

Multidimensional analysis of Gammaherpesvirus RNA expression reveals unexpected heterogeneity of gene expression

Lauren M. Oko¹, Abigail K. Kimball², Rachael E. Kaspar², Ashley N. Knox¹, Tim Chang³, Benjamin Alderete³, Linda F. van Dyk^{1*}, Eric T. Clambey^{2*#}

Affiliations:

¹ Department of Immunology and Microbiology, ² Department of Anesthesiology, University of Colorado Denver | Anschutz Medical Campus, Aurora, CO, 80045, USA

³ MilliporeSigma, a business of Merck KGaA, Darmstadt, Germany (Seattle, WA)

* Co-Corresponding authors:

Eric T. Clambey, eric.clambey@ucdenver.edu, 303-724-7783 (phone)

Linda F. van Dyk, linda.vandyk@ucdenver.edu, 303-724-4207 (phone)

Lead author for MS correspondence

Short title: Single-cell heterogeneity of Gammaherpesvirus RNA expression

ABSTRACT

Virus-host interactions are frequently studied in bulk cell populations, obscuring cell-to-cell variation. Here we investigate endogenous herpesvirus gene expression at the single-cell level, combining a sensitive and robust fluorescent in situ hybridization platform with multiparameter flow cytometry, to study the expression of gammaherpesvirus non-coding RNAs (ncRNAs) during lytic replication, latent infection and reactivation in vitro. This method allowed robust detection of viral ncRNAs of murine gammaherpesvirus 68 (γ HV68), Kaposi's sarcoma associated herpesvirus and Epstein-Barr virus, revealing variable expression at the single-cell level. By quantifying the inter-relationship of viral ncRNA, viral mRNA, viral protein and host mRNA regulation during γ HV68 infection, we find heterogeneous and asynchronous gene expression during latency and reactivation, with reactivation from latency identified by a distinct gene expression profile within rare cells. Further, during lytic replication with γ HV68, we find many cells have limited viral gene expression, with only a fraction of cells showing robust gene expression, dynamic RNA localization, and progressive infection. These findings, powered by single-cell analysis integrated with automated clustering algorithms, suggest inefficient or abortive γ HV infection in many cells, and identify substantial heterogeneity in viral gene expression at the single-cell level.

AUTHOR SUMMARY

The gammaherpesviruses are a group of DNA tumor viruses that establish lifelong infection. How these viruses infect and manipulate cells has frequently been studied in bulk populations of cells. While these studies have been incredibly insightful, there is limited understanding of how virus infection proceeds within a single cell. Here we present a new approach to quantify gammaherpesvirus gene expression at the single-cell level. This method allows us to detect cell-to-cell variation in the expression of virus non-coding RNAs, an important and understudied class of RNAs which do not encode for proteins. By examining multiple features of virus gene expression, this method further reveals significant variation in infection between cells across multiple stages of infection. These studies emphasize that gammaherpesvirus infection can be surprisingly heterogeneous when viewed at the level of the individual cell. Because this approach can be broadly applied across diverse viruses, this study affords new opportunities to understand the complexity of virus infection within single cells.

INTRODUCTION

The *Herpesviridae* are a family of large dsDNA viruses that include multiple prominent human and animal pathogens [1]. Although these viruses infect different cell types, and are associated with diverse pathologies, they share conserved genes and two fundamental phases of infection: lytic replication and latent infection [1]. Lytic replication is characterized by a cascade of viral gene expression, active viral DNA replication and the production of infectious virions. Conversely, latency is characterized by limited viral gene expression and the absence of de novo viral replication. While

latent infection is a relatively quiescent form of infection, the herpesviruses can reactivate from latency, to reinitiate lytic replication.

Among the herpesviruses, the gammaherpesviruses (γ HV) are lymphotropic viruses that include the human pathogens Epstein-Barr virus (EBV) [2] and Kaposi's sarcoma associated herpesvirus (KSHV) [3]. Murine gammaherpesvirus 68 (γ HV68, or MHV-68; ICTV nomenclature *Murid herpesvirus 4*, MuHV-4), is a well-described small animal model for the γ HVs [4]. While these viruses establish a lifelong infection that is often clinically inapparent, immune-suppressed individuals are particularly at risk for γ HV-associated malignancies [5].

