Dissecting Herpes Simplex Virus 1-Induced Host Shutoff at the RNA Level

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Abstract

Herpes simplex virus 1 (HSV-1) induces a profound host shut-off during lytic infection. The virion host shut-off (*vhs*) protein plays a key role in this process by efficiently cleaving host and viral mRNAs. Furthermore, the onset of viral DNA replication is accompanied by a rapid decline in host transcriptional activity. To dissect relative contributions of both mechanisms and elucidate gene-specific host transcriptional responses throughout the first 8h of lytic HSV-1 infection, we employed RNA-seq of total, newly transcribed (4sU-labelled) and chromatin-associated RNA in wild-type (WT) and Δ*vhs* infection of primary human fibroblasts. Following virus entry, v*hs* activity rapidly plateaued at an elimination rate of around 30% of cellular mRNAs per hour until 8h p.i. In parallel, host transcriptional activity dropped to 10-20%. While the combined effects of both phenomena dominated infection-induced changes in total RNA, extensive gene-specific transcriptional regulation was observable in chromatin-associated RNA and was surprisingly concordant between WT and Δ*vhs* infection. Both induced strong transcriptional up-regulation of a small subset of genes that were poorly expressed prior to infection but already primed by H3K4me3 histone marks at their promoters. Most interestingly, analysis of chromatin-associated RNA revealed *vhs*-nuclease-activity-dependent transcriptional down-regulation of at least 150 cellular genes, in particular of many integrin adhesome and extracellular matrix components. This was accompanied by a *vhs*-dependent reduction in protein levels by 8h p.i. for many of these genes. In summary, our study provides a comprehensive picture of the molecular mechanisms that govern cellular RNA metabolism during the first 8h of lytic HSV-1 infection.

Importance

The HSV-1 virion host shut-off (*vhs*) protein efficiently cleaves both host and viral mRNAs in a translation-dependent manner. In this study, we model and quantify changes in *vhs* activity as well as virus-induced global loss of host transcriptional activity during productive HSV-1 infection. In general, HSV-1-induced alterations in total RNA levels were dominated by these two global effects. In contrast, chromatin-associated RNA depicted gene-specific transcriptional changes. This revealed highly concordant transcriptional changes in WT and *Δvhs* infection, confirmed DUX4 as a key transcriptional regulator in HSV-1 infection and depicted *vhs*-dependent, transcriptional down-regulation of the integrin adhesome and extracellular matrix components. The latter explained seemingly gene-specific effects previously attributed to *vhs*-mediated mRNA degradation and resulted in a concordant loss in protein levels by 8h p.i. for many of the respective genes.

Introduction

Herpes simplex virus 1 (HSV-1), one of eight herpesviruses infecting humans, is widely known for causing cold sores but also associated with life-threatening diseases such as encephalitis [1, 2]. A key characteristic of HSV-1 lytic infection is the induction of a profound host shut-off that is predominantly induced at the RNA level. The virion host shut-off (*vhs*) endonuclease plays a crucial role in this process. *Vhs* is delivered by the tegument of the incoming virus particles and, together with *de novo* expressed *vhs* protein, rapidly starts cleaving both cellular and viral mRNAs in a translation-initiation-dependent manner [3-8]. Later on in infection, *vhs* nuclease activity is dampened by the concerted action of at least two viral proteins, i.e. UL48 (VP16) and UL49 (VP22) [9-11], with the viral UL47 protein (VP13/14) potentially also being involved [12]. In addition to *vhs*-mediated mRNA degradation, HSV-1 shuts down host gene expression by efficiently recruiting RNA polymerase II (Pol II) and elongation factors from the host chromatin to the replicating viral genomes [13-15]. This results in an extensive loss of Pol II occupancy from host chromatin starting with the advent of viral DNA replication by 2-3h post infection (h p.i.) [16]. Furthermore, HSV-1 induces proteasome-dependent degradation of Pol II later on (>12h p.i.) in infection [17]. Finally, extensive RNA degradation upon cleavage by the *vhs* nuclease also appears to contribute to the transcriptional shut-off by 24h of infection [18].

Both *vhs*-mediated mRNA degradation and global inhibition of transcription substantially alter the host transcriptome during productive infection. Virus-induced alterations in total RNA levels can be a consequence of either of these two global phenomena or due to gene-specific changes in RNA stability or transcription. Their relative contributions, however, could so far not be distinguished. Recently, Pheasant *et al*. presented a genome-scale RNA-seq study analyzing nuclear-cytoplasmic

compartmentalization of viral and cellular transcripts during lytic HSV-1 infection [19]. They proposed that the translational shut-off induced by HSV-1 is primarily a result of *vhs*-induced nuclear retention and not degradation of infected cell mRNA. Furthermore, they suggested differential susceptibility of transcripts to *vhs* RNA cleavage activity. We previously performed 4-thiouridine (4sU) labeling followed by sequencing (4sU-seq) to characterize *de novo* transcription and RNA processing in hourly intervals during the first 8h of lytic HSV-1 infection of primary human foreskin fibroblasts (HFF) (Fig 1A) [20, 21]. This revealed extensive poly(A) read-through transcription into downstream intergenic regions resulting from disruption of transcription termination (DoTT) for the majority of but not all cellular genes. Due to nuclear retention of the respective aberrant transcripts, DoTT also notably contributes to host shut-off [21]. Furthermore, read-in transcription from upstream genes commonly results in the seeming induction of genes. DoTT and read-in transcription thus confounds the analysis of changes in cellular RNA levels and host transcriptional activity during HSV-1 infection.

To dissect the effects of *vhs*-mediated RNA degradation and global loss in transcriptional activity during lytic HSV-1 infection on a genome-wide scale, we now performed total RNA-seq and 4sU-seq time-course analysis on HFF infected with a *vhs*-null mutant virus in which *vhs* has been inactivated by replacement of amino acids 251-489 with LacZ (Δ*vhs*) [22]. Here, we used the same experimental setting and standardized conditions as previously employed for wild-type (WT) HSV-1 infection (Fig 1A) [20]. Furthermore, we analyzed subcellular RNA fractions (cytoplasmic, nucleoplasmic and chromatin-associated RNA) at 0 and 8h p.i. of WT and Δ*vhs* infection (Fig 1B). Mathematical modelling of RNA synthesis and *vhs*-mediated RNA decay revealed that *vhs* activity rapidly plateaued upon WT HSV-1

118 infection with *vhs* continuously degrading about 30% of cellular mRNAs per hour until
119 at least 8h p.i. In contrast, total RNA changes in Δ*vhs* infection were dominated by
120 the global loss in Pol II activity. Changes in total mRNA levels upon HSV-1 infection
121 are thus shaped by differences in basal transcription and RNA turnover rates
122 between the individual genes. In contrast, chromatin-associated RNA provided an
123 unbiased picture of gene-specific transcriptional changes. This revealed an
124 extensive, previously unsuspected *vhs*-dependent transcriptional down-regulation of
125 the integrin adhesome and extracellular matrix (ECM). Notably, this included the key
126 *vhs*-sensitive genes reported by Pheasant *et al*. Accordingly, increased reduction of
127 total mRNA levels for these genes is not due to increased susceptibility to *vhs*-
128 mediated RNA decay of the respective transcripts, but rather due to additional, *vhs*-
129 cleavage-activity-dependent effects on their transcription. V*hs*-dependent down-
130 regulation of transcriptional activity resulted in reduced protein levels of many of the
131 respective genes already at 8h p.i. in WT but not in Δ*vhs* infection as confirmed by
132 quantitative whole-proteome mass spectrometry.

133 **Results**

134 **Genome-wide RNA-seq analysis in WT and Δ*vhs* infection**

135 To dissect the role of *vhs*, global inhibition of Pol II activity and host gene-specific
136 regulation during productive HSV-1 infection, we employed the same experimental
137 set-up for Δ*vhs* infection as for our previous transcriptome analyses on WT HSV-1
138 infection [20]. We infected HFF with Δ*vhs* at a high MOI of 10 and performed 4sU-seq
139 in hourly intervals and total RNA-seq every two hours during the first 8h of infection
140 (2 biological replicates; Fig 1A). Consistent with our previous findings [20, 23] and
141 with the modest attenuation of Δ*vhs* in HFF, HSV-1-induced DoTT affected the same
142 genes in Δ*vhs* infection but was less prominent compared to WT infection (Fig 1C, S1

143 Dataset). Since read-in transcription into downstream genes due to HSV-1-induced
144 DoTT from upstream genes can be mistaken for “induction” of these downstream
145 genes [20], we excluded genes with read-in transcription from all following analyses
146 (see methods for details). This resulted in a set of 4,162 genes without read-in
147 transcription for which RNA fold-changes comparing infection vs. mock and their
148 significance were determined using DESeq2 [24]. Unless otherwise noted, all fold-
149 changes shown in the following are always in comparison to mock infection from the
150 corresponding experiments.

151 DESeq2 normalization assumes that there are no global changes in RNA levels
152 between conditions. This is not the case in HSV-1 infection due to *vhs*-mediated RNA
153 degradation and the global loss of transcriptional activity. A possible approach to
154 normalize to decreasing RNA levels during infection uses RNA spike-ins [19, 25, 26].
155 However, such a normalization effectively only decreases fold-changes by a constant
156 factor (or a constant additive term for log2 fold-changes) for all genes. It does not
157 affect the correlation between fold-changes (evaluated as Spearman rank correlation
158 r_s in the following), which is scale-invariant. As the analyses here were all performed
159 without such normalization, the fold-changes discussed here always represent
160 relative changes compared to other genes regarding their relative contribution to
161 overall RNA levels. Accordingly, this means that genes identified here as non-
162 regulated only exhibit the global reductions in RNA levels or transcription that equally
163 affect all genes. This enables the identification of gene-specific changes in RNA
164 abundance and transcription levels that differ from the majority of genes.

165 **Delineating *vhs*-mediated RNA degradation and loss of transcriptional activity**

166 Gene expression fold-changes in 4sU-RNA were highly correlated between Δ*vhs* and
167 WT infection when comparing the same time points, confirming the high degree of

standardization between the two independent experiments (Fig 1D-F). The only exceptions were the first two 4sU-seq time points (0-1 and 1-2h p.i., Spearman rank correlation $r_s \leq 0.3$), when essentially no (n≤2) cellular genes were differentially expressed in both WT and Δ*vhs* infection (multiple testing adjusted p≤0.001, |log2 fold-change| ≥1). This was expected as fold-changes were only very small (median |log2 fold-change| ≤0.1) and dominated by experimental noise. The highest correlations between 4sU-seq fold-changes in WT and Δ*vhs* infection compared to mock were observed at 4-5h and 5-6h p.i. ($r_s \approx 0.8$, Fig 1D,E). Correlations decreased towards the end of the time-course in particular for genes down-regulated in WT (Fig 1F), consistent with the well described effects of *vhs* on cellular RNA levels late in infection [27]. Notably, the later stages of Δ*vhs* infection (from 6-7h p.i.) were better correlated to slightly earlier stages (4-5h, 5-6h p.i.) of WT infection, indicating slightly slower progression of Δ*vhs* infection.

