1 **Redefining De Novo Gammaherpesvirus Infection Through High-Dimensional, Single-**

2 **Cell Analysis of Virus and Host**

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28 **SUMMARY**

29

30 Virus infection is frequently characterized using bulk cell populations. How these findings

31 correspond to infection in individual cells remains unclear. Here, we integrate high-dimensional

32 single-cell approaches to quantify viral and host RNA and protein expression signatures using

33 de novo infection with a well-characterized model gammaherpesvirus. While infected cells

34 demonstrated genome-wide transcription, individual cells revealed pronounced variation in

35 gene expression, with only 9 of 80 annotated viral open reading frames uniformly expressed in

36 all cells, and a 1000-fold variation in viral RNA expression between cells. Single-cell analysis

37 further revealed positive and negative gene correlations, many uniquely present in a subset of

38 cells. Beyond variation in viral gene expression, individual cells demonstrated a pronounced,

39 dichotomous signature in host gene expression, revealed by measuring host RNA abundance

40 and post-translational protein modifications. These studies provide a resource for the high-

41 dimensional analysis of virus infection, and a conceptual framework to define virus infection as

42 the sum of virus and host responses at the single-cell level.

43

44 **HIGHLIGHTS**

45 • CyTOF and scRNA-seq identify wide variation in gene expression between infected cells.

46 • Host RNA expression and post-translational modifications stratify virus infection.

47 • Single cell RNA analysis reveals new relationships in viral gene expression.

48 • Simultaneous measurement of virus and host defines distinct infection states.

49 **INTRODUCTION**

50

51   Gammaherpesviruses (γHVs) are a subfamily of the *Herpesviridae* characterized by their

52 ability to establish latency in lymphoid cells and their association with numerous malignancies

53 (Barton et al., 2011; Cesarman, 2014; Zamora, 2011). The γHVs include the human viruses

54 Kaposi's sarcoma-associated herpesvirus (KSHV, or human herpesvirus 8) and Epstein-Barr

55 virus (EBV), and murine gammaherpesvirus 68 (MHV68 or γHV68; ICTV nomenclature, *murid*

56 *herpesvirus 4*, MuHV-4), a model system for its human virus counterparts (Barton et al., 2011;

57 Dong et al., 2017; Virgin et al., 1997). MHV68 provides a genetically tractable system, capable

58 of inducing a range of outcomes *in vitro* and *in vivo* (Barton et al., 2011; Forrest and Speck,

59 2008; Suarez and van Dyk, 2008).

60   Like other herpesviruses, the MHV68 lifecycle is characterized by two major stages: latent

61 and lytic infection (Pellett and Roizman, 2013). During latent infection, viral gene expression is

62 limited, with no *de novo* viral replication (Pellett and Roizman, 2013). While latent γHV infection

63 is associated with lifelong infection and malignancies, the γHVs critically rely on lytic infection

64 for virus replication and transmission between hosts. Lytic infection is characterized by robust

65 viral gene expression and the production of new infectious virions. The conventional view of

66 lytic infection is that of a synchronous cascade of gene expression that results in genome-wide

67 transcription. The paradigm for lytic gene expression was established in bulk cell populations,

68 stratifying viral gene expression into three broad categories: immediate-early (IE), early (E),

69 and late (L) gene expression (Johnson et al., 2010; Pellett and Roizman, 2013; Rochford et al.,

70 2001). IE genes are expressed in the absence of new viral protein synthesis, E genes require

71 new viral protein synthesis but do not require new viral DNA synthesis, and L genes require

72 new viral DNA synthesis (Ahn et al., 2002; Ebrahimi et al., 2003; Johnson et al., 2010;

73 Martinez-Guzman et al., 2003; Pellett and Roizman, 2013). Lytic gene expression can also be

74 stratified by kinetic class, affording a revised perspective on viral gene expression (Cheng et

75 al., 2012).

76   The advent of high-throughput, single-cell based methods has revealed a more complex

77 picture of herpesvirus infection, from latent infection (Messinger et al., 2019; Oko et al., 2019;

78 Shnayder et al., 2018), to lytic infection (Drayman et al., 2019; Oko et al., 2019; Wyler et al.,

79 2019), reactivation from latency (Adang et al., 2006; Oko et al., 2019) and viral tropism (Sen et

80 al., 2014). Recently, we identified unanticipated heterogeneity between infected cells during

81 lytic infection, with wide variance in viral gene expression, from cells with robust viral gene

82 expression to cells with little to no detectable expression of either viral non-coding RNAs or

83 mRNAs (Oko et al., 2019). These studies suggest that lytic infection may be more

84 heterogeneous in individual cells than the perspective revealed by analysis of bulk populations.

85   Though stages of infection are defined based on viral gene expression, lytic infection is

86 also associated with profound alterations in host gene expression. First, many herpesviruses,

87 including KSHV, EBV, MHV68, varicella zoster virus (VZV), and Herpes simplex virus (HSV),

88 induce widespread degradation of host RNAs (i.e. host shutoff) during lytic infection, a

89 phenomenon associated with decreased expression of cellular housekeeping genes in either

90 bulk cell populations (Glaunsinger, 2015; Smiley, 2004; Waterboer et al., 2002) or individual

91 cells (Oko et al., 2019). Viruses have also evolved diverse mechanisms to manipulate host

92 processes (e.g. cell cycle, apoptosis, protein synthesis and signal transduction) (Chang et al.,

93 2016). For example, the ORF36 gene of MHV68 and EBV encodes a viral kinase that

94 phosphorylates H2AX, a proximal marker of the DNA damage response, to facilitate viral

95 replication (Tarakanova et al., 2007). Recent studies have further demonstrated that the EBV

96 and KSHV ORF36 homologs target additional host proteins including SAMHD1 and S6K to

97 impact virus restriction and protein synthesis, respectively (Bhatt et al., 2016; Zhang et al.,

98 2019). These studies emphasize that measuring host processes commonly targeted by

99 multiple viruses [including host shutoff (Glaunsinger, 2015), DNA damage responses

100   (Weitzman and Fradet-Turcotte, 2018) and innate signaling (Lee et al., 2015)], may provide an

101   important, complementary strategy to define viral infection.

102   Here we provide a resource to redefine lytic infection from a single-cell perspective, using

103   cytometry by time-of-flight (CyTOF, or mass cytometry) and single cell RNA-seq to achieve

104   high-dimensional, single-cell analysis of viral and host gene expression. These studies reveal:

105   i) the highly variable nature of viral gene expression between individual cells, with only a small

106   subset of genes expressed across all cells, ii) the utility of measuring host parameters as a

107   discriminator to identify infection state, and iii) new insights into infection and gene expression

108   relationships, previously obscured by bulk cell analysis.

109   **RESULTS**

110

111   *CyTOF analysis identifies a distinct protein expression profile for virally-infected cells.*

112   Recently, we discovered significant variability in viral gene expression between cells

113   during MHV68 infection (Oko et al., 2019). These studies identified a prominent fraction of cells

114   with limited viral gene expression, a phenomenon that could arise due to asynchronous

115   infection, infection with restricted viral gene expression (e.g. latency), or cells resistant to

116   infection. To define the complexity of virus infection in productively infected cells, we

117   established a system to purify cells based on expression of the MHV68 latency-associated

118   nuclear antigen (LANA) protein, a viral gene product expressed during lytic and latent infection

119   (Rochford et al., 2001). NIH 3T12 fibroblasts, a highly permissive cell line for MHV68 lytic

120   infection, were infected with a wild-type MHV68 recombinant expressing a LANA::β-lactamase

121   gene fusion (WT MHV68.LANAβlac), followed by FACS purification (Diebel et al., 2015; Nealy

122   et al., 2010) and high-dimensional single-cell analysis using CyTOF and single cell RNA-

123   sequencing (scRNA-seq).

124   First, we analyzed virally-infected cells by CyTOF, to obtain a high-dimensional single-

125   cell analysis of protein expression (Kimball et al., 2018). NIH 3T12 fibroblasts were harvested

126   at 16 hours post-infection (hpi), a time at which no cytopathic effect or new progeny virus is

127   released, and sort purified into LANAβlac+ (LANA+) and LANAβlac- (LANA-) fractions (Figure

128   S1A). On average, >94% of cells were LANA+ at this time, indicating active virus infection in

129   the vast majority of cells (Niemeyer et al., 2018). Mock-infected 3T12 cells were purified in

130   parallel. To ensure robust cross-comparison between these populations, each sample was

131   subjected to isotopic palladium-based barcoding, pooled together, stained with a panel of 23

132   isotopically-labelled antibodies, with samples collected on a Helios mass cytometer (Figure

133   1A). Mock-infected, LANA+ and LANA- cells had pronounced differences in clustering and

134   protein expression profiles (Figure S1B-D). LANA+ cells robustly expressed the virally-

135   encoded regulator of complement activation (vRCA) protein (Kapadia et al., 1999) and

136   phosphorylated histone H2AX (pH2AX), a target of the ORF36 viral kinase (Tarakanova et al.,

137   2007). LANA+ cells further showed increased expression of cell surface proteins (CD9, MHC I)

138   and cell cycle proteins (Ki-67, phosphorylated Rb [Serine 807/S811]), with modest induction of

139   signal transduction pathways (activated beta-catenin, pERK1/2, p-p38, pStat1, pStat3, pAKT

140   and pS6) compared to both LANA- and mock-infected populations (Figure S1C-D). These data

141   indicate that at the population level, LANA+ cells have a distinct protein expression signature

142   relative to mock-infected and LANA- cells.

143   Next we quantified the diversity of cellular phenotypes between LANA+ and LANA-

144   cells. When we applied PhenoGraph, an unsupervised clustering algorithm (Levine et al.,

145   2015), we identified 14 phenotypes across LANA+, LANA-, and mock-infected populations

146   (Figure 1B). The frequency of phenotypic clusters varied widely between LANA+ and LANA-

147   populations (Figure 1C), with 3 clusters exclusively present among LANA+ cells, 5 clusters

148   uniquely present among LANA- cells, and 6 clusters containing both LANA+ and LANA- cells

149   (Figure 1C). Clusters exclusively present among LANA+ cells (clusters 1-3, at the bottom of

150   Figure 1C) were characterized by maximal expression of vRCA and pH2AX, and increased

151   expression of pRB, pERK1/2, pAKT, and pS6 relative to other cell clusters (Figure 1C).

152   Expression of other parameters, including CD9, CD29, CD44, CD63, Ki-67 and IkBα did not

153   strictly correlate with either LANA+ or LANA- cells, indicating that these proteins were not

154   solely regulated as a function of virus infection (Figure 1C). While high expression of vRCA

155   and pH2AX was a strong discriminator between clusters exclusively in LANA+ versus LANA-

156   events, approximately half of LANA+ events expressed low levels of either vRCA or pH2AX,

157   overlapping with LANA- cells (Figure 1C-D). These data indicate that while many cells

158   expressing the LANAβlac fusion protein had increased virus and host protein expression, there

159   was a substantial fraction of LANA+ cells that could not be readily distinguished from LANA-

160   cells (clusters colored purple, Figure 1D). These data further identify phosphorylation of H2AX

161   (i.e. pH2AX) as a robust marker of virus-induced phenotypic changes at the single-cell level.

162

163   *LANA+ cells show heterogeneous protein and post-translational modifications during lytic*

164   *infection.*

165   To better understand phenotypic heterogeneity among cells with active viral gene

166   expression (i.e. LANA+ cells), we quantified phenotypic diversity among a large number of

167   LANA+ cells, clustering cells based on expression of nine cell surface-expressed proteins

168   (BST2, CD9, CD29, CD44, CD63, Ly6A/E, Ly6C, MHCI, and vRCA). This focused clustering

169   analysis identified 15 phenotypic clusters within LANA+ cells, which were then visually

170   inspected based on vRCA, pH2AX, Ly6C, and BST2 expression (Figure 2A-C). LANA+ cells

171   could be subdivided into cells with high and low viral gene expression, with vRCA and pH2AX

172   expression tightly overlapping. In contrast, Ly6C and BST2 protein expression was variable

173   across vRCA^{high} pH2AX^{high} and vRCA^{low} pH2AX^{low} cell fractions, indicating that these proteins

174   did not simply correlate with virus infection (Figure 2B-C). Stratification of LANA+ cells into

175   pH2AX^{high} and pH2AX^{low} subsets (Figure S2A) demonstrated that pH2AX^{high} cells had

176   increased expression of vRCA, MHC class I, and multiple phosphorylation-dependent

177   modifications including pRb, active β-catenin, pERK1/2, pStat1 and pAKT compared to

178   pH2AX^{low} cells (Figure 2D). pH2AX^{high} cells also had modestly decreased expression of CD9,

179   CD29, Ly6A/E, Ly6C and IkBα relative to pH2AX^{low} events (Figure 2D). Consistent with the

180   tight correlation between vRCA and pH2AX expression, LANA+ vRCA^{high} cells had increased

181   expression of pH2AX, MHC class I, pRb, active β-catenin, pERK1/2, pStat1 and pAKT

182   compared to vRCA^{low} cells and modestly reduced IkBa expression (Figure S2B-E). These

183   studies indicate that vRCA and pH2AX are strong markers of progressive infection among

184   LANA+ events, with phosphorylated Rb a robust secondary marker associated with a

185   pH2AX^{high} vRCA^{high} phenotype. These results further indicate that heterogeneous progression

186   through infection can be revealed by monitoring host protein expression at the single-cell level.

187

188   *MHV68-infected cells with a pH2AX^{high} vRCA^{high} cell phenotype express high viral mRNA and*

189   *low host actin RNA.*

190   We next investigated how pH2AX and vRCA expression corresponded with viral and

191   host RNA expression at the single-cell level, using the PrimeFlow methodology to

192   simultaneously measure protein and RNAs (Oko et al., 2019). This analysis focused on two

193   RNAs which we previously reported showed heterogeneous expression during MHV68

194   infection (Oko et al., 2019): i) ORF18, a viral RNA transcribed with late kinetics and ii) Actb, a

195   host RNA encoding beta-actin that is known to be targeted for degradation by the MHV68 SOX

196   endonuclease (Covarrubias et al., 2009).

197   As expected, mock-infected cells had no vRCA expression and a low frequency of

198   pH2AX+ events (Figure 3A). In contrast, MHV68-infected 3T12 cultures exhibited multiple

199   populations, including: 1) pH2AX- vRCA- cells (population 1, gray box, Figure 3A bottom

200   panel), 2) pH2AX+ vRCA- cells (population 2, light blue box), 3) pH2AX- vRCA+ cells

201   (population 3, orange box), and 4) pH2AX+ vRCA+ cells (population 4, red box). As these

202   studies did not use purified LANA+ cells, in contrast to the CyTOF studies, the presence of

203   these four populations suggests that these cultures contained a mixture of infected and

204   uninfected cells.

205   We next queried RNA expression within cells subdivided by pH2AX and vRCA

206   expression, using ORF18 expression as a direct readout of virus infection and Actb

207   degradation as a secondary readout of virus infection. Cells with a pH2AX- vRCA- phenotype

208   (gray box, Figure 3A-B) were present in mock- and MHV68-infected cultures, with the vast

209   majority of cells characterized by an ORF18- Actb+ phenotype and no evidence of infection

210   (Figure 3B-C). In contrast, cells with a pH2AX+ vRCA- phenotype (light blue box, Figure 3A-B)

211 contained ~1/3 of cells that were ORF18+ with variable expression of Actb (Figure 3B-C),

212 identifying infection based on viral RNA expression in the absence of a viral protein. Cells with

213 a pH2AX- vRCA+ phenotype (orange box, Figure 3A-B) were dominated by ORF18+ Actb- and

214 ORF18- Actb- cells, demonstrating direct measures of infection by RNA and protein. Lastly,

215 pH2AX+ vRCA+ cells (red box, Figure 3A-B) were predominantly ORF18+ Actb-, identifying

216 viral RNA and viral protein expression in the context of host RNA degradation. These data

217 demonstrate that pH2AX+ vRCA+ cells show progressive infection at the RNA and protein

218 level, whereas cells expressing only vRCA or pH2AX represent intermediate infection

219 phenotypes as measured by viral RNA expression and host RNA degradation.