Herpesvirus gene expression is extremely well-characterized in bulk populations. Despite increasing evidence for single-cell heterogeneity in gene expression [6-8], there remains limited understanding of herpesvirus infection at the single-cell level [9-12]. Here, we tracked endogenous viral and host RNAs using a sensitive, robust fluorescent in situ hybridization assay combined with multiparameter flow cytometry (PrimeFlow™) [13] to analyze the expression and inter-relationships of viral ncRNA, viral mRNA and cellular mRNA at the single-cell level during γ HV latency, reactivation and lytic replication. These studies revealed unanticipated heterogeneity of infection, emphasizing how single-cell analysis of virus infection can afford significant new insights into the complexity of γ HV infection.

RESULTS

Single-cell analysis of viral RNAs during lytic infection.

Traditional measurements of gene expression frequently rely on pooled cellular material, obscuring intercellular variation in gene expression. To better define expression of γ HV RNAs at the single cell level, we employed the PrimeFlowTM RNA assay [13] to study viral gene expression during murine gammaherpesvirus 68 (γ HV68) infection, a small animal γ HV [4, 13]. This method is a highly sensitive, extremely specific in situ hybridization assay, integrating Affymetrix-designed branched DNA technology with single-cell analysis powered by multiparameter flow cytometry. This method has been successfully used to detect both virus and host RNAs (e.g. in the context of HIV infected individuals [13, 14]).

We first tested the ability of PrimeFlowTM to measure multiple viral RNAs during lytic infection with γ HV68, including small non-coding RNAs (tRNA-miRNA encoding RNAs or TMERs [15]) and mRNAs. Mouse fibroblasts were infected with an intermediate multiplicity of infection (MOI=5 plaque forming units of virus/cell) resulting in a mixture of infected and uninfected cells. Under these conditions, TMER-5, one of the eight γ HV68 TMERs, and the γ HV68 gene 73, were readily detectable by conventional real-time PCR in γ HV68-infected, but not mock-infected, cultures (Fig. 1A, C). Parallel cultures were analyzed for RNA expression by PrimeFlowTM. Whereas mock-infected cells had no detectable expression of either the γ HV68 TMERs or gene 73, WT γ HV68-infected fibroblasts had a prominent population of TMER+ and gene 73+ cells, respectively (Fig. 1B and 1D). Infection of cells with a TMER-deficient γ HV68 (TMER-TKO [16]), in which TMER expression is ablated through promoter disruption,

revealed no detectable TMER expression (Fig. 1B), yet robust gene 73 expression (Fig. 1D). Parallel studies revealed ready detection of gene 18, another γ HV68 gene product (Fig. 1E). These studies show that PrimeFlowTM is a sensitive, robust and specific method to detect both viral non-coding and messenger RNAs during lytic infection, quantifying both the frequency of gene expression and expression on a per cell basis.

Heterogeneous gene expression during γ HV latency and reactivation from latency.

γ HV latency is characterized by limited gene expression. We next measured viral RNAs during latency and reactivation using the γ HV68-infected A20 HE2.1 cell line (A20. γ HV68), a drug-selected latency model with restricted viral gene expression that can reactivate following stimulation [17]. A20. γ HV68 cells are characterized by restricted viral gene expression, yet remain competent for reactivation from latency and the production of infectious virions following chemical stimulation with the phorbol ester, TPA [17, 18].

When we compared TMER expression between uninfected (parental, virus-negative A20) and infected (A20. γ HV68) cells by qRT-PCR, the viral ncRNA TMER-5 was exclusively detectable in A20. γ HV68 cells, with minimal changes between untreated and chemically-stimulated conditions (Fig. 2A). PrimeFlowTM analysis of TMER expression in untreated A20. γ HV68 cells revealed that a majority of these cells expressed the TMERs, as defined by a positive signal in samples subjected to the TMER probe relative to unstained cells (Fig. 2B). Untreated A20. γ HV68 cells contained a high frequency of cells expressing intermediate levels of TMERs (i.e. TMER^{mid} cells), with a significant signal enrichment above parental, virus-negative A20 cells (Fig. 2C).

While the frequency of TMER^{mid} cells remained relatively constant following TPA stimulation (compare “Untreated” versus “Stimulated”, Fig. 2C), stimulated A20.γHV68 cultures also contained a small fraction of cells with high levels of TMERs (i.e. TMER^{high} cells), not present in untreated cultures (Fig. 2C-D). Chemical stimulation is known to result in variable penetrance of reactivation in latently infected cell lines [17]. Based on this, we hypothesized that these rare, TMER^{high} cells may represent a subset of cells that are undergoing reactivation from latency.