In contrast to 4sU-RNA, fold-changes in total RNA obtained from WT and Δ*vhs* infection were only poorly correlated ($r_s \leq 0.11$, Fig 1G-J). Consistent with the cleavage activity of *vhs*, this was particularly prominent for genes down-regulated in WT infection. As 4sU-RNA was purified from total RNA, the poor correlation for total RNA fold-changes cannot be explained by poor reproducibility between the two independent experiments. We conclude that this instead reflects the expected strong impact of *vhs* cleavage activity on the cellular mRNAs. In principle, *vhs* cleavage activity should more strongly affect total mRNA levels of long-lived mRNAs than of short-lived mRNAs, as the former have much weaker *de novo* transcription relative to total RNA levels and are thus much more slowly replaced. On the contrary, HSV-1-induced global loss in transcriptional activity should more strongly affect total RNA levels of unstable, short-lived mRNAs. To test this hypothesis, we correlated the

observed changes in total RNA upon WT and Δ*vhs* infection with RNA half-lives of the respective transcripts in uninfected cells. RNA half-lives for all analyzed genes were measured in uninfected HFF based on newly transcribed to total RNA ratios as previously described (see also methods) [28]. Please note that in the following, we always refer to basal mRNA half-lives in uninfected cells, not during infection. This correlation analysis revealed the expected striking differences between WT and Δ*vhs* infection. In WT infection, total RNA fold-changes and mRNA half-lives were negatively correlated ($r_s = -0.38$ at 8h p.i., Fig 2A), i.e. total RNA levels of stable cellular mRNAs tended to decrease more strongly than of unstable mRNAs. This was already observable at 2h p.i. ($r_s = -0.31$) consistent with mRNA cleavage and degradation by tegument-delivered *vhs* protein. The negative correlation to RNA half-lives was also confirmed for total RNA fold-changes from the study of Pheasant *et al*. at 4h p.i. ($r_s = -0.36$), while at 12h p.i., a weaker, but still highly significant, negative correlation was observed (r_s = -0.15).

In Δ*vhs* infection, however, total RNA fold-changes and RNA half-lives were positively correlated from 4h p.i. onwards ($r_s = 0.55$ at 8h p.i., Fig 2B). Thus, total RNA levels of short-lived cellular RNAs were more strongly reduced than of long-lived ones. This effect is consistent with the well described gradual decline in global transcriptional activity starting around 3-4h p.i. [15, 20]. Accordingly, total RNA fold-changes in Δ*vhs* infection largely reflect the global loss in transcriptional activity during lytic HSV-1 infection rather than gene-specific regulation. The presence of negative correlations in WT infection, however, suggests that *vhs*-mediated RNA decay, not the global reduction in transcriptional activity on cellular genes, dominates total RNA fold-changes in lytic WT HSV-1 infection. Although a correlation of -0.38 may not appear high, it is surprisingly strong considering that the loss of

218 transcriptional activity that is also present in WT infection would lead to a positive, i.e.
219 opposite, correlation without *vhs* activity. Interestingly, negative correlations in WT
220 infection and positive correlations in Δ*vhs* infection to RNA half-lives were also
221 observed for total RNA fold-changes between 2 and 4h p.i., 4 and 6h p.i. and 6 and
222 8h p.i. (Fig 1C,D). *Vhs* cleavage activity thus continues to dominate changes in total
223 RNA levels at least until 8h p.i. Nevertheless, the much weaker negative correlation
224 at 12h p.i. observable in the data of Pheasant *et al*. are consistent with a near
225 complete loss of *vhs*-mediated cleavage activity at later times of infection by the
226 combined action of the viral VP16 and VP22 proteins [9-11].

227 To estimate the kinetics of *vhs* activity, we developed an ordinary differential equation
228 (ODE) model of HSV-1 infection that models (i) global changes in host transcriptional
229 activity, (ii) global changes in *vhs* endonuclease activity, (iii) subcellular
230 compartmentalization and nuclear export of transcripts, (iv) differences in basal
231 mRNA half-lives between genes, and (v) gene-specific transcriptional regulation (S2
232 Text). Using this model, we estimated the extent of loss in transcriptional activity from
233 our total RNA-seq time-course data in Δ*vhs* infection and the increase of *vhs*
234 endonuclease activity during WT HSV-1 infection from our total RNA time-course in
235 WT infection (S2 Text). Our results indicate that by 8h p.i. in Δ*vhs* infection
236 transcriptional activity dropped down to 10-20% of the level in uninfected cells (Fig
237 2E). Assuming an at least similar drop in transcriptional activity in WT infection, our
238 model suggests that at the height of *vhs* activity, ~30% of cellular RNA molecules are
239 lost per hour due to *vhs*-mediated RNA degradation (Fig 2F). This rate reached 26%
240 as early as 2h p.i. and remained fairly constant until 8h p.i. It is important to note that
241 our data exclude a significant drop in *vhs* activity until 8h p.i. as the drop in
242 transcriptional activity would otherwise have resulted in positive correlations between

243 total RNA fold-changes and mRNA half-lives in WT infection (S2 Text). Furthermore,
244 if the loss of transcriptional activity in WT infection were indeed dramatically higher
245 than in Δ*vhs* infection, *vhs*-mediated degradation would have to increase even faster
246 and to higher levels to achieve the observed negative correlations. In summary, our
247 model explains the wide range of total RNA fold-changes observed between genes in
248 HSV-1 infection simply by differences in basal RNA half-lives between genes in
249 uninfected cells and gene-specific transcriptional regulation. Although statistically
250 significant correlations were also observed between 4sU-RNA fold-changes and RNA
251 half-lives, these were relatively small in both WT ($r_s \geq -0.15$) and Δ*vhs* infection
252 ($r_s \leq 0.25$). Thus, changes in newly transcribed RNA obtained during 60min of 4sU-
253 labeling are also influenced by *vhs*-mediated decay and loss of transcriptional
254 activity, but substantially less strongly than for total RNA. We conclude that the poor
255 correlation in total RNA fold-changes between WT and Δ*vhs* infection is a direct
256 consequence of global effects of *vhs* on RNA stability throughout the first 8h of lytic
257 infection.

258 **Chromatin-associated RNA allows unbiased quantification of transcriptional**
259 **regulation during HSV-1 infection**

260 Since our analysis revealed some effect of *vhs*-mediated decay and loss of
261 transcriptional activity on 4sU-RNA, we analyzed subcellular RNA fractions
262 (cytoplasmic, nucleoplasmic and chromatin-associated RNA) from mock-, WT-, and
263 Δ*vhs*-infected cells at 8h p.i. (n=2; Fig 1B) to obtain an unbiased picture of
264 transcriptional activity in WT and Δ*vhs* infection. Here, subcellular fractions for mock-,
265 WT- and Δ*vhs*-infected cells were obtained and sequenced in the same experiment.
266 Only the data from mock and WT-infected cells have previously been published [21].
267 Known nuclear lincRNAs (MEG3, MALAT1, NEAT1) were enriched in nucleoplasmic

and chromatin-associated RNA and cytoplasmic lincRNAs (NORAD, VTRNA2-1) were enriched in cytoplasmic RNA (Fig 3A), confirming the efficient separation of the cytoplasmic and nuclear RNA fractions. Efficient separation of chromatin-associated RNA from nucleoplasmic RNA was confirmed by the strong overrepresentation of intronic reads in chromatin-associated RNA (Fig 3B). Please note that the increase in intronic reads in the nucleoplasmic RNA fraction in WT infection is due to extensive poly(A) read-through, which results in read-in transcription into downstream genes coupled with incomplete splicing and nuclear retention of read-through transcripts [20, 21]. This was also observed in Δ*vhs* infection, however less pronounced, consistent with the reduced levels of read-through transcription. Of note, the subcellular RNA fraction experiment also comprised total cellular RNA samples from WT and Δ*vhs* infection. The total RNA fold-changes here nicely matched the 8h time point in the respective WT ($r_s = 0.73$) and Δ*vhs* ($r_s = 0.82$) time-courses. This also confirmed the poor correlation of total RNA fold-changes between WT and Δ*vhs* infection ($r_s = 0.24$). Thus, it does not result from experimental bias between two independently performed time-course experiments. Furthermore, negative ($r_s = -0.36$) and positive ($r_s = 0.34$) correlations to RNA half-lives were again observed for WT and Δ*vhs* infection, respectively.

Since chromatin-associated RNA remains attached to the chromatin by the actively transcribing polymerases, it should not be accessible to *vhs*-mediated RNA cleavage and degradation. The absence of any significant correlation between fold-changes in chromatin-associated RNA and RNA half-life for both WT and Δ*vhs* infection ($r_s =$ -0.08 for WT and 0.07 for Δ*vhs* infection) confirms this assumption and provides further evidence for the efficient separation of the chromatin-associated RNA fraction. We thus focused on changes in chromatin-associated RNA to assess the effects of

293 HSV-1 infection and *vhs* on transcriptional regulation. Strikingly, comparison of
294 chromatin-associated RNA fold-changes revealed that changes in relative, gene-
295 specific transcriptional activity at 8h p.i. were extremely similar between WT and
296 Δ*vhs* infection ($r_s = 0.89$, Fig 3C). Thus, although the global loss in transcriptional
297 activity is higher in WT than Δ*vhs* infection due to a slower progression of Δ*vhs*
298 infection, gene-specific regulation of transcriptional activity for individual genes
299 remains mostly the same. The only exception was a set of 150 genes which were
300 transcriptionally down-regulated (beyond the general loss of transcriptional activity)
301 only in WT but not Δ*vhs* infection (magenta in Fig 3C). These are further analyzed
302 below.

303 Notably, 4sU-RNA fold-changes were better correlated to fold-changes in chromatin-
304 associated RNA ($r_s \approx 0.76$) than to nucleoplasmic ($r_s \approx 0.68$) or cytoplasmic RNA
305 ($r_s \approx 0.53$), while total RNA fold-changes were best correlated to cytoplasmic RNA
306 changes ($r_s \approx 0.74$). This indicates that even with a relatively long 4sU-labeling
307 duration of 60 min, 4sU-RNA to a large degree represents ongoing nascent
308 transcription on the chromatin level. We conclude that fold-changes in chromatin-
309 associated RNA provide an unbiased picture of transcriptional regulation in both WT
310 and Δ*vhs* infection.

311 **_Vhs_-dependent transcriptional down-regulation of the extracellular matrix and**
312 **integrin adhesome**

313 Differential gene expression analysis on chromatin-associated RNA identified 225
314 genes (5.4% of all genes) that were significantly down-regulated at the transcriptional
315 level (log2 fold-change ≤ -1, adj. $p \leq 0.001$) in both WT and in Δ*vhs* infection
316 compared to mock (blue in Fig 3C). This means that these down-regulated genes -
317 like genes only down-regulated in WT infection (magenta in Fig 3C) - show even

318 further reductions in transcription rates than non-regulated genes (gray and black in
319 Fig 3C). Notably, for the latter genes overall transcription rates also decrease but
320 only due to the general loss of host transcriptional activity in infection and they thus
321 show no apparent gene-specific regulation. The concordantly down-regulated genes
322 (blue in Fig 3C) were characterized by lower poly(A) read-through than non- or up-
323 regulated genes. Thus, their increased down-regulation cannot be explained by
324 negative effects of poly(A) read-through transcription on gene expression. Gene
325 Ontology (GO) [29] enrichment analysis for these genes did not yield any statistically
326 significant results. However, when comparing these genes to the INTERFEROME
327 database [30], we observed significant enrichment (adj. p ≤0.001) for genes down-
328 regulated by type II interferon.