220

221 *scRNA-seq reveals wide variation in the frequency and magnitude of viral RNA expression*

222 We next sought to obtain a global perspective on how MHV68 infection affects gene

223 expression using scRNA-seq. 3T12 fibroblasts were infected with an MOI of 0.5 PFU/cell using

224 two viruses with indistinguishable lytic replication [WT MHV68.LANAβlac and a viral cyclin-

225 deficient (cycKO) MHV68.LANAβlac (van Dyk et al., 2000)], with LANA+ cells sort purified at

226 16 hpi (Figure 1A). LANA+ cells were submitted for bulk RNA-seq and scRNA-seq analysis,

227 with a mean of 94,810 reads per cell for scRNA-seq (Figure S3A-C). While there was some

228 concordance between the most abundant host RNAs observed in bulk RNA-seq and scRNA-

229 seq, scRNA-seq analysis revealed wide variability among cells, a level of complexity obscured

230 in bulk RNA-seq analysis (Figure S3D). We next focused on scRNA-seq, given its capacity to

231 define intercellular variation and to avoid averaging of signal intensity across a population of

232 cells. Comparison of scRNA-seq data between WT and CycKO infected cells demonstrated a

233 similar distribution of cells and gene expression, with only five genes with average gene

234 expression differences greater than 2-fold (Table S1 and S2), consistent with their comparable

235 lytic replication in vitro (van Dyk et al., 2000). Therefore, subsequent analysis integrated

236 scRNA-seq data from LANA+ cells purified from WT and CycKO infection.

237   To understand MHV68 transcription at the single-cell level, we first quantified the

238   diversity and magnitude of viral transcripts. When viral RNAs (enumerated as unique

239   molecular identifiers, UMIs) were mapped to the MHV68 genome, 77 of the 80 annotated

240   MHV68 ORFs were detected. M10b, M10c and M12, three viral RNAs derived from MHV68

241   repeat structures were the only viral RNAs not detected (Virgin et al., 1997). LANA+ cells

242   expressed a wide range of viral genes (12-66 viral genes) per cell, with a median of 52 viral

243   genes per cell, based on detection of ≥ 1 UMI per viral gene per cell (Figure 4A). Mean UMI

244   per viral gene and percentage of cells expressing viral genes were positively correlated

245   (Spearman r coefficient = 0.9775), with 86.2% of LANA+ cells expressing the ORF73 RNA

246   (Figure 4B). Despite cell purification based on uniform LANA expression, total viral UMIs per

247   cell spanned a >1000-fold range (19-24,718 viral UMIs per cell) (Figure 4C). While the number

248   of viral genes detected per cell was positively correlated with total viral UMIs per cell, LANA+

249   cells exhibited a bimodal distribution of virus RNA^{high} (Virus^{high}) cells (>500 viral UMIs/cell) and

250   virus RNA^{low} (Virus^{low}) cells (<500 viral UMIs/cell) (Figure 4C-D). The distinction between

251   Virus^{high} and Virus^{low} cells was not simply explained by how many viral genes were detected

252   per cell, as some cells expressed the same number of viral genes yet showed a >10-fold

253   variance in total viral UMIs (e.g. the range of viral UMIs among cells that express 40 viral

254   genes per cell, Figure 4D).

255   To better understand viral transcription at the single-cell level, we quantified the

256   percentage of cells expressing each viral gene (Figure 4E) and mean UMI count within positive

257   cells (Figure 4F). The percentage of cells which expressed viral genes varied widely, with 9

258   viral genes (M3, ORF7, ORF17, ORF38, ORF45, ORF52, ORF58, M9, and ORF66) expressed

259   in the vast majority (98.63-100%) of cells (Figure 4E). These ubiquitous viral RNAs spanned

260   transcriptional categories [IE (ORF38), Early-Late (M3, ORF7), and L genes (ORF17, ORF45,

261   ORF52, ORF58, M9, ORF66)] and kinetic classes [class I (ORF38), class II (M3, ORF45,

262   ORF52, ORF58), class III (ORF17, M9, ORF66) and class IV (ORF7)] (Cheng et al., 2012). In

263   contrast, many viral genes were only detected in a subset of cells, including ORF50, an IE

264   gene that encodes Rta, a major transcriptional regulator of lytic replication (Figure 4E). The

265   range of expression for individual genes varied widely (Figure 4F). While many viral genes had

266   a mean UMI per viral gene between 1-10 UMIs per cell, the most highly expressed viral gene

267   ORF52 had a mean of >1000 UMIs per cell.

268

269   *Visualization of genome-wide viral gene expression at the single-cell level*

270   To investigate the complexity of gene expression across MHV68-infected cells, we next

271   visualized the absolute UMI count for each viral gene across all 1,605 LANA+ cells. Cells were

272   rank-ordered based on total viral UMIs detected per cell, from Virus^{low} to Virus^{high} cells across a

273   1000-fold range of viral UMIs per cell, with each row depicting expression for each MHV68

274   viral gene and each column depicting data from a single cell (Figure 5). This continuum of

275   gene expression revealed multiple patterns of gene expression. First, there were some viral

276   genes (e.g. ORF59 and ORF73βlac, which encodes LANA) which were detected in almost all

277   cells, with only modest differences in expression between cells with the fewest viral UMIs and

278   the greatest viral UMIs (Figure 5). Second, viral genes such as M3 and ORF52 progressively

279   increased in expression, proportional to the increase in viral UMIs (Figure 5). Third, viral

280   genes, including ORF48, ORF54, and ORF57, showed maximal expression in cells with

281   intermediate viral counts (Figure 5). By stratifying cells into either Virus^{high} or Virus^{low}

282   categories (a bifurcation identified in Figure 4C-D), we identified a subset of genes including

283   K3, ORF42 and ORF44 whose expression was sporadic in Virus^{low} cells and uniform in

284   Virus^{high} cells (Figure 5 and Figure S4). ORF68 was one of the only genes whose expression

285   was slightly higher among Virus^{low} cells compared to Virus^{high} cells (Figure 5 and Figure S4).

286   Gene expression signatures for Virus^{low} and Virus^{high} cells did not clearly track with any

287   transcriptional or kinetic class, and individual genes within these distinctions demonstrated

288   significant gene to gene variation (Figure S5A). These data identify the highly variable nature

289   of MHV68 transcription between individual cells during de novo infection.

290

291   *MHV68-infected cells can be stratified by differential abundance of virus and host RNAs*

292   To clarify the patterns of gene expression that we observed, we used unsupervised K-

293   means clustering to identify related cells. By clustering on viral and cellular RNA expression,

294   we identified 5 clusters among LANA+ cells (Figure 6A). 3-dimensional tSNE-based data

295   visualization further identified that an IE gene (ORF50) had maximal expression in clusters A

296   and B, an early-late (E-L) gene (ORF47) had maximal expression in clusters B and C, and a L

297   gene (M7) had maximal expression in clusters D and E (Figure 6B and Figure S5B). In

298   contrast, host Actb RNA, a target of virus-induced host shutoff mediated by the ORF37 gene,

299   showed maximal expression in clusters A and B, with low expression across clusters C

300   through E (Figure 6B-C). These data suggested that at this timepoint, LANA+ cells had

301   heterogeneous virus-induced host shutoff. Cells with a high percentage of viral UMIs were

302   predominant in clusters C through E, in contrast to cells with a high frequency of host UMIs

303   prevalent in clusters A and B (Figure 6D). Based on these distinctions, we divided cells into

304   virus-based and host-biased groups (Figure 6E-F).

305   The different proportions of viral and host UMIs between virus- and host-biased cells

306   could result from changes in either how many viral or host genes were expressed, or mean

307   expression per gene. Notably, the number of virus genes expressed (defined by mean UMI per

308   cell ≥ 1) was comparable between virus-biased and host-biased cells (Figure 6G). In contrast,

309   virus-biased cells were characterized by a pronounced decrease in the number of host RNAs

310   expressed, compared to host-biased cells (Figure 6H). When we analyzed gene expression

311   levels, virus-biased cells had increased viral gene expression per cell compared to host-biased

312   cells, with no significant difference in host gene expression among expressed genes (Figure

313   6I). These data indicate that virus-biased cells have increased viral gene expression, coupled

314   with a pronounced reduction in the number of detectable host RNAs. While cell cycle-

315   associated gene signatures were relatively comparable between virus-biased and host-biased

316   cells, host-biased cells expressed a number of interferon-response genes (Figure S6 and

317   Table S3), suggesting a potential role in resistance to infection.

318   Although stages of virus infection are typically defined by viral parameters only, the

319   observations of: i) Virus^{high} and Virus^{low} cells (Figure 4D), and ii) Virus-biased and host-biased

320   cells (Figure 6G), suggested that these two processes may be connected. Indeed, when we

321   analyzed the distribution of cells based on the sum of all host UMIs versus the sum of all viral

322   UMIs, we found three prominent populations (Figure 6J-K): 1) 72.8% of cells had high viral

323   RNA expression with reduced host RNA expression (i.e. Virus^{high} Host^{low}, in red, Figure 6K), 2)

324   11.1% of cells had a Virus^{high} Host^{high} phenotype (in orange, Figure 6K), and 3) 14.4% of cells

325   had a Virus^{low} Host^{high} phenotype (in blue, Figure 6K). The number of Virus^{low} Host^{high} cells

326   almost completely corresponded with the number of Virus^{low} events which we previously

327   identified (Figure 4D), with less than two percent of cells expressing a Virus^{low} Host^{low}

328   phenotype (Figure 6J). By comparing viral gene expression across these 3 populations, we

329   found that an IE gene (ORF50) and E-L gene (ORF47) had peak expression in Virus^{high}

330   Host^{high} cells, intermediate expression in Virus^{high} Host^{low} cells, with limited expression in

331   Virus^{low} Host^{high} cells (Figure 6L-M). In contrast, a L gene (M7) had high expression exclusively

332   in Virus^{high} Host^{low} cells (Figure 6N). Virus^{low} Host^{high} cells had lower expression across many

333   virus genes, with the exception of ORF68 which was modestly higher in Virus^{low} Host^{high} cells

334   compared to other cells (Figure 6O). Actb, a known target of virus-induced shutoff, retained

335   high expression in both Virus^{low} Host^{high} and Virus^{high} Host^{high} cells with a pronounced decrease

336   in Virus^{high} Host^{low} cells (Figure 6P). These data demonstrate that while many LANA+ cells

337   show signs of robust viral RNA expression coupled with reduced host RNAs, subsets of

338   LANA+ cells express high levels of host RNAs with differential viral RNA abundance.

339

340 *Correlation analysis reveals positive and negative correlations in MHV68 transcription at the*

341 *single-cell level.*

342   Finally, we sought to investigate the inter-relationships between viral genes, leveraging

343 the unique insights obtained from single-cell data. Initially, we generated a correlation matrix of

344 all viral gene-gene expression relationships querying all LANA+ cells. This correlation matrix

345 identified that the majority of gene-gene pairs had a positive correlation (83.6%, depicted in

346 blue), with fewer negative correlations (16.1%, depicted in red) (Figure S7). We next examined

347 gene-gene relationships in Virus^{high} or Virus^{low} cells. While the majority of gene-gene

348 relationships in Virus^{high} and Virus^{low} cells were positive correlations, Virus^{high} cells revealed an

349 increased frequency of negative correlations (29.1%) among a subset of viral genes (Figure 7A

350 and S7).

351   We next sought to investigate gene-gene relationships as of function of Virus^{high} or

352 Virus^{low} cells. On the one hand, there were some gene pairs whose relationship was preserved

353 between Virus^{high} and Virus^{low} cells, exemplified by ORF50 and ORF57, and ORF54 and

354 ORF57, both showing positive correlations across Virus^{high} and Virus^{low} cells (Figure 7B-C). On

355 the other hand, there were other gene pairs whose relationship was distinct between Virus^{high}

356 and Virus^{low} cells. These included ORF50 and M9, and ORF54 and ORF64, with minimal

357 correlation across total cells, a negative correlation in Virus^{high} cells, and minimal correlation

358 among Virus^{low} cells (Figure 7B). The complexity of gene-gene expression relationships was

359 clearly demonstrated by plotting gene-by-gene expression levels from total cells (Figure 7C-D).

360 Predicted correlations were exemplified by a positive correlation between host RNAs Actb and

361 Gapdh and a negative correlation between Actb and ORF64 (Figure 7C-D). While positive

362 correlations between ORF50 and ORF57, and ORF57 and ORF54 were consistent across all

363 events, the relationships between ORF50 and M9, ORF64 and ORF54 were more complex

364 (Figure 7D). These data emphasize that gene-gene coexpression relationships can be

365   obscured by combining cell subsets, and emphasize the value of single-cell approaches to

366   define gene expression inter-relationships within homogeneous cell populations.

367 **DISCUSSION**

368

369   In this manuscript, we present a strategy to investigate viral infection, simultaneously

370 quantifying viral and host RNA and protein expression in individual cells. This approach

371 provides a conceptual framework to understand how individual cells respond to virus infection

372 at the protein and RNA level, presents new insights into the complexity of virus infection from

373 virus and host perspective, and establishes high-dimensional datasets as a resource for further

374 analysis of virus-host interactions at the single-cell level. By focusing on a well-characterized

375 virus, tightly controlled infection conditions, and cells with uniform viral gene expression, these

376 studies emphasize the knowable complexity of infection in individual cells and the limitations of

377 studying infection in bulk cell populations.

378

379 *Implications*

380   Our studies illustrate the power of defining virus infection by quantifying viral and host

381 gene expression across individual cells and have significant implications for understanding

382 virus-host interactions. First, our data highlight that gene expression analysis in a population of

383 cells conceals wide variance in virus infection, specifically failing to identify cells with low gene

384 expression and obscuring negative feedback mechanisms. Second, our data emphasize that

385 defining virus infection strictly by viral gene expression ignores the essential contribution of the

386 host cell to this process, and that virus infection stage may be more appropriately considered

387 as the integrated impact of virus on the host. Quantitation of host RNAs and virus-dependent

388 post-translational host modifications were particularly informative in discriminating between

389 infection subtypes, while measures of protein abundance were less impactful. Third, our data

390 suggest that phenotypic variation between cells, or input virions, results in cell subsets with

391 restricted viral gene expression, a potentially novel strategy to identify negative regulators of

392 infection. While the relative contribution of host and virus variation to these diverse outcomes

393 remains to be addressed, there is an increasing breadth of literature in support of phenotypic

394 variation from both sides (e.g. (Avraham et al., 2015; Chaturvedi et al., 2020; Hansen et al.,

395 2018; Shaffer et al., 2017)). We propose that integrated single-cell analysis of virus and host

396 will be particularly informative to understand how viral and host genes regulate infection,

397 whether influencing viral or host gene expression, and/or altering the relative proportion of

398 permissive or resistant cells. Fourth, these studies provide an opportunity to reassess

399 fundamental concepts in virology, including how variation in virus (e.g. differing ratios in

400 particle:PFU ratio (Van Skike et al., 2018)) or the host cell integrate to impact infection

401 outcome. We anticipate that further studies, combined with further mining of these datasets by

402 the community, will identify new rules governing virus infection that may be pharmacologically

403 targeted.

404

405 *Insights into herpesvirus infection*

406   Herpesvirus infection is classically divided into lytic infection, that generates new

407 infectious virions, and latent infection, that facilitates long-term persistence in host cells while

408 avoiding immune detection. Lytic infection is frequently understood as a coordinated cascade

409 of gene expression, characterized by genome-wide transcription (Pellett and Roizman, 2013).

410 High resolution mapping of coding and non-coding viral RNAs (Cheng et al., 2012; O'Grady et

411 al., 2019) and new molecular insights into the regulation of viral RNA transcription and

412 abundance (Hartenian et al., 2020) have further refined models of lytic gene expression.

413 Despite these advances, most studies have analyzed bulk cell populations, obscuring

414 intercellular heterogeneity.

415   Here, we provide three new datasets, with two complementary high-dimensional single-

416 cell approaches, CyTOF and scRNAseq, to investigate *de novo* γHV infection of cells that are

417 highly permissive for lytic infection. These studies build on our recent analysis of MHV68 gene

418 expression during *de novo* infection, studies that revealed asynchronous or inefficient infection

419   among MHV68-exposed cells (Oko et al., 2019). Here, we extended these studies, purifying

420   actively-infected cells (defined by expression of the viral protein LANA, which is expressed

421   during lytic and latent infection) and deeply characterizing RNA and protein expression profiles

422   during *de novo* infection. By monitoring viral and host RNAs and proteins, these studies

423   defined robust markers of productive lytic infection and revealed heterogeneity in progression

424   following *de novo* infection.