To test this, we analyzed the properties of TMER^{mid} and TMER^{high} cells, comparing viral protein expression in untreated and stimulated A20.γHV68 cells. We analyzed: 1) a γHV68 expressed GFP-hygromycin resistance fusion protein (HygroGFP), under the control of a heterologous viral promoter (the CMV immediate early promoter) [17], and 2) the γHV68 regulator of complement activation (RCA), a viral protein encoded by the γHV68 gene 4 [19]. The vast majority of TMER^{mid} cells were negative for HygroGFP and RCA (i.e. HygroGFP- RCA-), regardless of whether the cells were present in untreated or stimulated cultures (Fig. 2E-F). Conversely, TMER^{high} cells, which were uniquely present in stimulated cultures, had a significantly increased frequency of HygroGFP+ cells with induction of RCA protein+ cells in a subset of cells when compared to TMER^{mid} cells present in either untreated or stimulated cultures (Fig. 2E-F). By using imaging flow cytometry, we further analyzed the subcellular localization of TMERs in TMER^{mid} cells compared with TMER^{high} RCA+ cells. TMERs were predominantly nuclear in both TMER^{mid} and TMER^{high} RCA+ cells, as defined by co-localization with DAPI fluorescence (Fig. 2G). These data demonstrate that the TMERs are expressed during latency, and that following reactivation-inducing stimulation,

TMERs are further induced in a rare subset of cells which are characterized by increased viral transcription and translation.

Detection of endogenous viral gene expression during KSHV latency and reactivation.

To extend these findings, we analyzed viral gene expression in the KSHV infected B cell tumor line, BCBL-1, focused on detection of an abundant viral ncRNA, the KSHV polyadenylated nuclear RNA (PAN, nut1, or T1.1) [20]. PAN RNA is known to be highly inducible upon induction of reactivation in KSHV latently infected B cell lymphoma cell lines [10, 20]. The frequency of PAN RNA+ cells was low in untreated BCBL-1 cells, with ~1% of cells spontaneously expressing this ncRNA (Fig. 3A-B). Despite the low frequency, this hybridization was clearly above background, as ncRNA defined on the KSHV- and EBV-negative B cell lymphoma cell line BL41 [21, 22] (Fig. 3A-B). Upon stimulation of BCBL-1 cells with the reactivation-inducing stimuli TPA and sodium butyrate, the frequency of PAN RNA+ cells significantly increased with expression in ~25% of cells (Fig. 3A-B). Although stimulation of BCBL-1 cells significantly increased the frequency of PAN RNA+ events compared to untreated cultures, PAN RNA expression on an individual cell basis was comparable between cells from untreated or stimulated cultures (Fig. 3C). As anticipated, stimulation of BCBL-1 cells was associated with increased viral DNA, consistent with stimulated cultures undergoing reactivation from latency (Supplemental Fig. 1).

We next analyzed the properties of BCBL-1 cells as a function of PAN RNA expression. In untreated cells, PAN RNA+ or RNA- cells had comparable cell size (define by forward scatter, FSC) and granularity (defined by side scatter, SSC). Gene

73 expression was low in untreated BCBL-1 samples, with signal intensity in PAN RNA- cells close to the background fluorescence observed in unstained samples. PAN RNA+ cells in untreated cultures had a modest increase in gene 73 expression relative to PAN RNA- cells (Fig. 3D-E). In stimulated BCBL-1 cultures, PAN RNA+ cells had a modest decrease in cell size (defined by forward scatter) and a trend towards reduced granularity (defined by side scatter) compared to PAN RNA- cells (Fig. 3F-G). Stimulated BCBL-1 cultures also had an increased gene 73 signal when compared to unstained samples (Fig. 3F), with PAN RNA+ cells again showing ~2-fold increase compared to PAN RNA- cells (Fig. 3F-G). These data demonstrate robust detection of PAN RNA by PrimeFlowTM, and further identify PAN RNA expression in a subset of both untreated and reactivation-induced BCBL-1 cells.

Detection of endogenous viral gene expression during EBV latency and reactivation.

EBV encodes two abundant non-coding RNAs, the EBV-encoded RNAs (EBERs) EBER1 and EBER2. We tested the ability of the PrimeFlowTM method to detect EBER in an EBV positive, Burkitt lymphoma type I latency cell line, Mutu I [23]. EBER expression was detected in a ~45% of Mutu I cells in either untreated or stimulated conditions, with no significant probe hybridization in the KSHV- and EBV-negative BL41 cell line (Fig. 4A). Stimulated Mutu I cells showed a modest, 2-fold increase in EBER expression on an individual cell basis, relative to untreated EBER+ cells (Fig. 4B). Based on these data, EBER expression in Mutu I cells appears to be constitutive, with stimulation under these conditions resulting in minimal consequences on either the frequency or per-cell expression of the EBERs.

