel macromolecular assembly in coexpressing cells. J Virol 69:7932-7941.

24. Mbong EF, Woodley L, Dunkerley E, Schrimpf JE, Morrison LA, Duffy C. 2012. Deletion of the herpes simplex virus 1 UL49 gene results in mRNA and protein translation defects that are complemented by secondary mutations in UL41. J Virol 86:12351-61.

- 721 25. Duffy C, Mbong EF, Baines JD. 2009. VP22 of herpes simplex virus 1 promotes
722 protein synthesis at late times in infection and accumulation of a subset of viral
723 mRNAs at early times in infection. J Virol 83:1009-17.
- 724 26. Lam Q, Smibert CA, Koop KE, Lavery C, Capone JP, Weinheimer SP, Smiley JR.
725 1996. Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion
726 host shutoff function. Embo J 15:2575-81. abs.html.
- 727 27. Sciortino MT, Taddeo B, Giuffre-Cuculietto M, Medici MA, Mastino A, Roizman B.
728 2007. Replication-competent herpes simplex virus 1 isolates selected from cells
729 transfected with a bacterial artificial chromosome DNA lacking only the UL49 gene
730 vary with respect to the defect in the UL41 gene encoding host shutoff RNase. J
731 Virol 81:10924-32.
- 732 28. Poppers J, Mulvey M, Khoo D, Mohr I. 2000. Inhibition of PKR activation by the
733 proline-rich RNA binding domain of the herpes simplex virus type 1 Us11 protein. J
734 Virol 74:11215-21.
- 735 29. He B, Gross M, Roizman B. 1997. The gamma(1)34.5 protein of herpes simplex
736 virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha
737 subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of
738 protein synthesis by double-stranded RNA-activated protein kinase. Proc Natl Acad
739 Sci U S A 94:843-8.
- 740 30. Sciortino MT, Parisi T, Siracusano G, Mastino A, Taddeo B, Roizman B. 2013. The
741 virion host shutoff RNase plays a key role in blocking the activation of protein
742 kinase R in cells infected with herpes simplex virus 1. J Virol 87:3271-6.
- 743 31. Hollinshead M, Johns HL, Sayers CL, Gonzalez-Lopez C, Smith GL, Elliott G. 2012.
744 Endocytic tubules regulated by Rab GTPases 5 and 11 are used for envelopment of
745 herpes simplex virus. EMBO J 31:4204-20.

- 746 32. Gaglia MM, Covarrubias S, Wong W, Glaunsinger BA. 2012. A common strategy for
747 host RNA degradation by divergent viruses. *J Virol* 86:9527-30.
- 748 33. Covarrubias S, Gaglia MM, Kumar GR, Wong W, Jackson AO, Glaunsinger BA.
749 2011. Coordinated destruction of cellular messages in translation complexes by the
750 gammaherpesvirus host shutoff factor and the mammalian exonuclease Xrn1. *PLoS*
751 *Pathog* 7:e1002339.
- 752 34. Burgess HM, Gray NK. 2012. An integrated model for the nucleo-cytoplasmic
753 transport of cytoplasmic poly(A)-binding proteins. *Commun Integr Biol* 5:243-7.
- 754 35. Kumar GR, Glaunsinger BA. 2010. Nuclear import of cytoplasmic poly(A) binding
755 protein restricts gene expression via hyperadenylation and nuclear retention of
756 mRNA. *Mol Cell Biol* 30:4996-5008.
- 757 36. Gray NK, Hrabalkova L, Scanlon JP, Smith RW. 2015. Poly(A)-binding proteins and
758 mRNA localization: who rules the roost? *Biochem Soc Trans* 43:1277-84.
- 759 37. Soliman TM, Sandri-Goldin RM, Silverstein SJ. 1997. Shuttling of the herpes
760 simplex virus type 1 regulatory protein ICP27 between the nucleus and cytoplasm
761 mediates the expression of late proteins. *J Virol* 71:9188-97.
- 762 38. Sacks WR, Greene CC, Aschman DP, Schaffer PA. 1985. Herpes simplex virus
763 type 1 ICP27 is an essential regulatory protein. *J Virol* 55:796-805.
- 764 39. Chen IH, Sciabica KS, Sandri-Goldin RM. 2002. ICP27 interacts with the RNA
765 export factor Aly/REF to direct herpes simplex virus type 1 intronless mRNAs to the
766 TAP export pathway. *J Virol* 76:12877-89.
- 767 40. Koffa MD, Clements JB, Izaurralde E, Wadd S, Wilson SA, Mattaj JW, Kuersten S.
768 2001. Herpes simplex virus ICP27 protein provides viral mRNAs with access to the
769 cellular mRNA export pathway. *Embo J* 20:5769-78.

- 770 41. Fontaine-Rodriguez EC, Knipe DM. 2008. Herpes simplex virus ICP27 increases
771 translation of a subset of viral late mRNAs. J Virol 82:3538-45.
- 772 42. Smith RWP, Anderson RC, Larralde O, Smith JWS, Gorgoni B, Richardson WA,
773 Malik P, Graham SV, Gray NK. 2017. Viral and cellular mRNA-specific activators
774 harness PABP and eIF4G to promote translation initiation downstream of cap
775 binding. Proc Natl Acad Sci U S A 114:6310-6315.
- 776 43. Kedersha N, Stoecklin G, Ayodele M, Yacono P, Lykke-Andersen J, Fritzler MJ,
777 Scheuner D, Kaufman RJ, Golan DE, Anderson P. 2005. Stress granules and
778 processing bodies are dynamically linked sites of mRNP remodeling. J Cell Biol
779 169:871-84.
- 780 44. Zhu H, Zheng C. 2020. The Race between Host Antiviral Innate Immunity and the
781 Immune Evasion Strategies of Herpes Simplex Virus 1. Microbiol Mol Biol Rev 84.
- 782 45. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of
783 image analysis. Nat Methods 9:671-5.
- 784