425   These studies initially focused on how MHV68 infection influences cellular responses at

426   the protein level using CyTOF. By comparing cells with or without signs of active infection, we

427   found multiple infection phenotypes. First, LANA+ cells contained a prominent cell fraction that

428   co-expressed high levels of a viral protein, vRCA, and phosphorylation of histone H2AX, a

429   virus-dependent event. vRCA^{high} pH2AX^{high} cells were only found in LANA+ cells, and were

430   further characterized by increased phosphorylation of host proteins (pRb, active β-catenin,

431   pERK1/2, pStat1 and pAKT) consistent with reports of γHV-induced modulation of the host

432   proteome (Chang et al., 2016). vRCA^{high} pH2AX^{high} cells also had a prominent RNA phenotype,

433   ORF18^{high} (late viral RNA) and Actb^{low} (host RNA indicator of virus-induced host shutoff). While

434   vRCA^{high} pH2AX^{high} ORF18 RNA^{high} Actb RNA^{low} cells showed the most progressive signs of

435   infection, we readily identified additional cell subsets expressing viral RNA in the absence of

436   viral protein or host-shutoff, potentially reflecting delayed infection or a disconnect between

437   transcription and translation. These studies identify vRCA and pH2AX as strong indicators of

438   robust and progressive virus infection, and indicate that including additional viral and host

439   indicators can distinguish among diverse infection stages.

440   We next turned to scRNA-seq to gain a broader understanding of virus and host RNA

441   expression during *de novo* infection. Though we anticipated that many LANA+ cells (i.e.

442   vRCA^{high} pH2AX^{high} cells) would express high viral RNAs and low host RNAs (exemplified by

443   ORF18 and Actb, respectively), the relative homogeneity of LANA+ cells at the RNA level was

444   unknown. One of the most striking outcomes of these studies was the wide range of viral and

445   host gene expression observed at the single-cell level, derived from infection with a low MOI,

446   and harvested at a single timepoint. LANA+ cells had a 1000-fold variance in total viral reads

447   per cell, with cells expressing between 12-66 viral genes per cell and widespread variance in

448   both the frequency and expression level of viral RNAs. While this analysis mapped RNAs to 77

449   of the 80 annotated MHV68 ORFs (Virgin et al., 1997) using a 3' based sequencing platform, it

450   is notable that: i) only one viral gene (ORF52) was detected in 100% of LANA+ cells, with ii)

451   only 9 viral genes (M3, ORF7, ORF17, ORF38, ORF45, ORF52, ORF58, M9, and ORF66)

452   expressed in a high percentage (>98.5%) of LANA+ cells. Though many of these genes were

453   highly expressed on a per cell basis, a high frequency of expression does not solely track with

454   high expression. For example, ORF73 RNA, which encodes for LANA, was detected in 86.2%

455   of cells with an average of 5 UMIs per cell. Lack of detection of ORF73 in LANA+ sort purified

456   cells may seem to be a paradox, as cells were purified based on LANA+ cells. This apparent

457   discordance may either be due to RNA dropout or to cells expressing LANA protein in the

458   absence of ORF73 RNA. For other genes, limited detection likely reflects extremely low

459   expression during lytic infection [e.g. M2, a latency-associated gene that has not historically

460   been detected during lytic infection (Rochford et al., 2001; Virgin et al., 1999)]. These results

461   suggest that lack of detection of some MHV68 genes is likely due to little to no expression

462   during lytic infection and not due to technical issues with scRNA-seq. More broadly, these

463   studies indicate that stratifying gene expression by the IE$\rightarrow$E$\rightarrow$L paradigm, or by kinetic class,

464   is insufficient to describe the complexity of viral transcription in individual cells, even in a highly

465   permissive cell type.

466   An important complementary perspective on virus infection was our capacity to assess

467   how MHV68 infection remodels host RNA expression. Virus-biased cells expressed 1.5 times

468   more viral UMIs than host UMIs, a striking result when considering that there are only 80

469   annotated viral ORFs (Virgin et al., 1997) compared to ~25,000 host genes (Guenet, 2005).

470   Virus:host RNA skewing was further driven by a dramatic reduction in host gene expression,

471   consistent with virus-induced host shutoff (Covarrubias et al., 2011). Host shutoff was readily

472   visualized by pronounced reduction in ActB RNA expression in vRCA^{high} pH2AX^{high} cells,

473   illustrating the value of monitoring host RNAs at the single-cell level as an indicator of virus

474   infection. When we assessed the global relationship between virus and host RNAs, we found

475   additional complexity. While most LANA+ cells (73%) showed a reciprocal relationship

476   expression of high viral RNAs and low host RNAs (i.e. Virus^{high} Host^{low}), there were substantial

477   cell fractions that were either Host^{high}Virus^{high} or Virus^{low} Host^{high}, suggesting delayed or

478   impaired virus-induced host shutoff (a process that requires transcription, translation and

479   nuclease-dependent degradation of RNAs (Covarrubias et al., 2011)).

480

481   *Potential relationships of virus infection phenotypes*

482   The identification of distinct infection phenotypes raises the question of how these

483   subsets are inter-related. Though definitive lineage relationships require sequential tracking of

484   individual infected cells, we postulate that the Virus^{high} Host^{high} phenotype identifies cells with

485   slower progression through lytic infection, preceding Virus^{high} Host^{low} cells. In contrast, we

486   speculate that Virus^{low} Host^{high} cells may represent a distinct biological outcome. Although

487   Virus^{low} cells expressed fewer viral genes, frequently at a lower magnitude, these cells

488   contained viral RNAs from all phases of infection, with 90% of Virus^{low} cells expressing IE

489   (ORF38), E or E-L (M3, ORF7, ORF68) or L (ORF17, ORF45, ORF52, ORF58, M9, ORF66)

490   genes; these genes span kinetic classes, indicating that there is not a specific block in infection

491   (Cheng et al., 2012). Virus^{low} cells did contain a few viral genes (e.g. ORF68) that are modestly

492   increased relative to Virus^{high} cells; nonetheless, the Virus^{low} gene signature would be

493   obscured in bulk cell analysis. The molecular basis of the Virus^{low} Host^{high} phenotype remains

494   to be determined but could arise from host or virus variation. Curiously, Virus^{low} cells

495   expressed multiple interferon-stimulated genes, but not type I interferons, suggesting a

496   potential cell-intrinsic block to lytic infection. The Virus^{low} Host^{high} phenotype may also arise

497   from virus-intrinsic differences (e.g. variation in particle:PFU differences (Klasse, 2015) or

498   virus-like vesicles (Gong et al., 2017)). Variable outcomes of γHV infection have been reported

499   in multiple contexts. MHV68 infection of endothelial cells results in a significant cell fraction of

500   cells undergoing persistent infection (Suarez and van Dyk, 2008). KSHV infection of fibroblasts

501   results in variable lytic or latent infection (Jang et al., 2016; Krishnan et al., 2004; Matthews et

502   al., 2011), and EBV infection in gastric epithelial cells includes both lytic and latent outcomes

503   (Hill et al., 2013; Ma et al., 2011; Nawandar et al., 2015). Despite the restricted gene

504   expression of Virus^{low} cells, expression of multiple viral genes strongly suggests that these

505   cells have the potential to impact biological outcomes and immune regulation.

506

507   *Virus gene-gene relationships revealed by scRNA-seq*

508   Virus infection involves the coordination of highly complex gene expression patterns, a

509   poorly understand process at the single-cell level. Given this knowledge gap, we leveraged the

510   unique insights of scRNA-seq to quantify genome-wide correlation matrices. Unexpectedly, we

511   found gene pairs which were differentially regulated between Virus^{high} or Virus^{low} subsets,

512   including pronounced negative correlations among Virus^{high} cells. The complexity of these

513   relationships was illustrated by examining correlations between ORF50 and other viral genes.

514   ORF50 encodes the Rta viral transactivator and is known to both induce and repress

515   transcriptional targets (Coleman et al., 2005; Damania et al., 2004; Hair et al., 2007; Pavlova

516   et al., 2005; Wu et al., 2000). Correlation analysis among Virus^{high} cells corroborated

517   established ORF50 targets (e.g. positive correlation with ORF57, negative correlation with

518   ORF73) and identified new potential relationships (e.g. positive correlation with ORF54 and M8

519   and negative correlation with ORF64-67). These findings emphasize the importance of defining

520   gene expression relationships in biologically homogeneous populations, something uniquely

521   afforded by single-cell analysis.

522   Our scRNA-seq analysis quantified viral RNA abundance in LANA+ cells, mapping

523   RNAs to the annotated MHV68 genome using polyA-primed sequencing. This resource

524   illustrates the complexity of viral RNA expression following *de novo* γHV infection and sets the

525   foundation for new insights in gene expression in individual cells, including how virion-

526   packaged RNAs shape expression, the relative contribution of transcription and RNA decay to

527   the transcriptome, and RNA:protein relationships in individual cells (Hartenian and

528   Glaunsinger, 2019; Purushothaman et al., 2015; Ruiz et al., 2019). This approach further

529   emphasizes that not all abundant viral RNAs are equally expressed at the single-cell level, and

530   argue for the importance of single-cell analysis to identify candidate immunogens based on

531   uniform, high expression.

532   In total, these studies provide a resource to understand the complexity of virus-host

533   interactions within individual cells. This work provides three databases from matched *de novo*

534   gammaherpesvirus infection of a single permissive cell type, from CyTOF to bulk RNA-seq to

535   scRNA-seq, and exemplify the power of these approaches to reveal new insights into virus-

536   host interaction during γHV infection. We anticipate that these data will serve as an important

537   benchmark for future studies of virus infection, providing a wealth of observations to further

538   revise our understanding of virus-host interactions.

560

561 **DECLARATION OF INTERESTS**

562 The authors declare no competing interests.

563 **AUTHOR CONTRIBUTIONS**

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582 Writing – review & editing:

583   **FIGURE LEGENDS**

584

585   **Figure 1. CyTOF analysis identifies a distinct protein expression profile for virally-**

586   **infected cells.** High-dimensional single-cell protein analysis of MHV68 infection by CyTOF. (A)

587   3T12 fibroblasts were either mock or MHV68-infected with WT MHV68.LANAβlac (MOI=0.5),

588   with indicated populations FACS purified according to LANA::β-lactamase expression at 16

589   hours pi. (B) PhenoGraph analysis of mock, LANA- and LANA+ cells subjected to CyTOF

590   analysis and visualized by tSNE-based dimensionality reduction identified 14 cell clusters

591   (colored by cluster ID). (C) Comparison of the frequency (left) and protein expression profile

592   (right) of PhenoGraph-defined clusters in LANA- and LANA+ samples. Clusters (in rows),

593   ranked from those exclusively in LANA- samples (top) to those exclusively in LANA+ samples

594   (bottom); asterisks indicate clusters statistically significantly different between conditions,

595   defined by unpaired t-test corrected for multiple comparisons using the Holm-Sidak method,

596   p<0.05. Data show mean ± SEM with individual symbols indicating individual sample values

597   (n=4 per group). Relative protein expression for each cluster (right panel) is indicated by

598   heatmap intensity, with protein markers divded by functional categories. (D) tSNE plot of

599   LANA- and LANA+ cells colored according to LANA- and LANA+ exclusivity (left), vRCA

600   expression (middle), and pH2AX expression (right panel). Red and blue lines identify events

601   exclusive to LANA+ or LANA- clusters (left panel). Ruler defines range of expression with

602   values calculated using the equation arcsinh (x/5) where x is raw expression value. Live, DNA+

603   cells (^{191}Ir+ ^{193}Ir+ ^{195}Pt-) were imported into PhenoGraph with 9,162 events total analyzed

604   (1,018 events from each sample; n=1, mock, n=4 each for LANA+ and LANA- samples),

605   clustered on 9 cell surface proteins (vRCA, BST2, CD9, CD29, CD44, CD63, Ly6A/E, Ly6C,

606   and MHCI). See also Figure S1.

607

608 **Figure 2. LANA+ cells show heterogeneous protein and post-translational modifications**

609 **during lytic infection.** CyTOF analysis of LANA+ MHV68-infected cells as in Figure 1. (A)

610 PhenoGraph analysis of 40,000 LANA+ cells (10,000 cells/sample, 4 samples) clustered by 9

611 cell surface proteins (vRCA, BST2, CD9, CD29, CD44, CD63, Ly6A/E, Ly6C, and MHCI)

612 identified 15 unique cellular clusters colored by cluster ID and displayed on a tSNE plot. (B)

613 LANA+ cells can be stratified into six categories based on expression of pH2AX, vRCA, Ly6C,

614 and BST2, with (C) LANA+ cells colored by expression levels for each protein marker. Ruler

615 defines range of expression with values calculated using the equation arcsinh (x/5) where x is

616 raw expression value. (D) LANA+ cells were divided into pH2AX^{Lo} and pH2AX^{Hi} events, and

617 compared for raw median expression. Data depict proteins whose expression was statistically

618 significantly different between pH2AX^{Lo} and pH2AX^{Hi} events. Data depict mean ± SEM with

619 individual symbols indicating values from independent samples (n=4/group). All samples were

620 analyzed for statistical significance using unpaired t tests, corrected for multiple comparisons

621 using the Holm-Sidak method, with statistical significance denoted as *p<0.05, ** p<0.01,

622 ***p<0.001. See also Figure S2.

623

624 **Figure 3. MHV68-infected cells with a pH2AX^{high} vRCA^{high} cell phenotype express high**

625 **viral mRNA and low host actin RNA**. Flow cytometric analysis of protein and RNA

626 expression in 3T12 fibroblasts infected with MHV68 (MOI=0.5, harvested 16 hpi). (A) Analysis

627 of vRCA and pH2AX protein expression in mock and MHV68-infected cultures identified four

628 populations denoted by numbers and color (1=Gray, 2=Blue, 3=Orange, 4=Red). (B) Analysis

629 of viral ORF18 and host Actin (Actb) mRNA expression across four populations of MHV68-

630 infected cultures, stratified by vRCA and pH2AX expression. Border color of biaxial plots

631 corresponds to the parent population stratified by protein expression in panel A. (C)

632 Quantitaiton of the frequency of cells based on RNA expression profile as in panel B, depicting

633   mean ± SEM from 3 independent experimental samples. Flow cytometric analysis was done

634   on singlets, following gating to remove doublets.

635

636   **Figure 4. scRNA-seq reveals wide variation in the frequency and magnitude of viral RNA**

637   **expression.** scRNA-seq analysis of MHV68 viral gene expression in LANA+ 3T12 fibroblasts

638   as in Figure 1A. (A) LANA+ cells show a wide range in how many viral genes are expressed on

639   a per-cell basis. Expression of a viral gene was defined conservatively as any viral gene with

640   ≥1 UMI detected per cell, with data showing the distribution of cells based on number of viral

641   genes expressed per cell. (B) Viral genes vary widely in mean UMI per cell and the frequency

642   of cells expressing an individual viral gene. Mean viral UMI per cell was calculated from

643   positive cells that expressed ≥1 UMI per gene, with five representative genes identified by

644   unique shading. Each dot represents expression for a single viral gene product for all 80

645   annotated MHV68 open reading frames. (C) The distribution of cells based on total viral UMIs

646   reveals a bimodal distribution of virus low and virus high cells. (D) Total viral UMIs per cell as a

647   function of the number of viral genes detected per cell (as in panel A). Cells demonstrate a

648   bimodal distribution of total viral UMIs with virus high and low cells, with a positive Spearman

649   correlation coefficient, rs, as indicated. (E) Frequency of cells expressing each viral gene, with

650   viral gene expression defined as ≥1 UMI for each viral gene. Dashed line indicates 50% of

651   events. (F) Mean UMI count per viral gene, defined by calculating mean value only from

652   positive cells (≥1 UMI for each viral gene), depicting mean ± SD. Data represent scRNA-seq

653   data from all LANA+ cells (n=1605 cells). nd, genes that were not detected. See also Figure

654   S3 and Table S1.

655

656   **Figure 5. Visualization of genome-wide viral gene expression at the single-cell level.**

657   scRNA-seq analysis of MHV68 gene expression in LANA+ 3T12 fibroblasts as depicted in

658   Figure 1A. Comprehensive visualization of viral gene expression on a single cell basis. Each

659   row depicts expression pattern for a different viral gene. Each column depicts expression

660   within an individual cell. Cells are rank ordered from the cell with the least (left) to the greatest

661   total viral UMI count (right), with corresponding total viral (filled orange line) and host (black

662   dots) UMIs per cell depicted in the bottom panel. Cells are further bisected into Virus low cells

663   (< 500 viral UMIs per cell) and Virus high cells (> 500 viral UMIs per cell) by a thick vertical

664   black line, from a total of 1605 cells. UMI counts are stratified based on the indicated heatmap

665   scale. See also Figure S4.