329 Interestingly, a set of 150 genes (3.6% of all genes) was significantly down-regulated
330 (log2 fold-change ≤ -1, adj. $p \leq 0.001$) in WT but not Δ*vhs* infection (marked
331 magenta in Fig 3C, S3 Dataset). *Vhs*-dependent down-regulation of these genes was
332 confirmed in nucleoplasmic RNA, 4sU-RNA from 6-7h p.i. onwards and in parts also
333 in total RNA from 6h p.i. onwards (Fig 3D). To validate *vhs*-dependent transcriptional
334 down-regulation by qRT-PCR, we harvested chromatin-associated RNA at 8h p.i WT
335 and Δ*vhs* infection (n=2 additional replicates, Fig 3E). Relative RNA levels in Δ*vhs*
336 vs. WT infection were measured for two genes (control) that showed no gene-specific
337 regulation (ARF4, CNBP, from the gray genes in Fig 3C) and two genes with *vhs*-
338 dependent transcriptional down-regulation (COL6A2, MMP1, from the magenta
339 genes in Fig 3C). In addition, we included MMP3 since it was one of the genes
340 whose strong reduction in total RNA levels in HSV-1 infection was shown to be *vhs*-
341 dependent by Pheasant *et al*. [19]. While MMP3 was not included in our primary
342 analysis due to its proximity to nearby genes, differential gene expression analysis for

343 *all* human genes on chromatin-associated RNA showed that MMP3 was also
344 transcriptionally down-regulated in a *vhs*-dependent manner (see Dataset S4 for the
345 extended set of 578 *vhs*-dependent genes). Like Pheasant *et al*., we used 18S rRNA
346 as internal reference for the qRT-PCR since it is not translated and thus not targeted
347 by *vhs*. As expected, ARF4 and CNBP both showed higher (~3-fold) chromatin-
348 associated RNA levels in Δ*vhs* than in WT infection. This is consistent with slower
349 progression of Δ*vhs* infection compared to WT and thus reduced global reduction in
350 host transcriptional activity. Nevertheless, loss of COL6A2, MMP1 and MMP3
351 transcription in WT compared to Δ*vhs* infection was considerably greater (8- to 15-
352 fold), thereby confirming their *vhs*-dependent transcriptional down-regulation.

353 Functional enrichment analysis of *vhs*-dependently down-regulated genes again
354 showed an enrichment for genes downregulated upon type II interferon exposure.
355 Strikingly, however, we also observed a strong functional enrichment for several GO
356 terms (adj. $p \leq 0.001$, S5 Dataset), in particular “extracellular matrix (ECM)
357 organization” (>32-fold enriched, adj. $p < 10^{-25}$). This included fibronectin (FN1),
358 integrin beta 1 (ITGB1), a subunit of integrin complexes binding fibronectin, and
359 several genes encoding for collagen alpha chains. Enrichment was also observed for
360 “focal adhesion”, i.e. the integrin-containing, multi-protein complexes that anchor the
361 cell to the ECM and connect it to the actin cytoskeleton [31, 32]. Of note, the
362 additional *vhs*-dependent genes, which we identified in the extended genome-wide
363 analysis, were also significantly enriched for focal adhesion and ECM organization
364 (>11-fold enriched, adj. $p < 10^{-23}$).

365 Composition of integrin adhesion complexes after induction by their canonical ligand
366 FN1 has been determined by several quantitative proteomics studies in mouse and
367 human cells, including HFF [33-38]. Horton *et al*. consolidated these data into a

368 meta-adhesome of 2,412 proteins found in at least one of six high-quality studies
369 [33]. Adhesome components identified in the individual proteomics studies as well as
370 the meta-adhesome were significantly enriched among genes down-regulated in a
371 *vhs*-dependent manner (Fig 4A, adj. p ≤0.001). The highest enrichment was found for
372 the integrin adhesome components identified in HFF (>10-fold enrichment, adj.
373 $p = 5.3 \times 10^{-20}$). Furthermore, genes of the HFF adhesome (143 genes included in
374 our analysis) showed a systematic shift in regulation between WT and Δ*vhs* infection
375 in total RNA, 4sU-RNA and all RNA fractions (Fig 4B). HFF adhesome components
376 tended not to be (or at least less) transcriptionally down-regulated in Δ*vhs* infection
377 compared to WT infection, while the remaining genes showed no systematic shift.
378 This shift was already visible from 4-5h onwards in 4sU- and total RNA and when
379 comparing later time points of Δ*vhs* infection to earlier time points of WT infection.
380 Thus, *vhs*-dependent transcriptional down-regulation is not an artefact of comparing
381 different progression stages in the WT and *vhs* mutant life cycles in 8h p.i. chromatin-
382 associated RNA. When inspecting the protein-protein association network for the
383 HFF adhesome (from the STRING database [39]), the strongest differences between
384 Δ*vhs* infection and WT infection were observed in the subnetwork around FN1 and
385 integrin subunits (Fig 4C).

386 To investigate whether *vhs*-dependent down-regulation required *vhs* endonuclease
387 activity, we performed RNA-seq of chromatin-associated RNA at 8h p.i. using a *vhs*
388 single-amino acid mutant (D195N) that no longer exhibited the mRNA decay activity
389 but still binds to the translation initiation factors eIF4H and eIF4B [40]. For
390 comparison, we also included the parental BAC-derived WT virus (WT-BAC) as well
391 as mock, WT and Δ*vhs* infection at 8h p.i. (see methods). This confirmed *vhs*-
392 dependent transcriptional regulation in an independent experiment (Fig 4D) and

demonstrated that it requires *vhs* nuclease activity (Fig 4E) as fold-changes in D195N infection were extremely well correlated to Δ*vhs* infection (Fig 4F). Of note, investigation of RNA-seq read alignments for genomic differences showed that the D195N point mutation was the only genome difference of the D195N mutant virus compared to WT-BAC. This confirms that the D195N mutant expresses the nuclease-null variant of *vhs,* rather than inadvertently no *vhs*. This analysis also confirmed the presence of the inactivating LacZ insertion in the Δ*vhs* mutant [41]. We conclude that components of the integrin adhesome and ECM are transcriptionally down-regulated during lytic HSV-1 infection by a *vhs*-nuclease-activity-dependent mechanism.

Gamma-herpesviruses also encode an mRNA-targeting ribonuclease, SOX, which is not homologous to *vhs*. Abernathy *et al*. recently showed that extensive mRNA cleavage by the murine gamma-herpesvirus 68 (MHV68) endoribonuclease muSOX and subsequent Xrn1-mediated mRNA degradation leads to transcriptional repression for numerous genes [18]. The same phenomenon was observed for several genes by qRT-PCR if the HSV-1 *vhs* protein was exogenously expressed for 24h. Abernathy *et al*. employed 4sU-seq of WT MHV68 infection and infection with a muSOX-inactivating MHV68 mutant (ΔHS) and identified 342 muSOX-dependent genes. Although they found no clear links to specific biological processes in their functional enrichment analysis, the KEGG pathway “focal adhesion” was significantly enriched among muSOX-dependent genes (19 of 342 genes). To investigate whether *vhs*-dependent transcriptional down-regulation of the integrin adhesome and ECM components might be mediated by general RNA degradation or represent a *vhs*-specific response, we analyzed the overlap of muSOX-dependent genes to our list of *vhs*-dependent genes (Fig 4G). Only 14 of the 150 (9.3%) *vhs*-dependent genes were orthologues to muSOX-dependent genes. While this overlap was statistically

significant (Fisher's exact test, $p = 4.27 \times 10^{-5}$), it is nevertheless small. Although this provides some evidence that *vhs*-dependent transcriptional down-regulation for most genes is distinct from general mRNA-decay-dependent transcriptional repression, more work is required to rule out effects of different cell, species or virus backgrounds.

A different explanation for the concerted down-regulation of a set of functionally related genes could be *vhs*-mediated RNA degradation of a key cellular transcriptional regulator. We thus performed a motif search in promoters of *vhs*-dependently down-regulated genes but surprisingly found no significantly enriched known or novel transcription factor binding motifs in the proximal promoter regions (-2,000 to +2,000 bp relative to the transcription start site). To recover more distal regulation, we also performed a motif search in open chromatin peaks from ATAC-seq data in uninfected cells [21] within 10, 25, or 50kb of *vhs*-dependently down-regulated genes. While this recovered several motif hits for the AP-1 transcription factor, no significant enrichment compared to all identified open chromatin peaks was observed. Interestingly, however, the first *vhs*-dependent gene significantly down-regulated in 4sU-RNA of WT infection at 2-3h p.i. was the ETS transcription factor ELK3, one of three ternary complex factors (TCFs) that act as cofactors of serum response factor (SRF) [42]. SRF has been shown to be vital for focal adhesion assembly in embryonic stem cells [43]. TCF-dependent genes identified from simultaneous knockouts of all three TCFs as well as SRF targets from ChIP-seq have previously been determined in mouse embryonic fibroblasts (MEFs) [44]. Though we found no significant enrichment for TCF-dependent genes or TCF-dependent SRF targets, SRF targets in general were significantly enriched (~2.25-fold) among *vhs*-dependent genes ($p = 3.9 \times 10^{-5}$). Nevertheless, only 42 (28%) of *vhs*-dependent

443 genes were SRF targets and 93% of SRF targets were not *vhs*-dependent in our
444 study, thus other regulatory mechanisms have to be involved. Further work is
445 required to clarify this issue.

446 Pheasant *et al*. observed large differences regarding the extent of *vhs*-induced loss
447 in total RNA levels between different cellular genes at 12h WT infection [19]. Using
448 qRT-PCR, they showed that this reduction was *vhs*-dependent based on two sets of
449 genes that exhibited either high (COL6A2, MMP3, MMP1) or low reduction (GAPDH,
450 ACTB, RPLP0) in total RNA levels in WT infection. As Actinomycin D treatment
451 showed a similar stability of corresponding mRNAs in uninfected cells, they
452 concluded that these differences were due to differences in the susceptibility of the
453 respective transcripts to *vhs* cleavage activity. By RNA-seq and PCR on chromatin-
454 associated rather than total cellular RNA, we demonstrated that all three of their
455 PCR-confirmed highly *vhs*-sensitive genes are actually transcriptionally down-
456 regulated in a *vhs*-dependent manner. Moreover, genes defined as efficiently
457 depleted during WT infection by Pheasant *et al*. (log2 fold-change in total RNA at 12h
458 p.i. WT infection < -5) were significantly enriched for ECM organization (>3-fold, adj.
459 $p = 7.4 \times 10^{-7}$). We thus hypothesized that a significant fraction of highly *vhs*-
460 sensitive genes identified by Pheasant *et al*. might actually be *vhs*-dependently
461 transcriptionally down-regulated. Indeed, both original and additional *vhs*-
462 dependently transcriptionally regulated genes identified in our genome-wide analysis
463 were strongly enriched among efficiently depleted genes determined by Pheasant *et*
464 *al*. (4.2 - 6.8-fold enrichment, $p < 10^{-27}$) and were among the most significantly
465 down-regulated genes in total RNA at 12h p.i. in WT infection (Fig 4H). We conclude
466 that *vhs*-dependent transcriptional down-regulation notably contributes to reduced
467 total mRNA levels of the respective genes later on in WT HSV-1 infection and

468 thereby explains the previously observed strong *vhs*-dependent reduction of their
469 mRNA levels.