666

667   **Figure 6: MHV68-infected cells can be stratified by differential abundance of virus and**

668   **host RNAs.** scRNA-seq analysis of MHV68-infected LANA+ cells for viral and host RNAs. (A-

669   E) 3D tSNE-based dimensionality reduction was used to visualize gene expression within

670   individual cells. (A) K-means clustering identified 5 unique cell clusters, as labelled. (B) Viral

671   and host RNAs show different patterns of expression, comparing viral immediate early (IE),

672   early-late (E-L) and late (L) genes with host beta actin (Actb). UMI counts visualized in

673   individual cells (symbols) as defined by key. (C) The ratio of host Actb to ORF37 UMIs,

674   calculated for each cell and overlaid onto tSNE-based visualization, varies widely across cells.

675   (D) Percent of viral (left) and host UMIs (right) per cell was calculated for each cell, portrayed

676   by shade of color overlaid onto tSNE visualization. (E) Cells were separated into two groups:

677   virus biased (red) and host biased (black) based on percent viral and host UMIs, and K-means

678   clustering. Host biased cells included clusters A and B with <20% virus UMIs on a per cell

679   basis. (F) Characteristics of virus and host biased cells based on scRNA-seq data. (G-H)

680   Number of (G) viral or (H) host genes with mean UMI expression ≥ 1, among either virus (open

681   red symbol) or host biased cells (dashed black line). (I) Violin plots depict the distribution of

682   mean gene expression among expressed genes (mean UMI expression ≥ 1), comparing viral

683   genes (left) or host genes (right) in either virus biased (open red symbol) or host biased cells

684   (open black line). Quartiles and median are depicted in each violin plot. Statistical comparisons

685   compared viral or host genes and their difference between virus and host biased cells.

686   ****$p<0.0001$, one way ANOVA with Tukey's multiple comparisons test. ns, not significant. (J-K)

687   Biaxial analysis of LANA+ cells (n=1605 cells) comparing total host UMI count (y axis) versus

688   total viral UMI count (x axis) identified three major cell populations, stratified by differential

689   expression of viral or host genes. (K) Identification of cells that are Virus^{low} Host^{high} (blue),

690   Virus^{high} Host^{high} (orange), and Virus^{high} Host^{low} (red). (L-P) Histogram overlays comparing

691   relative expression of (L-O) viral and (P) host genes for the three identified cell populations.

692   Each gene is denoted by its IE, E, or L class and by its kinetic class defined by (Cheng et al.,

693   2012). See also Figure S5-6, Table S2.

694

695   **Figure 7. Correlation analysis reveals positive and negative correlations in MHV68**

696   **transcription at the single-cell level.** The inter-relationship of viral gene expression was

697   interrogated using scRNA-seq data from MHV68-infected LANA+ cells. (A) Correlation matrix

698   of viral genes among Virus^{high} cells (>500 viral UMIs/cell), depicting Spearman correlation

699   coefficient, rs, for each gene pair according to the indicated heatmap. Positive correlation is

700   indicated by blue, negative correlation indicated by red. Correlation matrix depicts all viral

701   genes except for M10b and M10c (X indicates gene for which there were no values) and M12,

702   M13 and M14 (excluded due to negligible values). (B) A comparison of correlation coefficients

703   between viral gene pairs when analyzing all cells (top), Virus^{high} (middle) or Virus^{low} (bottom)

704   cells. Data identify discrepant correlations observed in different cell subsets. (C) Examples of

705   host and virus gene pairs with positive correlations, demonstrated by plotting scRNA-seq gene

706   expression values on biaxial plots across all LANA+ cells. (D) Examples of host and virus gene

707   pairs with a lack of correlation or negative correlation. Panels in C-D depict scRNA-seq values

708   for all LANA+ cells (n=1,605 cells), including both Virus^{high} and Virus^{low} events. Each plot

709   includes the Spearman correlation coefficient for the indicated gene pair defined across all

710   cells. See also Figure S7.

711 **STAR ★ METHODS**

712 **Contact for Reagent and Resource Sharing**

713 Further information and requests for resources and reagents should be directed to, and will be

714 fulfilled by, the Lead Contact, Linda van Dyk (Linda.vanDyk@cuanschutz.edu).

715

716 **Experimental Model and Subject Details**

717 Viruses

718 All experiments used WT MHV68 (strain WUMS; ATCC VR-1465) (Virgin et al., 1997), using

719 either bacterial artificial chromosome–derived WT MHV68 (Adler et al., 2000) or WT

720 MHV68.LANAβlac, which encodes a fusion between ORF73 and the beta-lactamase gene

721 (Nealy et al., 2010). Certain studies were done using a variant engineered to contain a

722 translational stop in the ORF72 (v-cyclin) gene, on the WT MHV68.LANAβlac background

723 (Niemeyer et al., 2018). MHV68 was grown and titrated by plaque assay on 3T12 fibroblasts,

724 as previously published (Diebel et al., 2015).

725 Cell Culture

726 NIH 3T12 cells (ATCC # CCL-164) were grown in Dulbecco's modified Eagle medium (DMEM,

727 Gibco, Life Technologies) supplemented with 5% fetal bovine serum (Atlanta Biologicals), 1x

728 Penicillin-Streptomycin-L-Glutamine (Gibco, Life Technologies). Cells were passaged using

729 0.05% trypsin (Gibco, Life Technologies) after washing with 1x PBS (Gibco, Life

730 Technologies). Cells were maintained at 37°C in a 5% CO_2 humidified incubator (Forma

731 Scientific).

732

733 **Method Details**

734 RNA sequencing and Analysis

735 3T12 fibroblasts were infected at an MOI=0.5 with a low volume inoculum for one hour, after

736 which inoculum was removed and cells were rinsed. Cells were harvested at 16 hours post-

737   infection (hpi) and stained with β-lactamase substrate as previously reported (Diebel et al.,

738   2015) using the CCF2-AM substrate (Invitrogen, Life Technologies). Cells were subjected to

739   fluorescence activated cell sorting (FACS), with cells gated on singlet cells that were positive

740   for the cleaved CCF2-AM substrate. Cleavage of CCF2-AM was defined as positive

741   fluorescence in the violet laser-excited 450nm emission channel obtained in WT

742   MHV68.LANAβlac or cycKO MHV68.LANAβlac-infected cultures, compared to parental WT

743   MHV68 infected cultures with no beta-lactamase fusion gene, used to define background

744   fluorescence. β-lactamase positive cells were sorted using a FACSAria Fusion (BD

745   Biosciences). Cells were spun down at 1,200 RPM (Sorvall RT60003), and resuspended in 1x

746   PBS (Gibco, Life Technologies). Cells were either prepared for CyTOF or RNA-sequencing by

747   the Genomics and Microarray Shared Resource Core at University of Colorado. Bulk RNA-seq

748   libraries were sequenced using Illumina HiSeq4000 platform with single-end reads (1 × 151),

749   with forty million reads per sample collected and resulting sequences filtered and trimmed to

750   remove low-quality bases (Phred score <15), and analyzed using a custom computational

751   pipeline consisting of open-source gSNAP, Cufflinks. Single cell RNA Sequencing (scRNA-

752   seq) (1000 cells per sample) was submitted for single cell barcoding and cDNA synthesis on a

753   10x Genomics Chromium Controller (10x Genomics), with recovered cell number and

754   sequencing depth for scRNA-seq described in Figure S3. cDNA was sequenced using the

755   NovaSEQ 6000 instrument with an S4 Flow Cell (Illumina). Sequence data was analyzed as

756   follows: Cellranger (2.0.2) count module was used for alignment, filtering, barcode counting

757   and UMI counting of the single cell FASTQs. tSNE coordinates were determined by

758   CellRanger.

759

760   PrimeFlow RNA Assay

761   3T12 fibroblasts were infected at an MOI=1.0 with a small-volume inoculation. Inoculum was

762   removed and rinsed-off with PBS after one hour and growth medium was replaced. Cells were

763   harvested 16 hpi and processed for flow cytometry using the PrimeFlow RNA Assay (Thermo

764   Fisher) as described previously (Oko et al., 2019). Briefly, cells were stained with a rabbit

765   antibody against the MHV68 ORF4 protein (vRCA), labeled with a Zenon R-phycoerythrin

766   rabbit IgG label reagent (Life Technologies) per manufacturer's protocol (Oko et al., 2019).

767   Following cell fixation, cells were then subjected to fluorescent barcoding, labeling distinct

768   experimental conditions with either Ghost Dye Violet 450 (Tonbo Biosciences), Ghost Dye

769   Violet 510 (Tonbo Biosciences) or leaving the cells unlabeled. Labelled samples were then

770   combined into a single tube and subjected to probe hybridization for viral and host RNAs or

771   proteins following the manufacturer's protocol. Flow cytometric analysis was done on an LSR II

772   instrument (BD Biosciences). Compensation values were initially based on antibody-stained

773   beads (BD Biosciences) and cross-validated using cell samples stained with individual

774   antibody conjugates, with compensation modified as needed post-collection using FlowJo.

775   PrimeFlow probes and antibodies included: i) a type 6 (AlexaFluor750)-mouse actb probe, ii) a

776   type 4 (AF488)-MHV68 ORF18 probe, iii) PE-labelled anti-vRCA antibody, and iv) AF647-

777   conjugated anti-mouse phospho-histone H2AX antibody (clone JBW301, that recognizes

778   phosphorylated serine 139 of H2AX).

779

780   CyTOF Sample Preparation and Data Acquisition

781   CyTOF antibodies were obtained from Fluidigm (previously DVS Sciences), provided by the

782   University of Colorado CyTOF Antibody Bank, supported by the Flow Cytometry Shared

783   Resource and the Human Immune Monitoring Shared Resource of the University of Colorado

784   Cancer Center. Samples were FACS purified based on LANA::β-lactamase dependent CCF2-

785   AM cleavage, into either LANAβlac+ or LANAβlac- fractions, transferred to 5mL screw cap

786   tubes, spun down, and stained for viability using 5 μM Cell-ID Cisplatin (Fluidigm) diluted in 1X

787   PBS (Rockland Immunochemicals) for 5 minutes at RT. Samples were then washed, fixed, and

788   each sample labelled with a series of unique palladium isotopic barcodes (Cell-ID 20-Plex

789   Palladium (Pd) Barcoding Kit, Fluidigm). After samples were washed and pooled, cells were

790   pre-treated with 2.4G2 Fc receptor blockade (Tonbo) using a 1:100 dilution of antibody. Cells

791   were incubated with a panel of antibodies, diluted in Maxpar Cell Staining Buffer (Fluidigm), to

792   detect cell surface proteins, washed, then incubated with secondary detection antibodies (anti-

793   FITC, anti-PE, anti-APC, anti-Biotin, and goat anti-rabbit isotopically labelled antibodies)

794   (Kimball et al., 2019). Samples were washed, fixed, and permeabilized (eBioscience Foxp3 /

795   Transcription Factor Staining Buffer Set, Thermo Fisher) before incubation with antibodies to

796   intracellular proteins. The sample was washed, fixed with freshly made 1.6%

797   paraformaldehyde diluted in Maxpar PBS (16% paraformaldehyde, Alfa Aesar), incubated in

798   0.125nM Iridium intercalator diluted in Maxpar PBS (Fluidigm), and stored at 4˚C until

799   acquisition. Samples were washed with 3ml of Maxpar Cell Staining Buffer, washed two times

800   with deionized water (Milli-Q®), resuspended in deionized water (Milli-Q) containing 1:9 EQ

801   Four Element Calibration Beads (Fluidigm), filtered through CellTrics 30um disposable filters

802   (Sysmex-Partec), and counted with a BIORAD TC20 automated cell counter. Data was

803   acquired on the Helios mass cytometer (Fluidigm). To ensure an appropriate flow rate though

804   the Helios, the sample volume was adjusted with the EQ Four Element Calibration Beads

805   (Fluidigm)/deionized water (Milli-Q) to dilute the sample to 1 million cells/mL at an acquired

806   event rate of 400,000-500,000 events/hr.

807

808   CyTOF sample normalization/debarcoding

809   Data were normalized with bead-based normalization using NormalizerR2013b_MacOSX and

810   then debarcoded using SingleCellDebarcoderR2013b_ MacOSX, using software downloaded

811   from the Nolan laboratory GitHub page (https://github.com/nolanlab). Normalized and

812   debarcoded data were subjected to traditional Boolean gating in FlowJo, identifying DNA+

813   events (^{191}Iridium (Ir)+ ^{193} Ir+) that were viable (^{195} Platinum(Pt)−), exported for downstream

814   analysis in PhenoGraph.

815

CyTOF Data Analysis

817 Normalized and debarcoded live DNA+ events were imported into cytofkit, and subjected to the

818 PhenoGraph algorithm. Clustering was done on either 9 cell surface proteins (Figures 1-2) or

819 all 23 proteins (Figure S1) . PhenoGraph was run with the following settings: 1) Merge method:

820 "min" (Figures 1, S1) or "ceiling: 10,000 events" (Fig. 2), 2) Transformation method:

821 "cytofAsinh", 3) Clustering method: "Rphenograph", and 4) Visualization method: "tSNE". All

822 other settings were selected by default. Median marker intensity for mock, LANA-, and LANA+

823 samples was calculated in FlowJo. Statistical significance was tested in GraphPad Prism

824 using an unpaired t test, with statistical significance as identified.

825

PhenoGraph visualization and analysis

827 PhenoGraph tSNE plots were visualized and customized in the R package "Shiny" (Kimball et

828 al., 2018). In the "Shiny" application cluster color was customized, tSNE plots were colored

829 according to the expression of various cellular markers, and dendrograms were generated.

830 The "cluster cell percentage" .csv file was used for comparing the distribution of events

831 between conditions and determining statistical significance.

832

RT-qPCR

834 RNA was isolated using the RNeasy Micro Kit (Qiagen) following manufacturer's

835 specifications. DNA contamination was removed using Turbo-DNAse (ThermoFisher)

836 according to the manufacturer's specifications. RT-qPCR for Ifnb1 was performed using a

837 primer/probe set (ThermoFisher) and the LightCycler 480 Probes Master (Roche) in 384-well

838 plates on the QuantStudio 7 Flex Instrument (Applied Biosystems, Life Technologies). RNA

839 was reverse transcribed with SuperScript II (Invitrogen) and qPCR was performed using iQ

840 SYBR Green SuperMix (Bio-Rad) in 384-well plates on the QuantStudio 7 Flex instrument

841   (Applied Biosystems, Life Technologies). qRT-PCR results were analyzed using the Pfaffl

842   method, as described by (Pfaffl, 2001), with standardization relative to 18S.

843

844   <u>Quantification and Statistical Analysis</u>

845   Data from the 10X Genomics Chromium Controller (10x Genomics) were analyzed with

846   CellRanger, including calculation of cell coordinates for 3D tSNE plots, visualized in Plotly.

847   Unbiased K means clustering was carried out using the DoKmeans function in Seurat. SeqGeq

848   was used for biaxial plotting of scRNAseq data. All flow cytometry data analysis was performed

849   using FlowJo V.10.0.8r1 with flow cytometry data shown as dot plots or averaged for bar

850   graphs. Statistical significance was tested using unpaired student's t test or one-way ANOVA

851   analysis with Tukey's multiple comparisons test. Statistical analysis and graphing were

852   performed with GraphPad Prism 7.0d or higher. Gene expression correlation was determined

853   with Spearman's correlation analysis in GraphPad Prism (8.4.2). CyTOF analysis was done

854   using PhenoGraph as described above or in (Kimball et al., 2018). p values less than 0.05

855   were considered significant.