470

471 **A common core of up-regulated genes in WT and Δ*vhs* infection**

472 Analysis of chromatin-associated RNA identified a set of 462 genes that were
473 significantly up-regulated in both WT and Δ*vhs* infection (log2 fold-change ≥ 1, adj.
474 $p \leq 0.001$, marked red in Fig 3C). Only 3 genes were up-regulated in WT but not or
475 2-fold less in Δ*vhs* infection. Thus, transcriptional up-regulation during HSV-1
476 infection is independent of *vhs*. Clustering analysis of *vhs*-independent up-regulated
477 genes identified four subgroups that were distinguished mostly by how strongly and
478 early in infection they were up-regulated (Fig 5A, S6 Dataset). In particular, a set of
479 24 genes (marked orange in Fig 5A) was up-regulated both very early and strongly in
480 WT and Δ*vhs* infection, with up-regulation of 21 of these genes (91.7%) detectable in
481 total RNA at 6h p.i. or earlier in both WT and Δ*vhs* infection. Not surprisingly, several
482 of these genes (e.g. RASD1, NEFM, NPTX2) have previously been identified as
483 highly up-regulated in HSV-1 infection by microarray analysis on total RNA [45, 46]
484 and 10 were significantly up-regulated in total RNA at 12h p.i. WT infection in the
485 Pheasant *et al*. data [19]. Up-regulation of all genes in the orange and blue clusters
486 was also confirmed in 4sU-RNA. No enrichment for GO terms was observed either
487 for individual clusters or all up-regulated genes, however the green and orange
488 cluster were enriched for interferon type I-up-regulated genes (adj. $p = 1.68 \times 10^{-5}$
489 and adj. $p = 0.0019$ for the green and orange cluster, respectively). Notably, 50% of
490 genes in the orange cluster were up-regulated by type I interferons (>4.5-fold
491 enrichment).

One characteristic feature of up-regulated genes in general and the orange cluster in particular was their low level of gene expression in uninfected cells (Fig 5B). Notably, 71% of genes in the orange cluster were not or only very lowly expressed (total RNA FPKM ≤1) in uninfected cells compared to 8% of all genes (Fisher's exact test $p < 10^{-13}$). In total, 76 (17, 22, 32, 5 from the orange, blue, green, and red cluster, respectively) up-regulated genes (16.5%) were poorly expressed in uninfected cells. HSV-1-induced up-regulation of genes not normally expressed has previously been reported for human alpha globin genes (HBA1, HBA2), which are normally only expressed in erythroid cells [47]. RNA-seq analysis of these two duplicated genes is complicated by their high sequence similarity (>99% on coding sequence, 5' UTRs and upstream of promoter [48]), as most reads can be mapped equally well to both genes and their promoter regions. Nevertheless, our data clearly confirmed that at least one of the two alpha globin genes is transcribed during HSV-1 infection as early as 2-3h p.i. and translated into protein at least from 4h p.i. (according to our previously published Ribo-seq data [20]). Our analysis suggests that similar up-regulation from no or low expression is observed for a number of other cellular genes. Since we used relatively strict criteria to exclude genes that only appeared to be expressed during infection due to read-in transcription, we also investigated more lenient criteria to identify the extent of induction for genes that are not expressed prior to infection (see methods for details). These criteria applied to 17 of the up-regulated genes (e.g. DLL1) and an additional 33 genes not included in our previous analysis. Manual inspection of these 33 genes confirmed transcriptional up-regulation only for 13 genes (IRF4, RRAD, FOSB, ARC, CA2, DIO3, DLX3, GBX2, ICOSLG, MAFA, MAFB, NGFR, PCDH19). Of these, 6 and 8 were up-regulated by type I and II interferons, respectively. In summary, only a small fraction of genes not expressed in uninfected fibroblasts is induced by HSV-1 infection.

518 To start investigating how the rapid up-regulation of these genes might be achieved,
519 we performed ChIPmentation [49] of H3K4me3 histone marks (2 replicates each in
520 uninfected cells and at 8h p.i. WT infection). H3K4me3 has been reported to regulate
521 assembly of the preinitiation complex for rapid gene activation [50]. Furthermore, a
522 bivalent chromatin modification pattern combining H3K4me3 and H3K27me3 has
523 been described in embryonic stem (ES) cells, which serves to keep silenced
524 developmental genes poised for activation [51]. Across all 4 samples, we identified
525 32,601 unique non-overlapping peak regions, which were strongly enriched around
526 gene promoters (S7 Dataset). In total, 98.7% of analyzed genes exhibited H3K4me3
527 peaks around the promoter in both replicates of uninfected cells. Notably, this also
528 applied to 21 of the 24 genes in the orange cluster (87.5%, see Fig 5C,D for
529 examples). Only NPTX1 and NPTX2 showed no significant H3K4me3 promoter peak
530 in either replicate of uninfected cells, but both showed peaks in at least one replicate
531 of infected cells. In total, 97.8% of all up-regulated genes and 92.1% of up-regulated
532 genes that were not or lowly expressed in uninfected cells (total RNA FPKM ≤1)
533 showed significant peaks in both replicates of uninfected cells. In summary, this
534 indicates strong, early, *vhs*-independent transcriptional up-regulation of a small
535 number of poorly expressed genes which are already poised for expression by
536 H3K4me3 marks at their promoters.

537 Recently, Full *et al.* reported that the germline transcription factor DUX4 (double
538 homeobox 4) and several of its targets are highly up-regulated by HSV-1 infection
539 [52]. We thus compared genes up- or down-regulated by doxycycline-inducible DUX4
540 [53] with genes transcriptionally regulated in HSV-1 infection (Fig 5E). We found that
541 HSV-1 up-regulated genes were significantly (Fisher's exact test, adj. $p \leq 0.001$)
542 enriched for DUX4 up-regulation and HSV-1 down-regulated genes were significantly

543 enriched for DUX4 down-regulation. Notably, the fraction of genes up-regulated by
544 DUX4 was similar (~36%) for all clusters of HSV-1 up-regulated genes, independent
545 of their expression in uninfected cells. Interestingly, however, genes that were
546 transcriptionally down-regulated in HSV-1 infection in a *vhs*-mediated manner were
547 less enriched for DUX4-mediated down-regulation than genes for which
548 transcriptional down-regulation was independent of *vhs.* Moreover, enrichment for
549 adhesome components was more pronounced among *vhs*-dependent genes not
550 down-regulated by DUX4 than among those down-regulated by DUX4. Thus, while
551 DUX4 is a major transcriptional regulator in HSV-1 infection, it is not responsible for
552 *vhs*-mediated down-regulation of the integrin adhesome.

553

554 **_Vhs_-dependent transcriptional down-regulation impacts on cellular protein**
555 **levels**

556 To investigate how changes in total RNA levels and transcription alter protein levels
557 in infected cells, we performed a Tandem Mass Tag (TMT)-based quantitative
558 proteomic analysis of WT- and Δ*vhs*-infected HFF at 0 and 8h p.i. (n=3 replicates). In
559 total, 7,943 proteins were identified (S8 Dataset). No filtering based on read-in
560 transcription was performed, as read-through transcripts are neither exported nor
561 translated [20, 21]. Protein fold-changes were poorly correlated to fold-changes in
562 total RNA, 4sU-RNA, or subcellular RNA fractions ($r_s \leq 0.21$) and generally tended to
563 be less pronounced. Both observations are consistent with the higher stability of
564 proteins compared to mRNAs (~5 times more stable in mouse fibroblasts [54]), thus
565 changes in *de novo* transcription and total RNA levels commonly take >8h to
566 significantly impact on protein levels. Consequently, protein fold-changes were very
567 well correlated between WT and Δ*vhs* infection (Fig 6A, $r_s = 0.96$) and only few

568 cellular proteins showed a significant difference between WT and Δ*vhs* infection. Due
569 to the less pronounced changes, we determined differentially expressed proteins with
570 a >1.5-fold change (adj. $p \leq 0.001$, Fig 6A). Most differentially expressed proteins
571 were concordantly regulated either down (1,444 genes, 73%) or up (499 genes,
572 25.3%) in both WT and Δ*vhs* infection. It should be noted that, similar to RNA-seq
573 data, protein fold-changes only represent relative changes in the presence of a global
574 loss in cellular protein levels. Thus, some up-regulated proteins may simply be
575 less/not down-regulated compared to most other proteins.

576 To evaluate consistency with previously identified HSV-1-regulated proteins, we
577 compared our concordantly up- or down-regulated proteins against significantly
578 regulated proteins (ANOVA p-value ≤ 0.001) from the proteomics time-course for 3,
579 6, 9 and 12h p.i. HSV-1 infection from the study of Kulej *et al*. [55]. This included
580 4,613 proteins and 53% of proteins from our study. While only 33 of the up-regulated
581 and 109 of the down-regulated proteins from our study were also significantly
582 regulated in the Kulej *et al.* study, the direction of regulation was consistent for both
583 sets of genes until 6h p.i. (Fig 6B). From 9h p.i., both up- and down-regulated
584 proteins from our study that were significantly regulated in the study of Kulej *et al.*
585 tended to be down-regulated to a similar extent. The relatively small overlap between
586 our study and the study by Kulej *et al.* is not surprising considering the generally low
587 overlap of previous proteomics studies on HSV-1 infection [55-57]. Most likely, this is
588 explained by differences between the employed cells and infection doses thereby
589 resulting in different infection kinetics.

590 Concordantly down-regulated proteins were significantly (adj. $p \leq 0.001$, S9 Dataset)
591 enriched for a number of GO terms, including “nucleotide-sugar biosynthetic process”
592 (>77-fold enriched), “canonical glycolysis” (>15-fold), “viral budding” (>9-fold) and

"activation of MAPK activity" (>4-fold). Interestingly, meta-adhesome (but not HFF adhesome) components were also significantly enriched (>1.9-fold), indicating that concordantly down-regulated proteins interact with the core adhesome, rather than are a part of it. Interestingly, concordantly up-regulated proteins were highly enriched for mitochondrial proteins (>7-fold, 186 proteins), but significantly depleted of meta-adhesome components. To test how many of these proteins were up-regulated following a significant increase in their total RNA levels, we determined genes that were significantly up-regulated in total RNA in both WT and *Δvhs* infection (19 genes). 5 of these were also up-regulated at protein level, including 4 genes that were up-regulated in chromatin-associated RNA (RASD1, SNAI1, CBX4, ITPR1). Thus, transcriptional up-regulation can have a small but measurable effect on protein levels by 8h p.i. Only few genes showed a significant differential effect (24 down-regulated in WT only, 6 up-regulated in Δ*vhs* only). Strikingly, the 24 proteins down-regulated in a *vhs*-dependent manner were strongly enriched for HFF adhesome components (>12-fold) and ECM organization (>23-fold). Accordingly, 9 of 14 (64%) proteins down-regulated only in WT infection and included in our RNA-seq analysis were down-regulated in chromatin-associated RNA in a *vhs*-dependent manner. An analysis of protein fold-changes for all *vhs*-dependently transcriptionally down-regulated genes (including our extended set) demonstrated that a significant number of respective proteins were either less down-regulated or (relatively) more up-regulated in Δ*vhs* infection than in WT infection (Fig 6C). Many of these were components of the integrin adhesome or were involved in ECM organization. Thus, *vhs*-dependent transcriptional down-regulation impacts protein levels of the respective genes already by 8h p.i.

618 **Discussion**

619 HSV-1 infection drastically alters host RNA metabolism at all levels by impairing host
620 mRNA synthesis, processing, export and stability. Here, we differentiate and quantify
621 their individual contributions to the RNA expression profile by combining RNA-seq of
622 total, newly transcribed (4sU-) and subcellular RNA fractions in WT and Δ*vhs*
623 infection. While it is important to note that the WT and Δ*vhs* time-course experiments
624 were performed independently, we carefully standardized the experimental
625 conditions, e.g. by infecting the same batch of cells following the same number of
626 splits after thawing as well as using the same batch of fetal bovine serum (FBS), to
627 achieve a maximum level of reproducibility. Indeed, all major results were confirmed
628 in experiments which were performed in parallel for WT and Δ*vhs* infection.

629 We developed a mathematical model to quantify both the loss of transcriptional
630 activity and the changes in *vhs* nuclease activity based on the correlations between
631 RNA half-lives and total RNA fold-changes during the first 8h of infection. This
632 showed a drop in transcriptional activity down to 10-20% of the original level by 8h
633 p.i. in Δvhs infection, consistent with the well-described general loss of Pol II from
634 host chromatin [14, 15]. The WT HSV-1 time-course depicted a rapid increase in *vhs*-
635 dependent degradation, with 20-30% of all cellular mRNAs degraded per hour by 2h
636 p.i., consistent with the well-described role of *vhs* upon viral entry. While *vhs* activity
637 did not further rise from 4h p.i. despite increasing *vhs* protein levels, it was constantly
638 maintained until 8h p.i. The kinetics of the viral life cycle are incorporated in our ODE
639 model via the functions describing *vhs* activity and cellular transcriptional activity. As
640 *vhs* activity and cellular transcriptional activity cannot be estimated simultaneously in
641 WT infection, we used the estimated changes of host transcriptional activity in WT
642 from *Δvhs* infection. However, no data from the *Δvhs* time-course was used in the

643 modelling of WT infection, only the estimated function describing the kinetics of host
644 transcriptional activity. Considering the slower progression of *⊿vhs* infection, we may
645 thus have underestimated the drop in transcriptional activity in WT infection.
646 However, if transcriptional activity drops even faster and further in WT infection, *vhs*
647 activity would have to increase even faster and to higher levels to explain the
648 observed negative correlations between RNA half-lives and total RNA fold-changes
649 in WT infection. It is important to note that our findings do not contradict previous
650 reports on the post-transcriptional down-regulation of *vhs* activity by its interaction
651 with VP16 and VP22 [9-11]. As previously already noted [9], counter-regulation of *vhs*
652 activity is not complete, but VP16 and VP22 clearly serve to prevent a further
653 detrimental increase in *vhs* activity during infection. Their activity thus explains the
654 plateau we observed for *vhs* activity despite substantially increasing *vhs* protein
655 levels. Moreover, application of our model to total RNA fold-changes at 12h p.i. WT
656 infection from the study of Pheasant *et al*. confirmed deactivation of *vhs* between 8
657 and 12h p.i.

658 Pheasant *et al*. also noted that *vhs*-dependent reduction in total RNA levels varied
659 widely between genes at 12h p.i. and hypothesized that this might indicate
660 differences in susceptibility to *vhs*-mediated degradation between transcripts [19].
661 Furthermore, they excluded an influence of basal transcription rates and RNA half-
662 lives for the three genes whose high *vhs*-sensitivity they confirmed by PCR.
663 However, we here show that all three genes they selected for experimental validation
664 are actually transcriptionally down-regulated in a *vhs*-dependent manner. Together
665 with the effects of *vhs* on RNA stability, this translates into a significant reduction in
666 the corresponding protein levels by 8h p.i. Accordingly, results from these three
667 genes cannot be extrapolated to genes down-regulated in total RNA *only* through

668 *vhs*-mediated RNA decay. Instead, our ODE model suggests that gene-specific
669 differences in mRNA half-lives substantially shape the variability in total mRNA
670 changes between genes at least until 8h p.i. This does not exclude a contribution of
671 other factors, e.g. *vhs*-induced nuclear retention of cellular mRNAs shown by
672 Pheasant *et al*. [19] or differences in translation rates between different mRNAs (and
673 thus translation-initiation-dependent mRNA cleavage by the *vhs* protein), which we
674 did not consider in our model. In particular, *vhs*-dependent transcriptional down-
675 regulation contributes substantially to the reduction in total RNA levels for the
676 respective genes. Furthermore, a recent study identified a set of 74 genes that
677 escape degradation by four herpesviral endonucleases, including *vhs* [58]. Almost all
678 of these genes were excluded from our analysis due to low expression (87%), read-in
679 transcription (7%), or proximity to nearby genes (3%). Two genes, however, which
680 were not excluded, (C19orf66, ARMC10) indeed did not show any significant change
681 in any of our data. Selective targeting of *vhs* to unstable mRNAs via AU-rich
682 elements in a translation-independent manner has also been reported [59]. We thus
683 do not exclude that some transcripts are more or less susceptible to *vhs*-mediated
684 decay than others. However, we conclude that strong *vhs*-dependent reductions in
685 total mRNA levels are not necessarily a consequence of increased susceptibility of
686 individual transcripts to *vhs*-mediated RNA cleavage.

687 In contrast to total cellular RNA changes, fold-changes in newly transcribed and, in
688 particular, chromatin-associated RNA were surprisingly similar between WT and
689 Δ*vhs* infection. This enabled us to decipher gene-specific transcriptional regulation
690 that is either dependent or independent of *vhs*. Although we performed the combined
691 total RNA- and 4sU-seq time-courses for both viruses in two separate experiments,

692 the high correlation of the 4sU-RNA fold-changes confirmed that it was valid to also
693 compare the corresponding total RNA-seq time-course data.

694 While the analysis of chromatin-associated RNA eliminated the bias originating from
695 *vhs* activity and the global loss in transcription, read-in transcription leading to
696 seeming, but non-functional, induction of genes has to be taken into account in all
697 gene expression profiling studies independent of the type of profiled RNA. By
698 excluding genes with evidence of read-in transcription from our analysis, we
699 ascertained that all identified induced genes represent true up-regulation and not
700 artefacts from read-in transcription. Notably, while most strongly up-regulated genes
701 identified in our study have been reported in previous studies on HSV-1-induced
702 differential host expression (e.g. RASD1 [19, 45, 60, 61]), several previously reported
703 genes, which were thought to be induced by HSV-1, are actually only seemingly
704 induced due to read-in transcription, e.g. ZSCAN4 [45, 62], SHH [60], and FAM71A
705 [19].

706 Around 30% of all up-regulated genes and 50% of the most strongly up-regulated
707 genes (orange cluster) were up-regulated by type I interferons (IFN). Moreover,
708 DUX4 was confirmed as a major transcriptional regulator in both WT and Δ*vhs*
709 infection for both up- and down-regulated genes (37% of up-regulated genes were
710 previously found to be up-regulated by DUX4 and 39% of down-regulated genes
711 were down-regulated by DUX4, Fig 5E). Although there was some overlap between
712 DUX4 and IFN-induced genes amongst the HSV-1-induced genes, it was not
713 significantly larger than expected at random. Interestingly, the DUX4 up-regulated
714 gene TRIM43 was recently identified as a herpesvirus-specific antiviral factor
715 independent of the type I interferon response [52]. This suggests that DUX4-

716 mediated regulation in HSV-1 infection may represent an alternative pathway which
717 augments the host intrinsic immune response.

718 A key finding of our study is the *vhs*-dependent, transcriptional down-regulation of
719 proteins involved in the integrin adhesome and ECM organization, which required *vhs*
720 nuclease activity. Suppression of ECM protein synthesis during HSV-1 infection has
721 already been reported over 30 years ago for the canonical integrin ligand FN1, type
722 IV procollagen, and thrombospondin [63]. Recently, this was confirmed for a few
723 other ECM components in human nucleus pulposus cells in both lytic and latent HSV-
724 1 infection [64]. *Vhs*-dependency of down-regulation was previously reported for FN1
725 [65], but was ascribed to the effect of *vhs* on FN1 RNA stability. This further
726 highlights the pitfalls in ascribing all *vhs*-dependent effects on total RNA levels solely
727 to *vhs*-mediated RNA decay. In contrast, our data demonstrates that *vhs*-dependent
728 down-regulation of specific genes is augmented by *vhs*-dependent repression of
729 transcription. Notably, while *vhs*-dependent down-regulation of the ECM and
730 adhesome can largely be confirmed in total RNA, it is challenging to distinguish it
731 from *vhs*-mediated mRNA degradation. The transcriptional effects only become
732 obvious when analyzing chromatin-associated RNA.

733 Interestingly, transcriptional down-regulation of ECM and integrin adhesome genes
734 was dependent on the nuclease activity of *vhs*. Recently, muSOX-mediated RNA
735 decay was reported to trigger transcriptional repression at late times of lytic MHV68
736 infection [18]. While HSV-1 *vhs* activity also triggered this phenomenon within 24h of
737 expression, the cellular genes transcriptionally regulated in a *vhs*-dependent manner
738 during the first 8h of HSV-1 infection showed little overlap to the genes affected by
739 the transcriptional effects of muSOX-induced RNA degradation. To date, the
740 molecular mechanism underlying the transcriptional shut-off induced upon extensive

741 cytoplasmic RNA degradation remains unclear. While we cannot fully exclude that
742 *vhs*-dependent transcriptional down-regulation of the integrin adhesome and ECM
743 components marks the advent of this effect, our data are more likely to be explained
744 by a distinct gene-specific function of *vhs* with more wide-spread transcriptional
745 repression only becoming relevant at later times of infection.