856

857   **Data and Software Availability**

858   CyTOF data have been deposited to FlowRepository.org and will be made publicly available

859   upon manuscript acceptance. RNA-Seq data have been deposited to NCBI GEO and will be

860   made publicly available upon manuscript acceptance.

861 **Key Resources Table**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-beta-Catenin (clone D13A1) – 165Ho	Fluidigm	Cat#: 3165027A, RRID: AB_2811247
Anti-Human/Mouse CD44 (clone IM7) – 171Yb	Fluidigm	Cat#: 3171003B
Anti-IkBa (amino-terminal antigen) (clone L35A5) – 164Dy	Fluidigm	Cat#: 3164004A, RRID: AB_2811249
Anti-Ki67 (clone B56) – 161Dy	Fluidigm	Cat#: 3161007B, RRID: AB_2811255
Anti-Mouse Ly-6A/E (Sca-1) (clone D7) – 169Tm	Fluidigm	Cat#: 3169015B, RRID: AB_2811233
Anti-Mouse Ly-6C (clone HK1.4) – 162Dy	Fluidigm	Cat#: 3162014B
Anti-Mouse MHC Class I (clone 28-14-8) – 144Nd	Fluidigm	Cat#: 3144016B, RRID: AB_2687831
Anti-Human cleaved PARP (clone F21-852) – 143Nd	Fluidigm	Cat#: 3143011A
Anti-p-p38 [T180/Y182] (clone D3F9) – 156Gd	Fluidigm	Cat#: 3156002A, RRID: AB_2661826
Anti-p4E-BP1 [T37/T46] (clone 236B4) – 149Sm	Fluidigm	Cat#: 3149005A, RRID: AB_2847866
Anti-pAkt [S473] (clone D9E) – 152Sm	Fluidigm	Cat#: 3152005A, RRID: AB_2811246
Anti-pERK 1/2 [T202/Y204] (clone D1314.4E) – 167Er	Fluidigm	Cat#: 3167005A, RRID: AB_2661834

Anti-pHistone H2A.X [Ser139] (clone JBW301) - 147Sm	Fluidigm	Cat#: 3147016A, RRID: AB_2847865
Anti-pRb [S807/811] (clone J112-906) – 150Nd	Fluidigm	Cat#: 3150013A, RRID: AB_2811254
Anti-pS6 [S235/S236] (clone N7-548) –172Yb	Fluidigm	Cat#: 3172008A, RRID: AB_2811251
Anti-pSHP2 [Y580] (clone D66F10) – 141Pr	Fluidigm	Cat#: 3141002A, RRID: AB_2811252
Anti-pStat1 [Y701] (clone 58D6) – 153Eu	Fluidigm	Cat#: 3153003A, RRID: AB_2661824
Anti-pStat3 [Y705] (clone 4/P-Stat3) – 158Gd	Fluidigm	Cat#: 3158005A, RRID: AB_2661827
Anti-APC (clone APC003) – 176Yb	Fluidigm	Cat#: 3176007B, RRID: AB_2811236
Anti-Biotin (clone 1D4-C5) – 170Er	Fluidigm	Cat#: 3170003B, RRID: AB_2811234
Anti-FITC (clone FIT-22) – 160Gd	Fluidigm	Cat#: 3160011B, RRID: AB_2811235
Anti-PE (clone PE-001) – 145Nd	Fluidigm	Cat#: 3145006B, RRID: AB_2687631
APC anti-mouse CD137 (Bst2, PDCA-1) (clone 927)	BioLegend	Cat#: 127015, RRID: AB_1967101
Biotin anti-mouse/rat CD29 (clone HMβ1-1)	BioLegend	Cat#: 102203, RRID: AB_312880

FITC anti-mouse CD9 (clone MZ3)	BioLegend	Cat#: 124807, RRID: AB_1279324
Goat Anti-Rabbit (polyclonal) – 175Lu	Fluidigm	Cat#: 3175002G
PE anti-mouse CD63 (clone NVG-2)	BioLegend	Cat#: 143903, RRID: AB_11203532
Purified Anti-Mouse CD16/CD32 (clone 2.4G2)	Tonbo	Cat#: 70-0161-U500, RRID: AB_2621487
vRCA Ab (goat polyclonal antiserum)	Dr. H.W. Virgin IV	Laboratory generated polyclonal antiserum
Alexa Fluor 647-conjugated anti-phospho Histone H2A.X [S139] (clone JBW301)	MilliporeSigma	Cat#: 05-636-AF647
Chemicals, Peptides, and Recombinant Proteins		
Paraformaldehyde (PFA), 16% w/v aqueous solution, methanol free	Alfa Aesar by Thermo Fischer	Cat#: 43368
1x PBS	Gibco, Rockland Immunochemicals	Cat#: 10010-023
Barcoding Perm Buffer (10X)	Fluidigm	Cat#: 201057
Cell-ID™ 20-Plex Pd Barcoding Set	Fluidigm	Cat#: PN S00114
Cell-ID™ Cisplatin	Fluidigm	Cat#: 201064
Cell-ID™ Intercalator-Ir	Fluidigm	Cat#: 201192B
Water, deionized distilled	Millipore	Cat#: EM3234
DMEM, high glucose	Gibco, Life Technologies	Cat#: 11965-118

eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization Concentrate and Diluent	Invitrogen by Thermo Fisher	Cat#: 00-5523-00
EQ™ Four Element Calibration Beads	Fluidigm	Cat#: 201078
Fetal Bovine Serum	Atlanta Biologicals	Cat#: S11550
iQ SYBR Green Supermix	Bio-Rad	Cat#: 170-8882
Lightcycler 480 Probes Master	Roche Molecular Systems	Ref#: 04707494001
Maxpar Cell Staining Buffer	Fluidigm	Cat#: 201068
Maxpar Fix I Buffer (5X)	Fluidigm	Cat#: S00115
Maxpar PBS	Fluidigm	Cat#: 201058
Penicillin-Streptomycin-Glutamate (100x)	Gibco, Life Technologies	Cat#: 10378016
Poly(I:C) HMW	Invivogen	Cat#: tlrl-pic
Proteinase K	Promega	Cat#: V3021
Sodium Chloride	ThermoFisher	Cat#: BP-358-212
SuperScript II Reverse Transcriptase	Invitrogen, Thermo Fisher	Cat#: 18064014
Trypsin-EDTA (0.05%), phenol red	Gibco, Thermo Fisher	Cat#: 25300-054
TURBO DNase (2 U/μL)	ThermoFisher	Cat#: AM2238
X-tremeGENE HP DNA transfection reagent	Sigma-Aldrich	Cat#: 6366236001
Critical Commercial Assays		

DNeasy Blood and Tissue Kit	Qiagen	Cat#: 69506
LiveBLAzer FRET-B/G Loading Kit with CCF2-AM	Life Technologies, ThermoFisher	Cat#: K1032
OneStep RT-PCR Kit	Qiagen	Cat#: 210212
PrimeFlow™ RNA Assay Kit	ThermoFisher	Cat#: 88-18005-210
RNeasy Micro Kit	Qiagen	Cat#: 74004
Zenon™ R-Phycoerythrin Rabbit IgG Labeling Kit	Invitrogen, ThermoFisher	Cat#: Z25355
Deposited Data		
Raw CyTOF data	This study	FlowRepository.org
Raw single cell RNAseq data	This study	GEO
Experimental Models: Cell Lines		
Murine 3T12 fibroblasts (BALB/3T12-3)	ATCC	ATCC CCL-164™
Experimental Models: Organisms/Strains		
Murine gammaherpesvirus 68 (Wild-type, WT MHV68)	L.F. van Dyk	(Virgin et al., 1997)
Murine gammaherpesvirus 68 with a LANA::βlac gene fusion (WT MHV68.blac)	L.F. van Dyk	(Nealy et al., 2010)
Murine gammaherpesvirus 68 cyclin-deficient virus with a LANA::βlac gene fusion (CycKO MHV68.blac)	L.F. van Dyk	(Niemeyer et al., 2018)
Oligonucleotides		
MHV68 ORF18 probe, Type 4 (AlexaFluor 488)	Thermo Fisher	VF4-60000512

Mouse Actb probe, Type 6 (AlexaFuor750)	Thermo Fisher	VB6-12823
Mouse 18S primer/probe set	Thermo Fisher	Cat#: Mm03928990_g1
Mouse 18S Quantitect Primer Assay	Qiagen	Cat#: QT00199367
Mouse Ifnb1 primer/probe set	Thermo Fisher	Cat#: Mm00439552_s1
Software and Algorithms		
Adobe Illustrator 24.1.3	Adobe	www.adobe.com/illustrator
Bead Normalization (NormalizerR2013b_MacOSX)	Github	https://github.com/nolanlab/bead-normalization
Cell Ranger	10x Genomics	https://www.10xgenomics.com
Cytofkit v3.8	Bioconductor	http://bioconductor.org/packages/release/bioc/html/cytofkit.html
FlowJo v10.4.2 and v10.6.1	FlowJo	https://www.flowjo.com
GraphPad Prism 7.0 and 8.0	GraphPad	https://www.graphpad.com
Matlab	MathWorks	https://www.mathworks.com/products/matlab.html
Microsoft Excel 15.28	Microsoft	www.microsoft.com
Microsoft Office 15.28	Microsoft	www.microsoft.com
R studio v1.1.453	R studio	https://www.rstudio.com/
SeqGeq 1.6.0	FlowJo	https://www.flowjo.com
Seurat	Satija lab	https://www.satijalab.org/seurat
Single Cell Debarcoder (SingleCellDebarcoderR2013b_ MacOSX)	Github	https://github.com/nolanlab/single-cell-debarcoder
XQuartz v2.7.11	XQuartz	https://www.xquartz.org

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REFERENCES

Adang, L.A., Parsons, C.H., and Kedes, D.H. (2006). Asynchronous progression through the lytic cascade and variations in intracellular viral loads revealed by high-throughput single-cell analysis of Kaposi's sarcoma-associated herpesvirus infection. J Virol. 80(20), 10073-10082. Published online 2006/09/29 DOI: 10.1128/JVI.01156-06.

Adler, H., Messerle, M., Wagner, M., and Koszinowski, U.H. (2000). Cloning and mutagenesis of the murine gammaherpesvirus 68 genome as an infectious bacterial artificial chromosome. J Virol. 74(15), 6964-6974. Published online 2000/07/11.

Ahn, J.W., Powell, K.L., Kellam, P., and Alber, D.G. (2002). Gammaherpesvirus lytic gene expression as characterized by DNA array. J Virol. 76(12), 6244-6256. Published online 2002/05/22 DOI: 10.1128/jvi.76.12.6244-6256.2002.

Avraham, R., Haseley, N., Brown, D., Penaranda, C., Jijon, H.B., Trombetta, J.J., Satija, R., Shalek, A.K., Xavier, R.J., Regev, A., et al. (2015). Pathogen Cell-to-Cell Variability Drives Heterogeneity in Host Immune Responses. Cell. 162(6), 1309-1321. Published online 2015/09/08 DOI: 10.1016/j.cell.2015.08.027.

Barton, E., Mandal, P., and Speck, S.H. (2011). Pathogenesis and host control of gammaherpesviruses: lessons from the mouse. Annu Rev Immunol. 29, 351-397. Published online 2011/01/12 DOI: 10.1146/annurev-immunol-072710-081639.

Bhatt, A.P., Wong, J.P., Weinberg, M.S., Host, K.M., Giffin, L.C., Buijnink, J., van Dijk, E., Izumiya, Y., Kung, H.J., Temple, B.R., et al. (2016). A viral kinase mimics S6 kinase to enhance cell proliferation. Proc Natl Acad Sci U S A. 113(28), 7876-7881. Published online 2016/06/28 DOI: 10.1073/pnas.1600587113.

Cesarman, E. (2014). Gammaherpesviruses and lymphoproliferative disorders. Annu Rev Pathol. 9, 349-372. Published online 2013/10/12 DOI: 10.1146/annurev-pathol-012513-104656.

Chang, P.C., Campbell, M., and Robertson, E.S. (2016). Human Oncogenic Herpesvirus and Post-translational Modifications - Phosphorylation and SUMOylation. Front Microbiol. 7, 962. Published online 2016/07/06 DOI: 10.3389/fmicb.2016.00962.

Chaturvedi, S., Klein, J., Vardi, N., Bolovan-Fritts, C., Wolf, M., Du, K., Mlera, L., Calvert, M., Moorman, N.J., Goodrum, F., et al. (2020). A Molecular Mechanism for Probabilistic Bet-hedging and its Role in Viral Latency. bioRxiv. DOI: https://doi.org/10.1101/2020.05.14.096560.

Cheng, B.Y., Zhi, J., Santana, A., Khan, S., Salinas, E., Forrest, J.C., Zheng, Y., Jaggi, S., Leatherwood, J., and Krug, L.T. (2012). Tiled microarray identification of novel viral transcript structures and distinct transcriptional profiles during two modes of productive murine gammaherpesvirus 68 infection. J Virol. 86(8), 4340-4357. Published online 2012/02/10 DOI: 10.1128/JVI.05892-11.

Coleman, H.M., Efstathiou, S., and Stevenson, P.G. (2005). Transcription of the murine gammaherpesvirus 68 ORF73 from promoters in the viral terminal repeats. J Gen Virol. 86(Pt 3), 561-574. Published online 2005/02/22 DOI: 10.1099/vir.0.80565-0.

Covarrubias, S., Gaglia, M.M., Kumar, G.R., Wong, W., Jackson, A.O., and Glaunsinger, B.A. (2011). Coordinated destruction of cellular messages in translation complexes by the gammaherpesvirus host shutoff factor and the mammalian exonuclease Xrn1. PLoS Pathog. 7(10), e1002339. Published online 2011/11/03 DOI: 10.1371/journal.ppat.1002339.

Covarrubias, S., Richner, J.M., Clyde, K., Lee, Y.J., and Glaunsinger, B.A. (2009). Host shutoff is a conserved phenotype of gammaherpesvirus infection and is orchestrated exclusively from the cytoplasm. J Virol. 83(18), 9554-9566. Published online 2009/07/10 DOI: 10.1128/JVI.01051-09.

Damania, B., Jeong, J.H., Bowser, B.S., DeWire, S.M., Staudt, M.R., and Dittmer, D.P. (2004). Comparison of the Rta/Orf50 transactivator proteins of gamma-2-herpesviruses. J Virol. 78(10), 5491-5499. Published online 2004/04/29 DOI: 10.1128/jvi.78.10.5491-5499.2004.

Diebel, K.W., Oko, L.M., Medina, E.M., Niemeyer, B.F., Warren, C.J., Claypool, D.J., Tibbetts, S.A., Cool, C.D., Clambey, E.T., and van Dyk, L.F. (2015). Gammaherpesvirus small noncoding RNAs are bifunctional elements that regulate infection and contribute to virulence in vivo. mBio. 6(1), e01670-01614. Published online 2015/02/19 DOI: 10.1128/mBio.01670-14.

Dong, S., Forrest, J.C., and Liang, X. (2017). Murine Gammaherpesvirus 68: A Small Animal Model for Gammaherpesvirus-Associated Diseases. Adv Exp Med Biol. 1018, 225-236. Published online 2017/10/21 DOI: 10.1007/978-981-10-5765-6_14.

Drayman, N., Patel, P., Vistain, L., and Tay, S. (2019). HSV-1 single-cell analysis reveals the activation of anti-viral and developmental programs in distinct sub-populations. Elife. 8. Published online 2019/05/16 DOI: 10.7554/eLife.46339.

Ebrahimi, B., Dutia, B.M., Roberts, K.L., Garcia-Ramirez, J.J., Dickinson, P., Stewart, J.P., Ghazal, P., Roy, D.J., and Nash, A.A. (2003). Transcriptome profile of murine gammaherpesvirus-68 lytic infection. J Gen Virol. 84(Pt 1), 99-109. Published online 2003/01/21 DOI: 10.1099/vir.0.18639-0.