746 An alternative explanation for the *vhs*-dependent repression of such a functionally
747 connected cellular network of genes is that *vhs* nuclease activity results in a rapid
748 depletion of transcripts of key, short-lived cellular transcription factor(s) governing
749 these genes. It is unclear, however, why only a single or very small number of
750 transcription factors would suffer so much more dramatically from *vhs* nuclease
751 effects. It is indeed surprising that *vhs*-mediated mRNA degradation does not cause
752 a similarly pronounced dysregulation downstream of short-lived transcription factors
753 involved in other processes. However, the surprisingly high correlation between fold-
754 changes in WT and Δ*vhs* infection observed in chromatin-associated RNA excludes
755 gross global effects of mRNA degradation of cellular transcriptional factors.
756 Furthermore, no enrichment of any known or novel transcription factor binding motif
757 could be identified in both proximal promoter regions or more distal open chromatin
758 regions identified by ATAC-seq. Promoter analysis applied to all expressed HFF
759 adhesome genes identified only one significant motif which was only observed in
760 <6% of genes, suggesting that there is no single key transcriptional regulator for the
761 integrin adhesome. Nevertheless, *vhs* may still directly interact with or target a major
762 cellular transcription factor that governs the expression of the integrin adhesome and
763 ECM via distal enhancers. Notably, ELK3, a TCF co-factor of SRF, was down-
764 regulated in a *vhs*-dependent manner early on in infection. While TCF-dependent
765 genes were not enriched among *vhs*-dependent genes, a ~2-fold enrichment of SRF

766 targets was observed. Since TCF-dependent genes were determined by triple
767 knockouts of all three TCFs [44], not all TCF-dependent genes likely depend on
768 ELK3. While our Western blot analysis of ELK3 protein abundance was inconclusive
769 (data not shown), quantitative proteomics suggested at least a weak change (1.6-
770 fold) between WT and Δ*vhs* infection. Thus, ELK3-dependent reduced recruitment of
771 SRF may still play a role. Alternatively, post-transcriptional processes, which have
772 been linked to transcriptional control of focal adhesions, may also be relevant for *vhs*-
773 dependent down-regulation. For instance, Rho signaling can result in nuclear
774 translocation of the SRF co-factor MRTF-A and prevention of this translocation
775 results in lower expression of cytoskeletal/focal adhesion proteins [66]. Furthermore,
776 up-regulation of nuclear actins lead to transcriptional down-regulation of a number of
777 adhesion proteins [67], such as ITGB1 and MYL9, which were also down-regulated in
778 a *vhs*-dependent manner in HSV-1 infection.

779 Untangling the molecular mechanisms underlying specific *vhs*-mediated down-
780 regulation of the integrin adhesome and ECM will be difficult without knowledge of
781 the responsible cellular transcription factor(s) and confounded by the pleotropic
782 effects of *vhs* nuclease activity. Nevertheless, we could show that *vhs*-dependent
783 transcriptional down-regulation has a clear impact on protein levels already by 8h p.i.,
784 as confirmed by quantitative whole cell proteomics. Proteins with strong *vhs*-
785 dependent reduction at 8h p.i. include matrix metallopeptidases MMP1-3, which are
786 involved in degradation of ECM proteins, their inhibitor TIMP1 as well as other MMP-
787 up-regulating or -interacting proteins (LUM, SPARC, THBS2).

788 In summary, our analyses provide a comprehensive, quantitative picture of the
789 molecular mechanisms that govern profound alterations in the host cell transcriptome
790 and proteome during lytic HSV-1 infection.

Materials and Methods

Cell culture and infections

Human fetal foreskin fibroblasts (HFF) were purchased from ECACC (#86031405) and cultured in DMEM with 10% FBS Mycoplex and 1% penicillin/streptomycin. HFF were utilized from passage 11 to 17 for all high-throughput experiments. This study was performed using WT HSV-1 strain 17 (data taken from previous studies [20, 21]) and its *vhs*-inactivated mutant (Δ*vhs*) [22]. In this mutant, *vhs* was inactivated by inserting the lacZ coding sequence at codon 251. It thus produces a 250-residue amino terminal *vhs* fragment and deletes residues 251-485. While the mutant may thus retain some unknown activity of *vhs*, its nuclease activity and thus all of its known functions are inactivated. Virus stocks were produced in baby hamster kidney (BHK) cells (obtained from ATCC) as described [20]. HFF were infected with HSV-1 24h after the last split for 15 min (for total RNA-seq, 4sU-seq and RNA-seq of subcellular fractions) or 1h (for RNA-seq of chromatin-associated RNA including the *vhs* D195N mutant), at 37°C using a multiplicity of infection (MOI) of 10. Subsequently, the inoculum was removed and fresh media was applied to the cells.

The *vhs* D195N mutant virus was constructed via *en passant* mutagenesis [68]. Mutagenesis templates were generated using PCR primers GTATATCTGGCCCGTACATCGATCT and GGTCAGTGTCCGTGGTGTACACGTACGCGACCGTGTTGGTGTGATAGAGGTTG GCGCAGGCATTGTCCGCCTCCAGCTGACCCGAGTTAAAGGATGACGACGATAA GTAGGG to amplify the kanamycin resistance cassette flanked by Isce-I restriction sites from vector pEP-Kan. Additional homologies for recombination were added to this product by PCR using primers GGTCAGTGTCCGTGGTGTAC and

816 TTCTGTATTCGCGTTCTCCGGGCCCTGGGGTACGCCTACATTAACTCGGGTCAG
817 CTGGAGGCGGACAATGCCTGCGCCAACCTCTATCACGTATATCTGGCCCGTAC
818 ATCGATCT before electroporation into *Escherichia coli* strain GS1783 containing the
819 pHSV(17+)Lox BAC [69]. BAC DNA was purified using the NucleoBond BAC 100 kit
820 (Macherey-Nagel #740579) and transfected for virus reconstitution into BHK-21 cells
821 with Lipofectamine 3000 (ThermoFisher #L3000-075).

822 *Preparation of RNA samples*

823 Sample preparation for 4sU-seq in Δ*vhs* infection was performed as reported
824 previously for WT HSV-1 [20]. In brief, 4-thiouridine (4sU) was added to the cell
825 culture medium for 60min at −1, 0, 1, 2, 3, 4, 5, 6, or 7h p.i. (2 × 15-cm dishes per
826 condition) during Δ*vhs* infection to a final concentration of 500μM (n=2 replicates).
827 Subsequently, the medium was aspirated and the cells were lysed with Trizol
828 (Invitrogen). Total RNA and newly transcribed RNA fractions were isolated from the
829 cells as described previously [28]. In an independent experiment, subcellular RNA
830 fractions (cytoplasmic, nucleoplasmic and chromatin-associated RNA) in mock and
831 8h p.i. of WT and Δ*vhs* infection were prepared as previously described (n=2
832 replicates) [21]. To assess the role of *vhs* nuclease activity in regulation of ECM and
833 integrin adhesome genes, chromatin-associated RNA in mock, WT, Δ*vhs, vhs* D195N
834 and WT-BAC infection at 8h p.i. (n=2 replicates) was prepared.

835 *Library preparation and RNA sequencing*

836 Sequencing libraries were prepared using the TruSeq Stranded Total RNA kit
837 (Illumina). rRNA depletion was performed after DNase treatment for total RNA and all
838 subcellular RNA fractions using Ribo-zero but not 4sU-RNA samples. Sequencing of
839 75bp paired-end reads was performed on a NextSeq 500 (Illumina) at the Core Unit
840 Systemmedizin (Würzburg).

Quantitative Reverse Transcription PCR

Chromatin-associated RNA was isolated in TRIzol as described above then DNase treated and purified with the Direct-zol RNA Miniprep Kit (Zymo #R2051). cDNA was synthesized using the 'Optional procedure' of the BioScript All-in-One cDNA Synthesis SuperMix (Biotool #B24403). Real-time PCR performed with SYBR Green qPCR Master Mix (Bimake #B21202) using the recommended three-step protocol and 1μM of the following primer pairs for each gene: 18S rRNA (GCAATTATTCCCCATGAACG, GGGACTTAATCAACGCAAGC), ARF4 (CCTTCTGCTTCTGCCCATCA, CGCATCTGCTTCTTGCCAAA), CNBP (AAACTGGTCATGTAGCCATCAAC, AATTGTGCATTCCCGTGCAAG), COL6A2 (GCAACGACTACGCCACCAT, GACCTTGATGATGCGGTTGA), MMP1 (TAGTGGCCCAGTGGTTGAAA, GGGCTGCTTCATCACCTTCA), and MMP3 (AGTCTCTGTGAATTGAAATGTTCG, AGTTCCCTTGAGTGTGACTCG). cDNA samples were diluted 1:10 in water for protein-coding genes and 1:1000 for 18S rRNA, then diluted 1:4 in the final reaction which was performed on a LightCycler® 96 (Roche). Cq values for each gene were calibrated to 18S rRNA for that sample and relative expression between samples calculated using the ΔΔCq method.

H3K4me3 ChIPmentation

The full description of H3K4me3 ChIPmentation is included in S10 Text.

Preparation of samples for proteomic analysis

HFF were infected with WT or Δ*vhs* HSV-1 for 8h at an MOI of 10. Infections were conducted in triplicate, with 4 uninfected controls (10 samples in total). Washed cells were snap-frozen in liquid nitrogen. Cells were lysed in by resuspending in 100μL 2% sodium dodecyl sulfate (SDS) and 50mM tetraethylammonium bromide (TEAB) pH 8.5 followed by 10min (30s on/off duty cycle) sonication in a Bioruptor® sonicator

(Diagenode). Lysates were quantified by BCA assay and 50µg of each sample was reduced and alkylated with 10mM tris(2-carboxyethyl)phosphine (TCEP) and 40mM iodoacetamide for 20min at room temperature in the dark. Samples were diluted to 500µL with 8M urea/50mM TEAB and applied to 30kDa Vivacon centrifugal ultrafiltration devices (Sartorius) and concentrated according to the manufacturer's instructions. Samples were resuspended and concentrated in 8M urea a further 3 times to remove residual SDS. There were a further 3 washes with digestion buffer (0.5% sodium deoxycholate (SDC), 50mM TEAB) before samples were resuspended in approximately 50µL digestion buffer with 1µg trypsin (Proteomics grade, Thermo Fisher). Filter units were then incubated in at 37°C overnight in a box partially filled with water to reduce evaporation. Peptides were recovered into a fresh tube by centrifugation and a further wash with 50µL digestion buffer. SDC was removed from each sample by precipitation with the addition of formic acid and two-phase partitioning with ethyl acetate. Peptides were then dried under vacuum. For TMT labelling samples were resuspended in 42µL 100mM TEAB and 0.4mg of each TMT reagent in 18µL anhydrous acetonitrile was added, vortexed to mix and incubated at room temperature for 1 hour. A small aliquot of each sample was analyzed by LC-MS to confirm labelling efficiency and samples were pooled 1:1 according to the total TMT reporter intensity in these QC runs. The pooled sample was then acidified and subjected to solid phase extraction (SPE) clean-up using 50mg tC18 cartridges (Waters) before drying under vacuum.

Basic pH Reversed-Phase fractionation.

Samples were resuspended in 40µL 200mM ammonium formate pH 10 and transferred to a glass HPLC vial. BpH-RP fractionation was conducted on an Ultimate 3000 UHPLC system (Thermo Scientific) equipped with a 2.1mm × 15cm, 1.7µm

Kinetex EVO column (Phenomenex). Solvent A was 3% acetonitrile, Solvent B was 100% acetonitrile, Solvent C was 200mM ammonium formate (pH 10). Throughout the analysis, Solvent C was kept at a constant 10%. The flow rate was 400µL/min and UV absorbance was monitored at 280nm. Samples were loaded in 90% Solvent A for 10 min before a gradient elution of 0–10% Solvent B over 10min (curve 3), 10-34% Solvent B over 21 min (curve 5), 34-50% Solvent B over 5min (curve 5) followed by a 10min wash with 90% Solvent B. 15s (100µL) fractions were collected throughout the run. Fractions containing peptide (as determined by 280nm light absorbance) were recombined across the gradient to preserve orthogonality with on-line low pH RP separation. For example, fractions 1, 25, 49, 73, 97 are combined and dried in a vacuum centrifuge and stored at -20°C until LC-MS analysis.

Mass Spectrometry

Samples were analysed on an Orbitrap Fusion instrument on-line with an UltiMate™ 3000 RSLCnano UHPLC system (Thermo Fisher). Samples were resuspended in 10µL 5% DMSO/1% trifluoroacetic acid (TFA) and 5µL of each fraction was injected. Trapping solvent was 0.1% TFA, analytical Solvent A was 0.1% formic acid, and Solvent B was acetonitrile with 0.1% formic acid. Samples were loaded onto a trapping column (300µm x 5mm PepMap cartridge trap, Thermo Fisher) at 10µL/min for 5min. Samples were then separated on a 50cm x 75µm i.d. 2µm particle size PepMap C18 column (Thermo Fisher). The gradient was 3-10% Solvent B over 10min, 10-35% Solvent B over 155min, 35-45% Solvent B over 9min followed by a wash at 95% Solvent B for 5min and re-equilibration at 3% Solvent B. Eluted peptides were introduced by electrospray to the MS by applying 2.1kV to a stainless-steel emitter (5cm x 30µm, Thermo Fisher). During the gradient elution, MS1 spectra were acquired in the Orbitrap, collision-induced dissociation (CID)-MS2 acquired in

the ion trap. Synchronous precursor selection (SPS)-isolated MS2 fragment ions were further fragmented using higher-energy collisional dissociation (HCD) to liberate reporter ions which were acquired in the Orbitrap (MS3).

Data Processing

Raw files were searched using Mascot (Matrix Science) from within Proteome Discoverer Ver 2.1 (Thermo Fisher) against the UniProt human database with appended common contaminants and UniProt HSV reference proteome. Peptide-spectrum match (PSM) false discovery rate (FDR) was controlled at 1% using Mascot Percolator. The reporter ion intensities of proteins with a High (1%) and Medium (5%) FDR were taken and subjected to LIMMA t-test in R. P-values were adjusted for multiple testing using the method by Benjamini and Hochberg [70]. Proteins with extremely high standard deviation between replicates in (>99 percentile) in either WT or Δ*vhs* infection were excluded from further analysis.

Processing of next-generation sequencing data

Sequencing reads were mapped against (i) the human genome (GRCh37/hg19), (ii) human rRNA sequences, and (iii) the HSV-1 genome (HSV-1 strain 17, GenBank accession code: JN555585) using ContextMap v2.7.9 [71] (using BWA as short read aligner [72] and allowing a maximum indel size of 3 and at most 5 mismatches). For the two repeat regions in the HSV-1 genome, only one copy each was retained, excluding nucleotides 1–9,213 and 145,590–152,222. ContextMap produces unique mappings for each read, thus no further filtering was performed. Read coverage was visualized using Gviz [73] after normalizing to the total number of mapped human reads and averaging between replicates. For identification of enriched H3K4me3 regions (=peaks), BAM files with mapped reads were converted to BED format using BEDTools [74] (v2.24.0) and peaks were determined from BED files using F-Seq with

default parameters [75]. Only peaks with length ≥500nt were considered. Unique non-overlapping peaks were identified by merging overlapping peaks across all samples using BEDTools. Overlaps of identified peaks to gene promoters were determined using ChIPseeker [76].

Analysis of transcription read-through and differential gene expression

Number of read fragments per gene were determined from the mapped 4sU-seq and RNA-seq reads in a strand-specific manner using featureCounts [77] and gene annotations from Ensembl (version 87 for GRCh37/hg19) [78]. All fragments (read pairs for paired-end sequencing or reads for single-end sequencing) overlapping exonic regions on the corresponding strand by ≥25bp were counted for the corresponding gene. Expression of protein-coding genes and lincRNAs was quantified in terms of fragments per kilobase of exons per million mapped fragments (FPKM) and averaged between replicates. Only fragments mapping to the human genome were counted for the number of mapped fragments as previously described [20]. Downstream and upstream transcription for genes was determined from 4sU-seq data as described [21], i.e. the FPKM in the 5kb windows down- or upstream of genes divided by the gene FPKM. Read-through transcription was quantified as the difference in downstream transcription between infected and uninfected cells, with negative values set to zero. Read-in transcription was calculated analogously as the difference in upstream transcription between infected and uninfected cells. For full details, see our previous publication [21]. Only genes were included in this paper that (i) had no upstream or downstream gene within 5kb, (ii) were expressed (FPKM ≥ 1 in 4sU-RNA) in uninfected cells or at least one time point of WT infection, and (iii) had at most 10% read-in transcription at any time during WT infection. For genes not expressed in uninfected cells (FPKM <1 in uninfected 4sU-RNA), at most 5% read-in

transcription during infection and at most 25% upstream transcription in uninfected cells was allowed. These restrictions were used to exclude genes that only appeared induced due to read-in transcription from an upstream gene. In total, 4,162 genes were included for the analyses in this manuscript. Differential gene expression analysis for these genes in total and 4sU-RNA and subcellular RNA fractions was performed based on gene read counts using DESeq2 [24] and p-values were adjusted for multiple testing using the method by Benjamini and Hochberg [70]. Additional candidate up-regulated genes with low or no expression in uninfected cells were determined using the following criteria: i) FPKM in uninfected 4sU- and total RNA ≤ 1; ii) FPKM in either 4sU-RNA or total RNA at any time of infection both ≥ 0.5 and ≥ 4-fold higher than in uninfected cells; iii) read-in transcription ≤ 20% at all time points. Candidate genes were subsequently validated by manual inspection of mapped reads for individual replicates in the IGV genome browser [79]. To identify the extended set of *vhs*-dependently transcriptionally down-regulated genes, we applied DESeq2 for all genes on RNA-seq of chromatin-associated RNA in mock, 8h p.i WT and *Δvhs* infection. Genes were defined as transcriptionally down-regulated in a *vhs*-dependent manner if they were significantly down-regulated in WT (log2 fold-change ≤ -1, adj. p-value ≤ 0.001), not down-regulated in *Δvhs* infection (log2 fold-change > -1) and there was at least a 2-fold increase in fold-changes in *Δvhs* compared to WT infection.

RNA half-lives

RNA half-lives were measured as described [28] from 4sU-RNA and total RNA measurements in uninfected cells from the WT time-course. For this purpose, we first calculated the ratio of gene expression (FPKM) values in 4sU- vs. total RNA in uninfected cells for all genes, normalized this ratio assuming a median RNA half-life

of 5h to determine the fraction of RNA newly transcribed in 1h hour for each gene (*N*(t), t=60*) and then calculated RNA half-life for each gene as $t_{1/2} = -t \ln 2 / \ln(1 - N^*(t))$.

Mathematical model

The ODE model of WT and Δ*vhs* infection is described in S2 Text.

Clustering, enrichment and network analysis

Hierarchical clustering was performed in R [80] using Euclidean distances and Ward's clustering criterion [81]. Gene Ontology (GO) [29] annotations for genes were obtained from EnrichR [82] and lists of interferon I, II, and III up- or down-regulated genes (at least 2-fold) were obtained from the INTERFEROME database [30]. Genes regulated by doxycycline-inducible DUX4 were taken from the study of Jagannathan *et al*. (Supplementary Table 1; up-regulated: log2 fold-change ≥ 1, false discovery rate (fdr) ≤ 0.001; down-regulated: log2 fold-change ≤ -1, fdr ≤ 0.001) [53]. TCF-dependent genes and SRF targets in MEFs were taken from the study by Gualdrini *et al*. [44]. Odds-ratios and significance of enrichment compared to the background of 4,162 genes was determined using Fisher's exact test in R [80] and p-values were adjusted for multiple testing using the method by Benjamini and Hochberg [70]. Human protein-protein associations were downloaded from the STRING database [39] (version 10.5) using NDEx [83] and visualized in Cytoscape [84]. Only associations with a score ≥350 are shown.

Comparison of muSOX and vhs-dependent genes

Fold-changes for WT and ΔHS MHV68 infection were taken from the study of Abernathy *et al*. [18] and downloaded from Gene Expression Omnibus (GSE70481). Mouse and human gene symbols were mapped to their orthologues in the respective other species using the Mouse/Human orthology table from the Mouse Genome

Informatics (MGI) database [85]. muSOX-dependent genes were defined according to the criteria applied by Abernathy *et al*.: down-regulated in WT (log2 fold-change ≤ -1 and fdr ≤ 0.1) but not in ΔHS infection (log2 fold-change > -1 or fdr > 0.1). *vhs*-dependent genes were defined according to our criteria described above.

Transcription factor binding motif search

Promoter motif search for *vhs*-dependently down-regulated genes was performed using HOMER in proximal promoter regions (-2,000 to +2,000 bp relative to the transcription start site). [86]. Potential transcription binding factor sites in uninfected cells were furthermore identified using ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing [87]) data of uninfected cells from our previous study (n=2 replicates) [21]. ATAC-seq data were mapped against hg19 as previously described [21] and open chromatin peaks were determined using MACS2 [88]. Blacklisted regions for hg19 (accession ENCFF001TDO) were downloaded from ENCODE [89] and peaks called in regions overlapping with blacklisted regions were removed from further analysis. Furthermore, only peaks occurring in both replicates were considered for motif search. Motif search was then performed using HOMER for open chromatin peaks within 10, 25, and 50kb, respectively, of *vhs*-dependently down-regulated genes.

Data Availability

All sequencing data are available in the Gene Expression Omnibus (GEO) under the following IDs: 4sU-seq and total RNA-seq data of WT infection, GSE59717; 4sU-seq and total RNA-seq data of Δ*vhs* infection, GSE129715; RNA-seq of total, cytoplasmic, nucleoplasmic and chromatin-associated RNA in WT and Δ*vhs* infection, GSE129582; RNA-seq of chromatin-associated RNA in WT, Δ*vhs*, vhs

D195N and WT-BAC infection, GSE140068; and H3K4me3 ChIPmentation, GSE132920.

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Figure captions

Figure 1: Experimental set-up and correlation of gene expression changes

(A-B) Experimental set-up of the 4sU-seq and total RNA time-courses (A) and sequencing of subcellular RNA fractions (B) in HSV-1 WT and *Δvhs* infection. The time-course experiments for the two viruses were performed as two independent experiments. Infections for the subcellular RNA fractions were performed within the same experiment. Data for WT infection for both experiments have already been published [20, 21]. (C) Median read-through values (y-axis) are linearly correlated to variance in log2 fold-changes (x-axis) for the 4sU-seq time-courses in WT (cyan) and Δ*vhs* infection (purple). The gray line indicates a linear fit to WT samples. (D-J) Comparison of log2 fold-changes in gene expression (infected vs. mock) between WT infection (x-axis) and Δ*vhs* infection (y-axis) for 4sU-seq RNA from 4-5h p.i. (D), 5-6h p.i. (E) and 7-8h p.i. (F) as well as for total RNA from 2h (G) 4h (H), 6h (I) and 8h p.i. (J). Points are color-coded according to density of points: from red = high density to blue = low density. Spearman rank correlation r_s is shown on the top-left of each panel.