Forrest, J.C., and Speck, S.H. (2008). Establishment of B-cell lines latently infected with reactivation-competent murine gammaherpesvirus 68 provides evidence for viral alteration of a DNA damage-signaling cascade. J Virol. 82(15), 7688-7699. Published online 2008/05/23 DOI: 10.1128/JVI.02689-07.

Glaunsinger, B.A. (2015). Modulation of the Translational Landscape During Herpesvirus Infection. Annu Rev Virol. 2(1), 311-333. Published online 2016/03/10 DOI: 10.1146/annurev-virology-100114-054839.

Gong, D., Dai, X., Xiao, Y., Du, Y., Chapa, T.J., Johnson, J.R., Li, X., Krogan, N.J., Deng, H., Wu, T.T., et al. (2017). Virus-Like Vesicles of Kaposi's Sarcoma-Associated Herpesvirus Activate Lytic Replication by Triggering Differentiation Signaling. J Virol. 91(15). Published online 2017/05/19 DOI: 10.1128/JVI.00362-17.

Guenet, J.L. (2005). The mouse genome. Genome Res. 15(12), 1729-1740. Published online 2005/12/13 DOI: 10.1101/gr.3728305.

Hair, J.R., Lyons, P.A., Smith, K.G., and Efstathiou, S. (2007). Control of Rta expression critically determines transcription of viral and cellular genes following gammaherpesvirus infection. J Gen Virol. 88(Pt 6), 1689-1697. Published online 2007/05/09 DOI: 10.1099/vir.0.82548-0.

946 Hansen, M.M.K., Desai, R.V., Simpson, M.L., and Weinberger, L.S. (2018). Cytoplasmic
947 Amplification of Transcriptional Noise Generates Substantial Cell-to-Cell Variability. Cell Syst.
948 7(4), 384-397 e386. Published online 2018/09/24 DOI: 10.1016/j.cels.2018.08.002.

949 Hartenian, E., Gilbertson, S., Federspiel, J.D., Cristea, I.M., and Glaunsinger, B.A. (2020).
950 RNA decay during gammaherpesvirus infection reduces RNA polymerase II occupancy of host
951 promoters but spares viral promoters. PLoS Pathog. 16(2), e1008269. Published online
952 2020/02/08 DOI: 10.1371/journal.ppat.1008269.

953 Hartenian, E., and Glaunsinger, B.A. (2019). Feedback to the central dogma: cytoplasmic
954 mRNA decay and transcription are interdependent processes. Crit Rev Biochem Mol Biol.
955 54(4), 385-398. Published online 2019/10/28 DOI: 10.1080/10409238.2019.1679083.

956 Hill, E.R., Koganti, S., Zhi, J., Megyola, C., Freeman, A.F., Palendira, U., Tangye, S.G., Farrell,
957 P.J., and Bhaduri-McIntosh, S. (2013). Signal transducer and activator of transcription 3 limits
958 Epstein-Barr virus lytic activation in B lymphocytes. J Virol. 87(21), 11438-11446. Published
959 online 2013/08/24 DOI: 10.1128/JVI.01762-13.

960 Jang, G.H., Lee, J., Kim, N.Y., Kim, J.H., Yeh, J.Y., Han, M., Ahn, S.K., Kang, H., and Lee, M.
961 (2016). Suppression of lytic replication of Kaposi's sarcoma-associated herpesvirus by
962 autophagy during initial infection in NIH 3T3 fibroblasts. Arch Virol. 161(3), 595-604. Published
963 online 2015/12/02 DOI: 10.1007/s00705-015-2698-2.

964 Johnson, L.S., Willert, E.K., and Virgin, H.W. (2010). Redefining the genetics of murine
965 gammaherpesvirus 68 via transcriptome-based annotation. Cell Host Microbe. 7(6), 516-526.
966 Published online 2010/06/15 DOI: 10.1016/j.chom.2010.05.005.

967 Kapadia, S.B., Molina, H., van Berkel, V., Speck, S.H., and Virgin, H.W.t. (1999). Murine
968 gammaherpesvirus 68 encodes a functional regulator of complement activation. J Virol. 73(9),
969 7658-7670. Published online 1999/08/10.

970 Kimball, A.K., Oko, L.M., Bullock, B.L., Nemenoff, R.A., van Dyk, L.F., and Clambey, E.T.
971 (2018). A Beginner's Guide to Analyzing and Visualizing Mass Cytometry Data. J Immunol.
972 200(1), 3-22. Published online 2017/12/20 DOI: 10.4049/jimmunol.1701494.

973 Kimball, A.K., Oko, L.M., Kaspar, R.E., van Dyk, L.F., and Clambey, E.T. (2019). High-
974 Dimensional Characterization of IL-10 Production and IL-10-Dependent Regulation during
975 Primary Gammaherpesvirus Infection. Immunohorizons. 3(3), 94-109. Published online
976 2019/07/30 DOI: 10.4049/immunohorizons.1800088.

977 Klasse, P.J. (2015). Molecular determinants of the ratio of inert to infectious virus particles.
978 Prog Mol Biol Transl Sci. 129, 285-326. Published online 2015/01/18 DOI:
979 10.1016/bs.pmbts.2014.10.012.

980 Krishnan, H.H., Naranatt, P.P., Smith, M.S., Zeng, L., Bloomer, C., and Chandran, B. (2004).
981 Concurrent expression of latent and a limited number of lytic genes with immune modulation
982 and antiapoptotic function by Kaposi's sarcoma-associated herpesvirus early during infection
983 of primary endothelial and fibroblast cells and subsequent decline of lytic gene expression. J
984 Virol. 78(7), 3601-3620. Published online 2004/03/16 DOI: 10.1128/jvi.78.7.3601-3620.2004.

985 Lee, H.R., Amatya, R., and Jung, J.U. (2015). Multi-step regulation of innate immune signaling
986 by Kaposi's sarcoma-associated herpesvirus. Virus Res. 209, 39-44. Published online
987 2015/03/23 DOI: 10.1016/j.virusres.2015.03.004.

988 Levine, J.H., Simonds, E.F., Bendall, S.C., Davis, K.L., Amir el, A.D., Tadmor, M.D., Litvin, O.,
989 Fienberg, H.G., Jager, A., Zunder, E.R., et al. (2015). Data-Driven Phenotypic Dissection of
990 AML Reveals Progenitor-like Cells that Correlate with Prognosis. Cell. 162(1), 184-197.
991 Published online 2015/06/23 DOI: 10.1016/j.cell.2015.05.047.

992 Ma, S.D., Hegde, S., Young, K.H., Sullivan, R., Rajesh, D., Zhou, Y., Jankowska-Gan, E.,
993 Burlingham, W.J., Sun, X., Gulley, M.L., et al. (2011). A new model of Epstein-Barr virus
994 infection reveals an important role for early lytic viral protein expression in the development of
995 lymphomas. J Virol. 85(1), 165-177. Published online 2010/10/29 DOI: 10.1128/JVI.01512-10.

996 Martinez-Guzman, D., Rickabaugh, T., Wu, T.T., Brown, H., Cole, S., Song, M.J., Tong, L.,
997 and Sun, R. (2003). Transcription program of murine gammaherpesvirus 68. J Virol. 77(19),
998 10488-10503. Published online 2003/09/13 DOI: 10.1128/jvi.77.19.10488-10503.2003.

999 Matthews, N.C., Goodier, M.R., Robey, R.C., Bower, M., and Gotch, F.M. (2011). Killing of
1000 Kaposi's sarcoma-associated herpesvirus-infected fibroblasts during latent infection by
1001 activated natural killer cells. Eur J Immunol. 41(7), 1958-1968. Published online 2011/04/22
1002 DOI: 10.1002/eji.201040661.

1003 Messinger, J.E., Dai, J., Stanland, L.J., Price, A.M., and Luftig, M.A. (2019). Identification of
1004 Host Biomarkers of Epstein-Barr Virus Latency IIb and Latency III. mBio. 10(4). Published
1005 online 2019/07/04 DOI: 10.1128/mBio.01006-19.

1006 Nawandar, D.M., Wang, A., Makielski, K., Lee, D., Ma, S., Barlow, E., Reusch, J., Jiang, R.,
1007 Wille, C.K., Greenspan, D., et al. (2015). Differentiation-Dependent KLF4 Expression
1008 Promotes Lytic Epstein-Barr Virus Infection in Epithelial Cells. PLoS Pathog. 11(10),
1009 e1005195. Published online 2015/10/03 DOI: 10.1371/journal.ppat.1005195.

1010 Nealy, M.S., Coleman, C.B., Li, H., and Tibbetts, S.A. (2010). Use of a virus-encoded
1011 enzymatic marker reveals that a stable fraction of memory B cells expresses latency-
1012 associated nuclear antigen throughout chronic gammaherpesvirus infection. J Virol. 84(15),
1013 7523-7534. Published online 2010/05/21 DOI: 10.1128/JVI.02572-09.

1014 Niemeyer, B.F., Oko, L.M., Medina, E.M., Oldenburg, D.G., White, D.W., Cool, C.D., Clambey,
1015 E.T., and van Dyk, L.F. (2018). Host Tumor Suppressor p18(INK4c) Functions as a Potent
1016 Cell-Intrinsic Inhibitor of Murine Gammaherpesvirus 68 Reactivation and Pathogenesis. J Virol.
1017 92(6). Published online 2018/01/05 DOI: 10.1128/JVI.01604-17.

1018 O'Grady, T., Feswick, A., Hoffman, B.A., Wang, Y., Medina, E.M., Kara, M., van Dyk, L.F.,
1019 Flemington, E.K., and Tibbetts, S.A. (2019). Genome-wide Transcript Structure Resolution
1020 Reveals Abundant Alternate Isoform Usage from Murine Gammaherpesvirus 68. Cell Rep.
1021 27(13), 3988-4002 e3985. Published online 2019/06/27 DOI: 10.1016/j.celrep.2019.05.086.

1022 Oko, L.M., Kimball, A.K., Kaspar, R.E., Knox, A.N., Coleman, C.B., Rochford, R., Chang, T.,
1023 Alderete, B., van Dyk, L.F., and Clambey, E.T. (2019). Multidimensional analysis of
1024 Gammaherpesvirus RNA expression reveals unexpected heterogeneity of gene expression.
1025 PLoS Pathog. 15(6), e1007849. Published online 2019/06/06 DOI:
1026 10.1371/journal.ppat.1007849.

1027 Pavlova, I., Lin, C.Y., and Speck, S.H. (2005). Murine gammaherpesvirus 68 Rta-dependent
1028 activation of the gene 57 promoter. Virology. 333(1), 169-179. Published online 2005/02/15
1029 DOI: 10.1016/j.virol.2004.12.021.

1030 Pellett, P.J., and Roizman, B. (2013). *Herpesviridae*. In: Fields Virology.

1031 Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR.
1032 Nucleic Acids Res. 29(9), e45. Published online 2001/05/09 DOI: 10.1093/nar/29.9.e45.

1033 Purushothaman, P., Thakker, S., and Verma, S.C. (2015). Transcriptome analysis of Kaposi's
1034 sarcoma-associated herpesvirus during de novo primary infection of human B and endothelial
1035 cells. J Virol. 89(6), 3093-3111. Published online 2015/01/02 DOI: 10.1128/JVI.02507-14.

1036 Rochford, R., Lutzke, M.L., Alfinito, R.S., Clavo, A., and Cardin, R.D. (2001). Kinetics of murine
1037 gammaherpesvirus 68 gene expression following infection of murine cells in culture and in
1038 mice. J Virol. 75(11), 4955-4963. Published online 2001/05/03 DOI: 10.1128/JVI.75.11.4955-
1039 4963.2001.

1040 Ruiz, J.C., Hunter, O.V., and Conrad, N.K. (2019). Kaposi's sarcoma-associated herpesvirus
1041 ORF57 protein protects viral transcripts from specific nuclear RNA decay pathways by
1042 preventing hMTR4 recruitment. PLoS Pathog. 15(2), e1007596. Published online 2019/02/21
1043 DOI: 10.1371/journal.ppat.1007596.

1044 Sen, N., Mukherjee, G., Sen, A., Bendall, S.C., Sung, P., Nolan, G.P., and Arvin, A.M. (2014).
1045 Single-cell mass cytometry analysis of human tonsil T cell remodeling by varicella zoster virus.
1046 Cell Rep. 8(2), 633-645. Published online 2014/07/22 DOI: 10.1016/j.celrep.2014.06.024.

1047 Shaffer, S.M., Dunagin, M.C., Torborg, S.R., Torre, E.A., Emert, B., Krepler, C., Beqiri, M.,
1048 Sproesser, K., Brafford, P.A., Xiao, M., et al. (2017). Rare cell variability and drug-induced
1049 reprogramming as a mode of cancer drug resistance. Nature. 546(7658), 431-435. Published
1050 online 2017/06/14 DOI: 10.1038/nature22794.

1051 Shnayder, M., Nachshon, A., Krishna, B., Poole, E., Boshkov, A., Binyamin, A., Maza, I.,
1052 Sinclair, J., Schwartz, M., and Stern-Ginossar, N. (2018). Defining the Transcriptional
1053 Landscape during Cytomegalovirus Latency with Single-Cell RNA Sequencing. mBio. 9(2).
1054 Published online 2018/03/15 DOI: 10.1128/mBio.00013-18.

1055 Smiley, J.R. (2004). Herpes simplex virus virion host shutoff protein: immune evasion
1056 mediated by a viral RNase? J Virol. 78(3), 1063-1068. Published online 2004/01/15 DOI:
1057 10.1128/jvi.78.3.1063-1068.2004.

1058 Suarez, A.L., and van Dyk, L.F. (2008). Endothelial cells support persistent gammaherpesvirus
1059 68 infection. PLoS Pathog. 4(9), e1000152. Published online 2008/09/13 DOI:
1060 10.1371/journal.ppat.1000152.

1061 Tarakanova, V.L., Leung-Pineda, V., Hwang, S., Yang, C.W., Matatall, K., Basson, M., Sun,
1062 R., Piwnica-Worms, H., Sleckman, B.P., and Virgin, H.W.t. (2007). Gamma-herpesvirus kinase
1063 actively initiates a DNA damage response by inducing phosphorylation of H2AX to foster viral
1064 replication. Cell Host Microbe. 1(4), 275-286. Published online 2007/11/17 DOI:
1065 10.1016/j.chom.2007.05.008.

1066 van Dyk, L.F., Virgin, H.W.t., and Speck, S.H. (2000). The murine gammaherpesvirus 68 v-
1067 cyclin is a critical regulator of reactivation from latency. J Virol. 74(16), 7451-7461. Published
1068 online 2000/07/25 DOI: 10.1128/jvi.74.16.7451-7461.2000.

1069 Van Skike, N.D., Minkah, N.K., Hogan, C.H., Wu, G., Benziger, P.T., Oldenburg, D.G., Kara,
1070 M., Kim-Holzapfel, D.M., White, D.W., Tibbetts, S.A., et al. (2018). Viral FGARAT ORF75A

promotes early events in lytic infection and gammaherpesvirus pathogenesis in mice. PLoS Pathog. 14(2), e1006843. Published online 2018/02/02 DOI: 10.1371/journal.ppat.1006843.

Virgin, H.W.t., Latreille, P., Wamsley, P., Hallsworth, K., Weck, K.E., Dal Canto, A.J., and Speck, S.H. (1997). Complete sequence and genomic analysis of murine gammaherpesvirus 68. J Virol. 71(8), 5894-5904. Published online 1997/08/01.

Virgin, H.W.t., Presti, R.M., Li, X.Y., Liu, C., and Speck, S.H. (1999). Three distinct regions of the murine gammaherpesvirus 68 genome are transcriptionally active in latently infected mice. J Virol. 73(3), 2321-2332. Published online 1999/02/11.

Waterboer, T., Rahaus, M., and Wolff, M.H. (2002). Varicella-zoster virus (VZV) mediates a delayed host shutoff independent of open reading frame (ORF) 17 expression. Virus Genes. 24(1), 49-56. Published online 2002/04/04 DOI: 10.1023/a:1014086004141.

Weitzman, M.D., and Fradet-Turcotte, A. (2018). Virus DNA Replication and the Host DNA Damage Response. Annu Rev Virol. 5(1), 141-164. Published online 2018/07/12 DOI: 10.1146/annurev-virology-092917-043534.

Wu, T.T., Usherwood, E.J., Stewart, J.P., Nash, A.A., and Sun, R. (2000). Rta of murine gammaherpesvirus 68 reactivates the complete lytic cycle from latency. J Virol. 74(8), 3659-3667. Published online 2000/03/23 DOI: 10.1128/jvi.74.8.3659-3667.2000.

Wyler, E., Franke, V., Menegatti, J., Kocks, C., Boltengagen, A., Praktiknjo, S., Walch-Ruckheim, B., Bosse, J., Rajewsky, N., Grasser, F., et al. (2019). Single-cell RNA-sequencing of herpes simplex virus 1-infected cells connects NRF2 activation to an antiviral program. Nat Commun. 10(1), 4878. Published online 2019/10/28 DOI: 10.1038/s41467-019-12894-z.

Zamora, M.R. (2011). DNA viruses (CMV, EBV, and the herpesviruses). Semin Respir Crit Care Med. 32(4), 454-470. Published online 2011/08/23 DOI: 10.1055/s-0031-1283285.

Zhang, K., Lv, D.W., and Li, R. (2019). Conserved Herpesvirus Protein Kinases Target SAMHD1 to Facilitate Virus Replication. Cell Rep. 28(2), 449-459 e445. Published online 2019/07/11 DOI: 10.1016/j.celrep.2019.04.020.
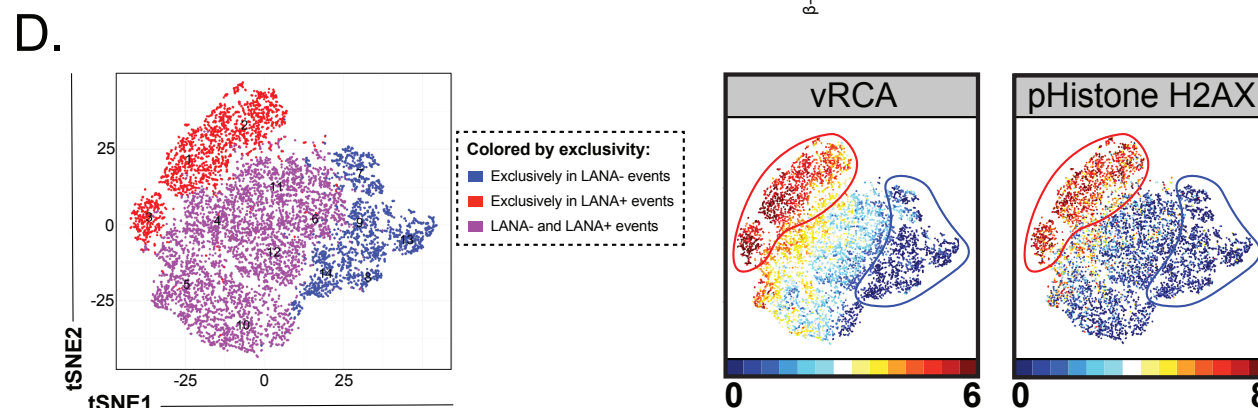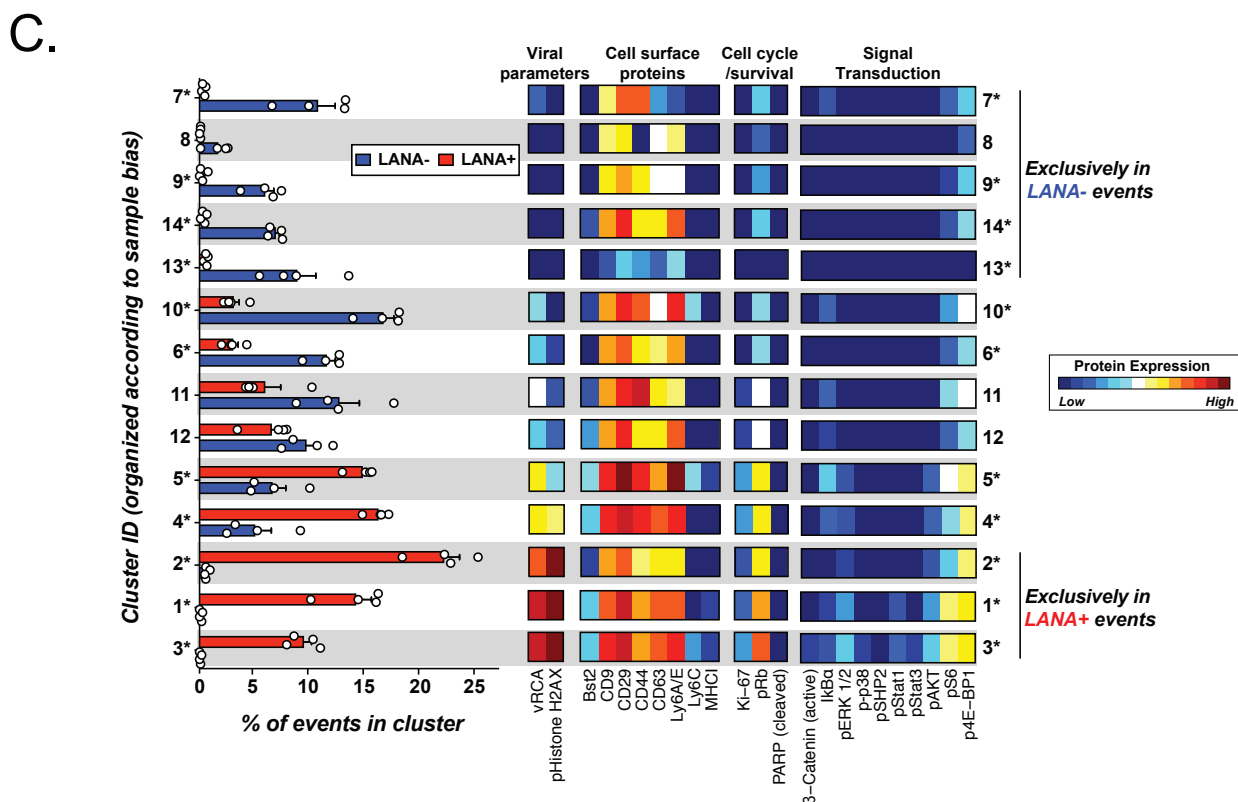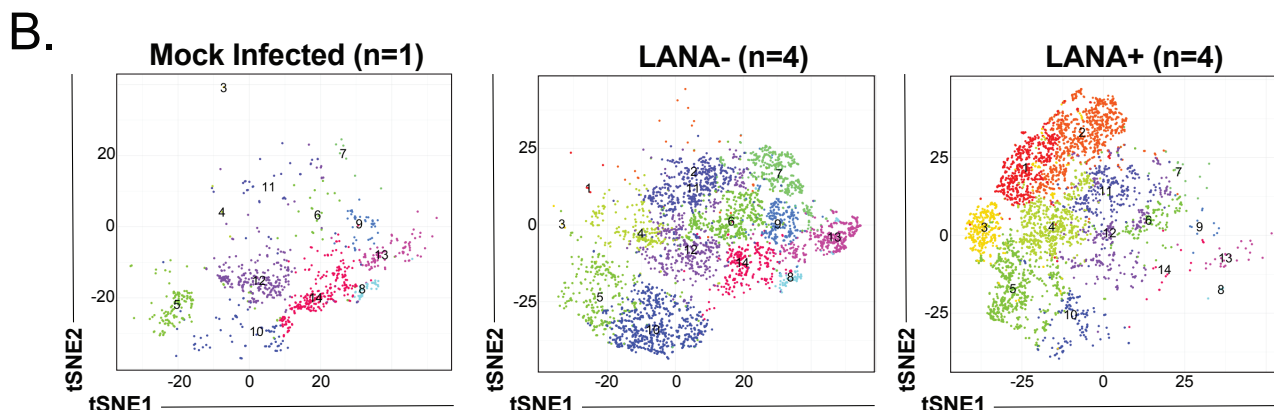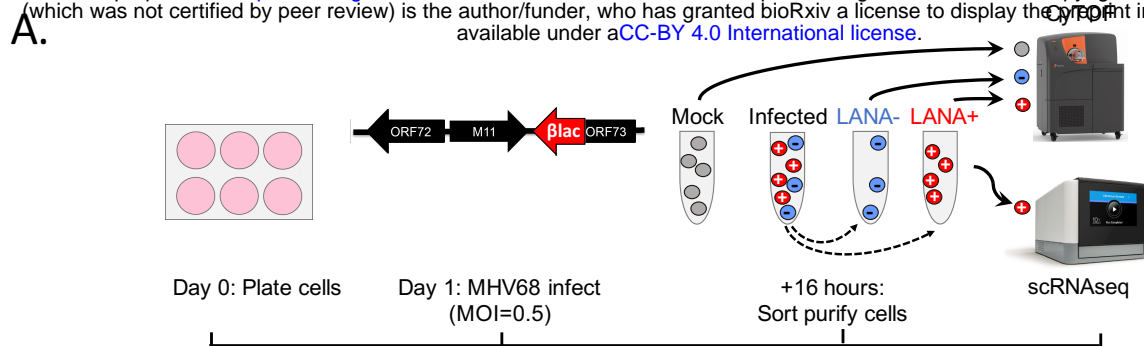
Figure 1
Berger et al
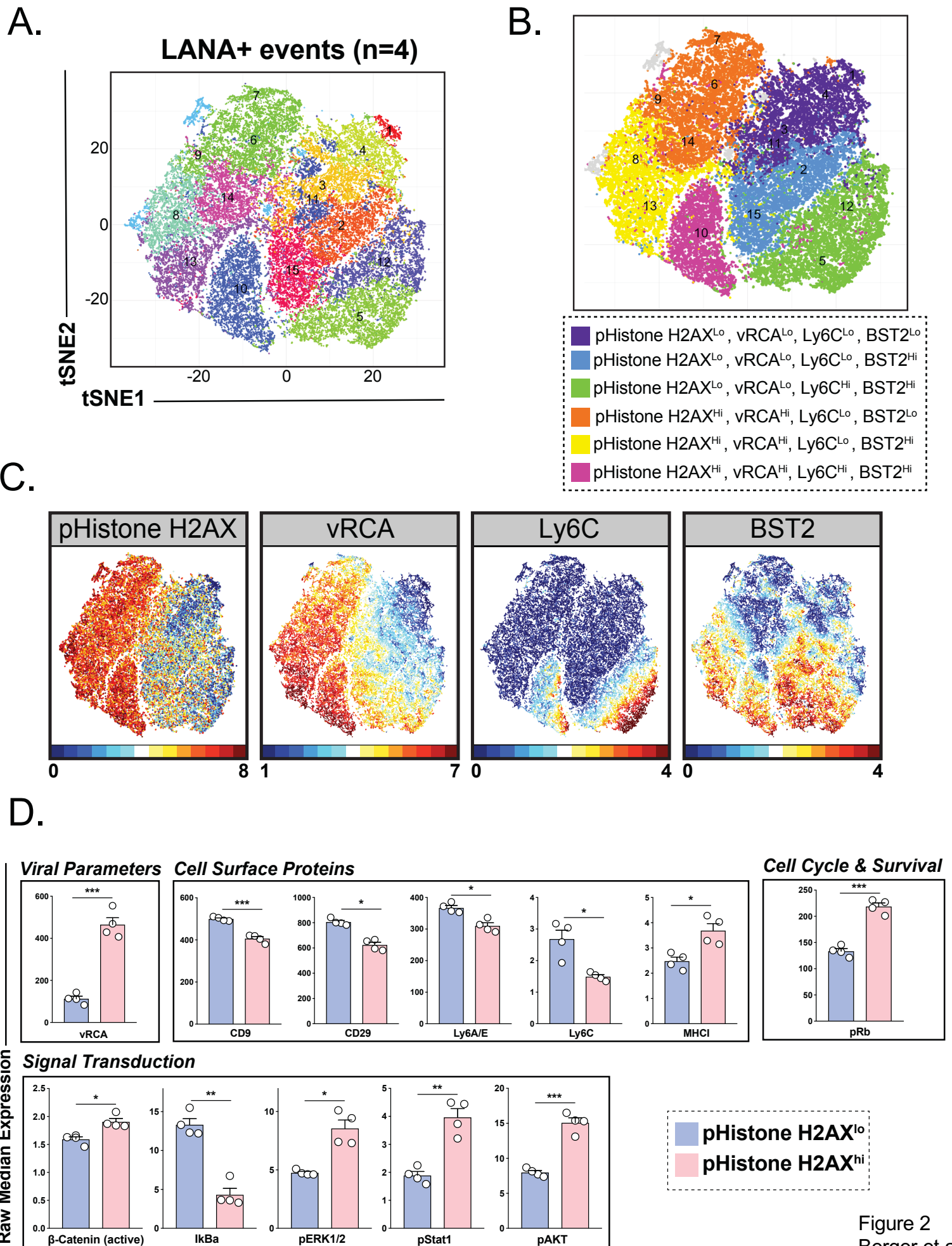
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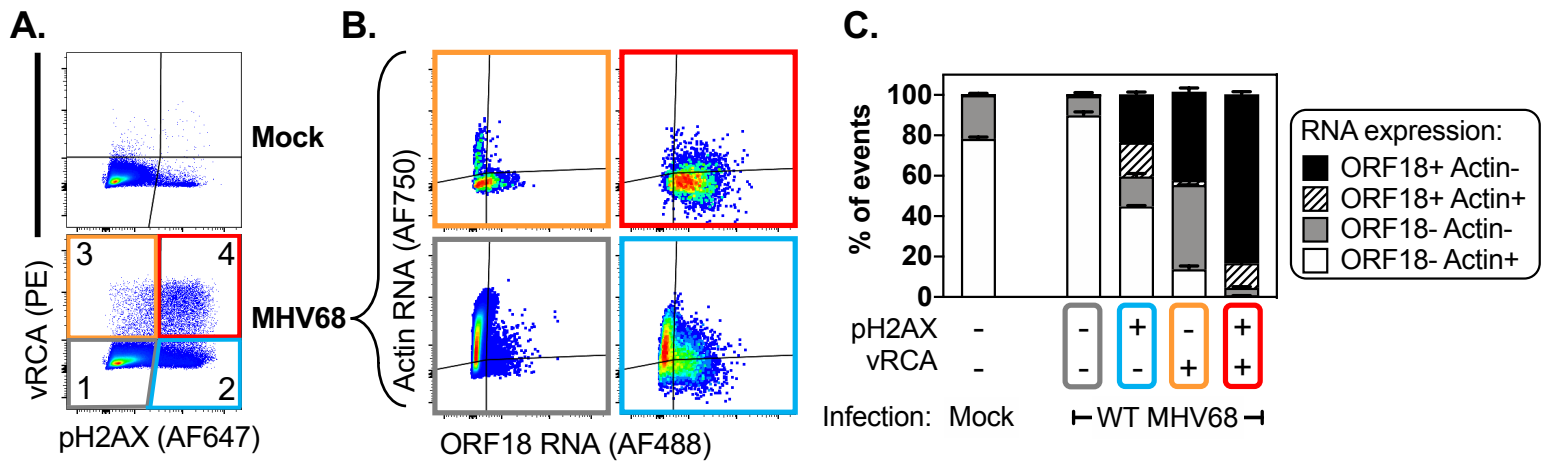
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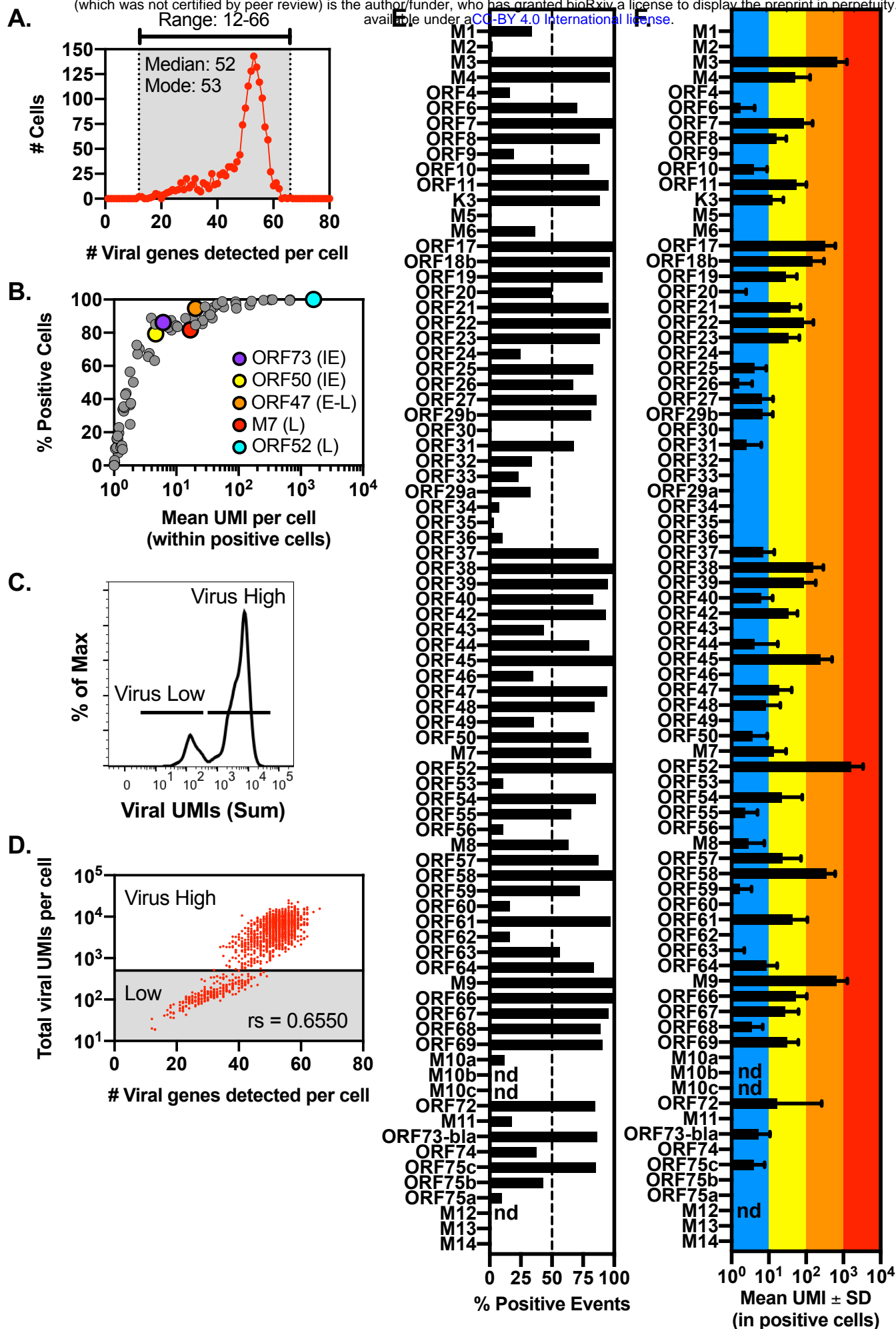
Scale: 0   1-10   11-100   101-1000   >1000 UMIs/cell
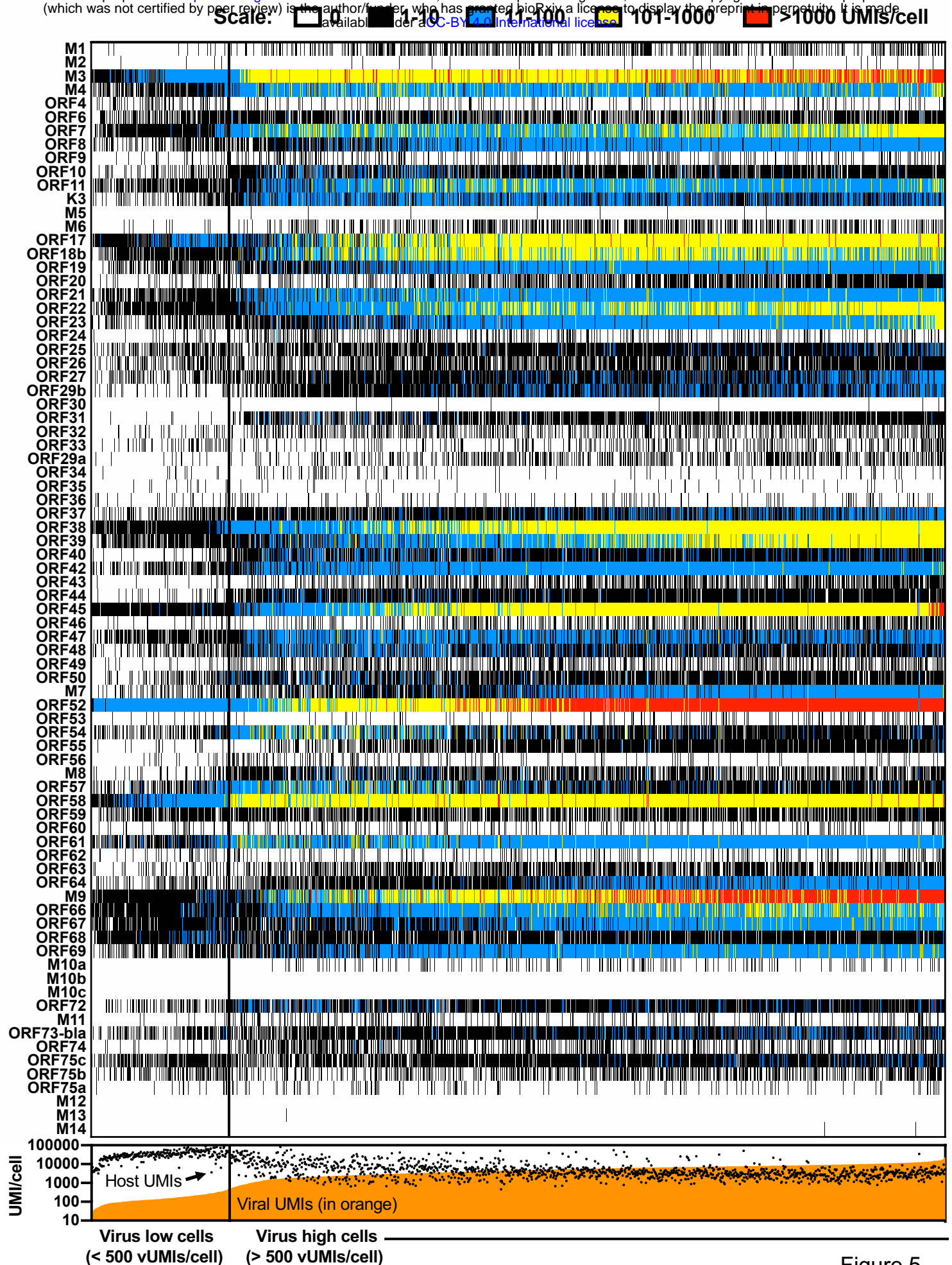
Figure 5