Figure 2: Effects of *vhs* activity and loss of transcriptional activity

(A-B) Comparison of log2 fold-changes in total RNA at 2, 4, 6 and 8h p.i. vs. mock (x-axis), respectively, against RNA half-lives (y-axis) for WT (A) and Δ*vhs* (B) infection. Background indicates density of points: from dark red=high density to cyan=low density. Spearman rank correlation r_s and p-value for significance of correlation is shown on the top of each panel. (C,D) Comparison of log2 fold-changes in total RNA at 8h p.i. vs. 6h p.i. (x-axis) against RNA half-lives (y-axis) for WT (C) and Δ*vhs* (D) infection. Negative correlations for WT infection indicate ongoing *vhs* activity between 6 and 8h p.i. Positive correlations for Δ*vhs* infection are indicative of increasing loss of transcription during this time. (E) Decrease in transcriptional activity relative to

1445 uninfected cells (y-axis) during HSV-1 infection (x-axis=h p.i.) estimated with our
1446 mathematical model from total RNA-seq data in Δ*vhs* infection (see S2 Text). (F)
1447 Development of *vhs* activity over time as estimated with our mathematical model from
1448 total RNA-seq data in WT infection (assuming the same decrease in transcriptional
1449 activity as for Δ*vhs* infection, see S2 Text). x-axis indicates h p.i. and y-axis shows
1450 the rate of cellular mRNA loss per hour (in %) due to *vhs* activity.

1451 **Figure 3: Transcriptional changes in WT and Δ*vhs* infection**

1452 (A) Log2 gene expression (FPKM) ratios for nucleoplasmic vs. cytoplasmic RNA and
1453 chromatin-associated vs. cytoplasmic RNA for three well-described nuclear lincRNAs
1454 (MEG3, MALAT1, NEAT1) and two cytoplasmic lincRNAs (NORAD, VTRNA2-1).
1455 (B) Percentage of intronic reads (= 100 x no. intronic reads / (no. intronic reads + no.
1456 exonic reads)) for cytoplasmic, nucleoplasmic and chromatin-associated RNA in
1457 mock, WT and Δ*vhs* infection shows an enrichment of intronic reads in chromatin-
1458 associated RNA. Parts of this figure for mock and WT infection were also shown in
1459 previous publications [21, 90]. (C) Comparison of log2 fold-changes in chromatin-
1460 associated RNA at 8h p.i. between WT (x-axis) and Δ*vhs* (y-axis) infection. Genes
1461 up- (log2 fold-change ≥ 1, adj. $p \leq 0.001$) or down-regulated (log2 fold-change ≤ -1,
1462 adj. $p \leq 0.001$) in both WT and Δ*vhs* infection are indicated in red and blue,
1463 respectively. Genes transcriptionally down-regulated in a *vhs*-dependent manner
1464 (log2 fold-change ≤ -1, adj. $p \leq 0.001$ in WT; log2 fold-change > -1 in Δ*vhs* infection
1465 as well as > 2-fold difference in regulation) are marked in magenta. (D) Heatmap of
1466 log2 fold-changes in 4sU-RNA, total RNA and subcellular RNA fractions in WT and
1467 Δ*vhs* infection for *vhs*-dependently down-regulated genes (magenta in C). Genes
1468 were clustered according to Euclidean distances and Ward's clustering criterion (see
1469 methods). (E) qRT-PCR measurements for relative transcription of *vhs*-dependently

down-regulated genes COL6A2, MMP1, and MMP3 and control genes ARF4 and CNBP, which exhibit no gene-specific regulation, in Δ*vhs* vs. WT HSV-1-infected cells at 8h p.i. Means from n=2 replicates are plotted with standard deviations as error bars.

Figure 4: V*hs*-dependent transcriptional down-regulation of the ECM and integrin adhesome

(A) *Vhs*-dependently transcriptionally down-regulated genes are significantly enriched for integrin adhesome components identified in six proteomics studies in HFF, MKF (3 studies), A375, MEF and K562 cells [33-38] and the meta-adhesome compiled by Horton *et al*. [33]. Barplot shows log10 of multiple testing corrected p-values from Fisher's exact test. (B) Boxplots showing the distribution of log2 fold-changes in 4sU-RNA, total RNA and subcellular RNA fractions in WT (red) and Δ*vhs* infection (blue) for components of the integrin adhesome identified in HFF [36] (top panel) and all other genes (bottom panel). This shows a clear shift between WT and Δ*vhs* infection for the HFF integrin adhesome but not the remaining genes. (C) Protein-protein associations from the STRING database [39] for the HFF integrin adhesome. Colors indicate the log2 ratio between fold-changes in Δ*vhs* infection and WT infection (see color bar on top). Red indicates less down-regulation or more up-regulation in Δ*vhs* infection than in WT infection and blue the opposite. Yellow borders highlight FN1, the canonical ligand of integrin adhesion complexes, and integrin subunits. The network was visualized with Cytoscape [84]. (D-F) Comparison of log2 fold-changes in chromatin-associated RNA for the repeat experiment of WT and Δ*vhs* infection at 8h p.i. (D), infection with the D195N mutant and its parental BAC-derived virus (WT-BAC) at 8h p.i. (E) as well as D195N and Δ*vhs* infection (F). Colors indicate the regulation in chromatin-associated RNA in our original experiment (see Fig 3C). red =

1496 up-regulated in both WT and Δ*vhs* infection, blue = down-regulated in both, magenta
1497 = down-regulated in a Δ*vhs* dependent manner, black = not regulated. (G) Venn
1498 diagram comparing human orthologues of muSOX-dependent genes identified by
1499 Abernathy *et al.* [18] against *vhs*-dependent genes identified in our study. The
1500 overlap of muSOX-dependent genes to the genes included in our analysis is also
1501 shown. Fisher's exact test was performed on the numbers in the light and dark cyan
1502 and green fields ($p = 4.27 \times 10^{-5}$). (H) Vulcano plot showing log2 fold-changes in
1503 total RNA in HSV-1 infection at 12h p.i. and multiple testing adjusted p-values from
1504 the study of Pheasant *et al.* [19]. The original and extended set of *vhs*-dependently
1505 transcriptionally down-regulated genes from our study are marked in magenta and
1506 violet, respectively. Genes defined as efficiently depleted by *vhs* by Pheasant *et al.*
1507 (log2 fold-change < -5) are left of the red vertical line. *Vhs*-dependent genes are
1508 among the most significantly down-regulated genes. Gene symbols are shown for the
1509 four genes with lowest adjusted p-values.
1510
1511 **Figure 5: *vhs*-independent transcriptional up-regulation of lowly expressed**
1512 **genes**
1513 (A) Heatmap of log2 fold-changes in 4sU-RNA, total RNA and subcellular RNA
1514 fractions for genes up-regulated in both WT and Δ*vhs* infection (red in Fig 3C).
1515 Genes were clustered according to Euclidean distances and Ward's clustering
1516 criterion (see methods). Four clusters were obtained at a distance threshold of 30
1517 and are indicated by colored bars (orange, blue, green, red). (B) Boxplots of the
1518 distribution of expression values (FPKM) in uninfected cells from 4sU-RNA, total RNA
1519 and subcellular RNA fractions show low or no expression of strongly up-regulated
1520 genes (orange cluster) in uninfected cells compared to other up-regulated clusters
1521 (blue, green, red) and remaining genes. (C-D) Strongly up-regulated genes with low

1522 expression in uninfected cells, such as DLL1 (C, negative strand) and GADD45G (D,
1523 positive strand), are already primed for up-regulation by H3K4me3 marks at their
1524 promoters. Tracks show read coverage (normalized to total number of mapped
1525 human reads; averaged between replicates) in uninfected and WT 4sU-RNA for
1526 selected time points (gray and cyan, top 3 tracks) and H3K4me3 ChIPmentation in
1527 uninfected cells and at 8h p.i. WT infection (green, bottom 2 tracks). Peaks identified
1528 in each replicate are shown separately below H3K4me3 read coverage tracks. Gene
1529 annotation is indicated on top. Boxes represent exons and lines introns. Genomic
1530 coordinates are shown on the bottom. For 4sU-seq data only read coverage on the
1531 same strand as the gene is shown (+ = positive strand, - = negative strand).
1532 H3K4me3 ChIPmentation is not strand-specific. (E) Barplots showing the fraction of
1533 transcriptionally regulated genes in HSV-1 infection that are either up- (red) or down-
1534 (blue) regulated by doxycycline-inducible DUX4 [53]. Results are shown separately
1535 for genes up-regulated in both WT and Δ*vhs* infection, the four clusters of up-
1536 regulated genes (indicated colors refer to the cluster colors in Fig 5A), genes down-
1537 regulated in both WT and Δ*vhs* infection as well as genes down-regulated in a *vhs*-
1538 dependent manner in WT infection. Horizontal dashed lines indicate the fraction of all
1539 analyzed genes regulated by DUX4. Numbers on top of bars indicate p-values
1540 (corrected for multiple testing) for a Fisher's exact test comparing the fraction of
1541 DUX4 up- or down-regulated genes between each group of HSV-1 regulated genes
1542 to the background of all genes (black: adj. $p \leq 0.001$, gray: not significant).
1543
1544 **Figure 6: Impact of HSV-1 infection on protein levels**
1545 (A) Comparison of log2 fold-changes in protein levels at 8h p.i. between WT (x-axis)
1546 and Δ*vhs* (y-axis) infection. Up- or down-regulated proteins (≥ 1.5-fold change, adj.
1547 $p \leq 0.001$) in both WT and Δ*vhs* infection are indicated in red and blue, respectively.

Proteins down-regulated in a *vhs*-dependent manner (≥ 1.5-fold down-regulated, adj. $p \leq 0.001$ in WT; less than 1.5-fold down-regulated in Δ*vhs* infection as well as >1.5-fold difference in regulation) are marked in magenta. Green indicates proteins that are up-regulated in Δ*vhs* infection but not in WT infection with a >1.5-fold difference in fold-changes. (B) Boxplots of log2 fold-changes of normalized protein iBAQ intensities from Kulej *et al.* [55] for HSV-1 infection vs. mock for proteins that are either significantly up- (red in A) or down-regulated (blue in A) in our study and significantly regulated between any pair of time points in the Kulej *et al.* time-course (ANOVA $p < 0.001$). P-values for Wilcoxon rank sum test comparing log2 fold-changes for the respective time points from the Kulej *et al.* study between our up- and down-regulated proteins are indicated on top. (C) Comparison of protein log2 fold-changes for *vhs*-dependently transcriptionally regulated genes (marked magenta and violet as in 4H) and other genes (color-coded according to density of points: from red = high density to blue = low density). Gene symbols are shown for genes with a ≥ 2-fold increase in protein fold-changes in Δ*vhs* infection compared to WT infection.