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A. K-means clustering

B.

ORF50 (IE)　　ORF47 (E-L)　　M7 (L)　　Actb (Host)

Legend: 0 | 0-1 | 1-10 | 10-20 | 20-50 | 50-100 | 100-200

C. ActB:ORF37 Ratio

UMI Ratio: <10, 10-19, 20-20, 30-39, 40-49, 50-59, 60-69, 70-79, 80-89, 90-100, >100

D. % Viral UMIs　　% Host UMIs

UMI Percent: <10, 10-19, 20-29, 30-39, 40-49, 50-59, 60-69, 70-79, 80-89, 90-100

E.
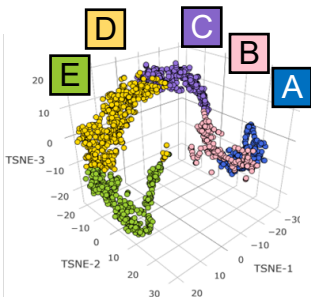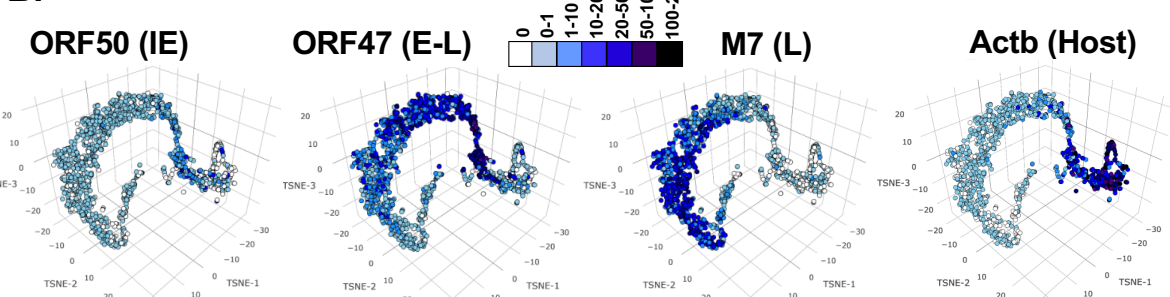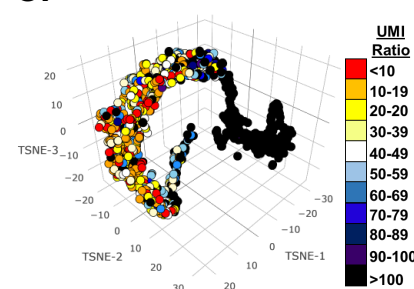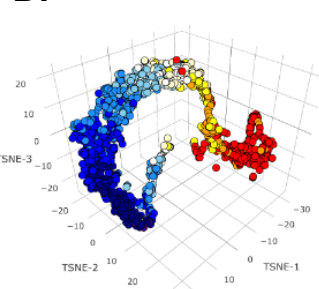
Virus Biased　　Host Biased
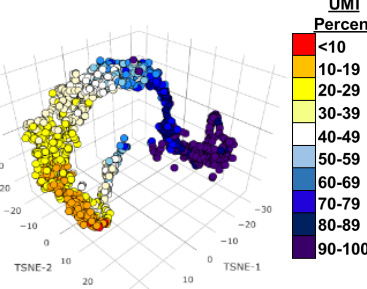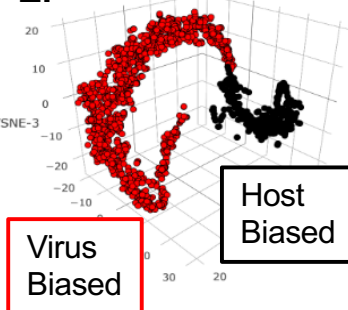
F.

Cell Groups	Cell #	Total UMIs	Viral UMIs	Host UMIs	UMI Ratio (Virus:Host)	% Viral UMIs	% Host UMIs
Virus Biased	1176	12,214,549	7,614,463	4,600,086	1.655	62.339	37.661
Host Biased	429	13,048,564	506,293	12,542,271	0.04	3.880	96.120

G. # of Viral Genes with Mean Expression ≥ 1 — Group: Virus Biased, Host Biased

H. # of Host Genes with Mean Expression ≥ 1 — Group: Virus Biased, Host Biased

I. Mean Gene Expression for Expressed Genes (Mean ≥ 1) — **** ns — Group: Virus Biased, Host Biased, Virus Biased, Host Biased — Genes: Viral, Host

J. Host Genes (Sum) vs MHV68 Genes (Sum) — 14.4, 11.1, 1.68, 72.8

K. Host Genes (Sum) vs MHV68 Genes (Sum)

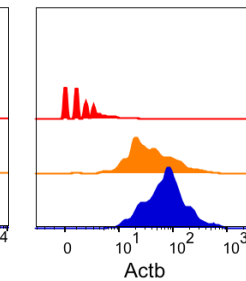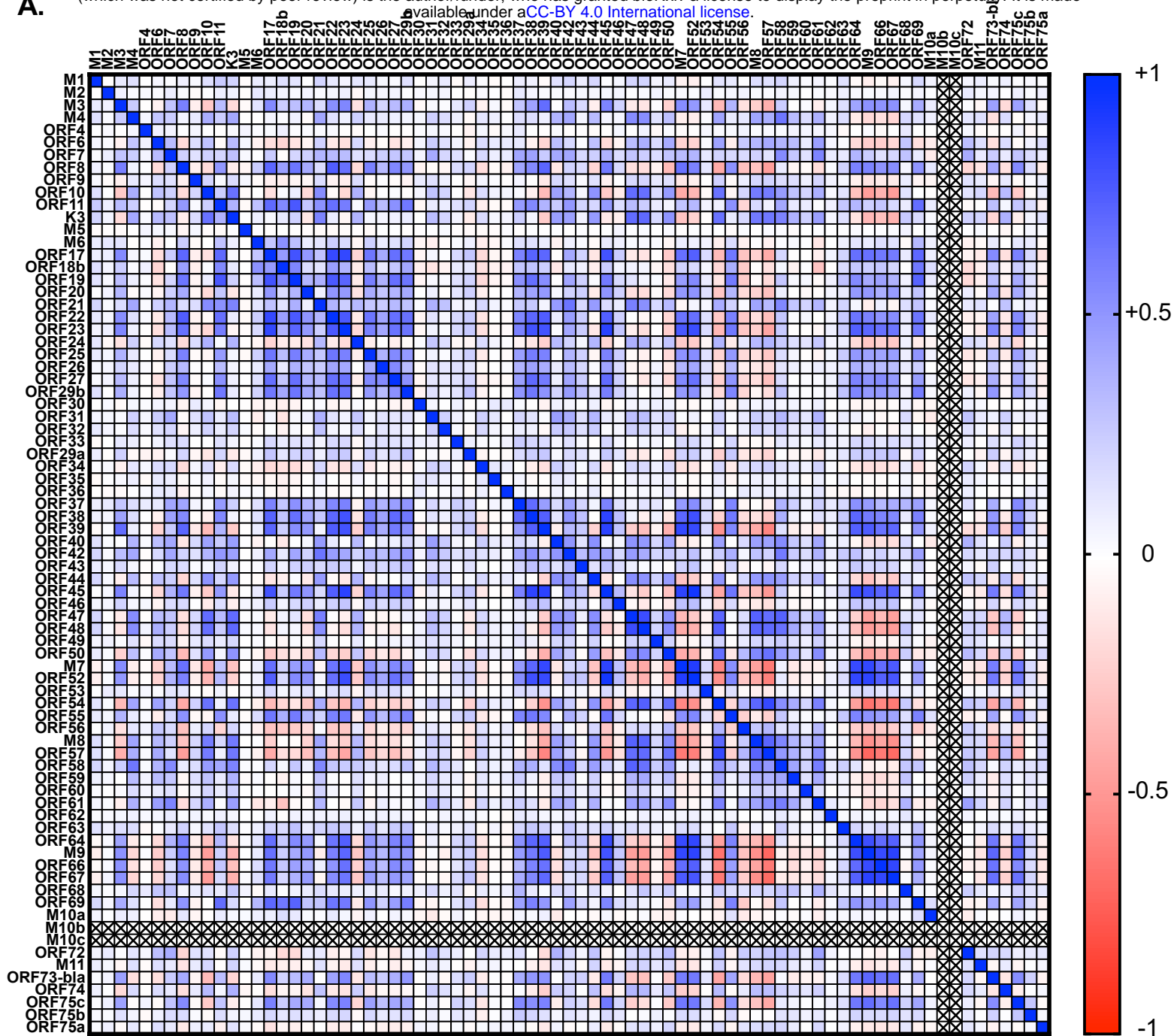**L.** ORF50 (IE, I)　**M.** ORF47 (E-L, II)　**N.** M7 (L, IV)　**O.** ORF68 (E, III)　**P.** Actb
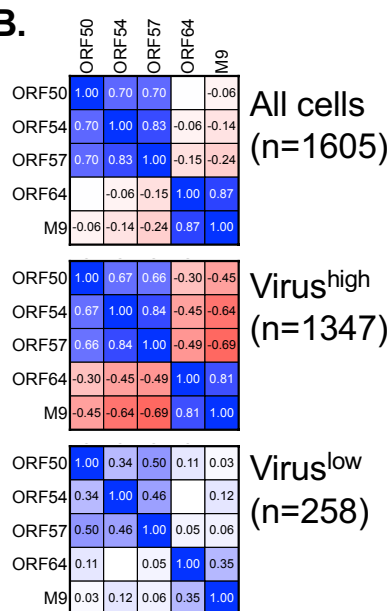
Virus^low Host^high　Virus^high Host^high　Virus^high Host^low

Figure 6
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Figure 7
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