Single-cell analysis of actin mRNA degradation as a readout of virus-induced host shutoff.

Many herpesviruses, including γ HV68, EBV and KSHV, induce host shutoff during lytic replication and reactivation from latency, a process characterized by dramatic decreases in host mRNAs [24, 25]. Consistent with published reports [24], qRT-PCR analysis of a cellular housekeeping gene, β -actin (Actb), showed reduced actin mRNA in γ HV68 lytically-infected fibroblasts by 18 hours pi (Fig. 5A). While mock-infected samples had a uniformly positive population of actin RNA^{high} cells detectable by PrimeFlowTM, γ HV68-infected fibroblast cultures demonstrated a bimodal distribution of actin RNA^{high} and actin RNA^{low} cells (Fig. 5B). The actin RNA^{low} population had a fluorescent signal that was only modestly above background fluorescence (defined by the “No probe” sample), suggesting an all-or-none phenomenon in which cells either had no change in actin RNA levels or had pronounced actin RNA degradation. Simultaneous analysis of TMER and actin RNA expression revealed that actin RNA^{low} cells were frequently TMER^{high}, with actin^{high} cells frequently TMER^{negative} at this time (Fig. 5C).

To determine whether actin RNA regulation could also be observed during γ HV68 latency and reactivation, we measured actin RNA levels in A20. γ HV68 cells. Parental, virus-negative A20 cells and A20. γ HV68 cells had relatively comparable actin RNA levels by qRT-PCR, in both untreated and stimulated cells (Fig. 5D). Given that host shutoff is expected to primarily occur in rare, reactivating cells, we measured actin RNA degradation relative to TMER expression by the PrimeFlowTM method. Untreated

A20. γ HV68 cultures had no discernable population of TMER^{low} actin RNA events, whereas stimulated cultures were characterized by a rare population of TMER^{high} actin RNA^{low} cells (Fig. 5E). We further compared actin RNA expression between TMER^{mid} and TMER^{high} cells, in untreated versus stimulated cultures using our previously defined subpopulations (Fig. 2). While TMER^{mid} cells from either untreated or stimulated cultures were predominantly actin RNA+, TMER^{high} cells from stimulated cultures showed a significant increased frequency of actin RNA^{low} events (Fig. 5F-G). These studies reveal actin RNA as a sensitive indicator of virus-induced host shutoff, and demonstrate this as an all-or-none phenomenon that can be readily queried at the single-cell level.

Heterogeneity of gene expression during de novo lytic replication.

Next, we revisited our analysis of gene expression during de novo lytic infection of fibroblasts, to examine co-expression relationships between viral ncRNA (TMERs), viral mRNA (the γ HV68 gene 73), viral protein (RCA protein) and cellular actin mRNA degradation [19, 24]. Fibroblast cultures were infected with an intermediate multiplicity of infection to produce a mixture of uninfected and infected cells, and then subjected to the PrimeFlowTM method.

To enable an unbiased, automated analysis of gene expression profiles in γ HV68 lytically infected cells relative to mock infected cells, data were subjected to the automated clustering algorithm X-shift [26], to identify potential subpopulations of cells with heterogeneous gene expression in these cultures. By sampling 1,000 cells from multiple mock- and virus-infected cultures, the X-shift algorithm consistently identified 7

major clusters of cells (Fig. 6A) defined by varying gene expression patterns. While some of the clusters were exclusively found in mock-infected cultures, virus-infected cultures contained three broad types of cell clusters: 1) uninfected cells, with no viral gene expression and normal actin RNA, 2) fully infected cells, with robust expression of the TMERs, gene 73, actin RNA downregulation and frequent expression of the RCA protein, and 3) intermediate populations characterized by variable expression of the TMERs and gene 73 (Fig. 6A).

To validate these findings using a more conventional method, we compared TMER and gene 73 RNA co-expression on a biaxial plot. By comparing mock-infected, WT-infected and TMER-TKO-infected cultures, this analysis revealed five populations of gene expression (Fig. 6B), including cells with: 1) no detectable expression of either viral RNA (TMER- gene 73-), 2) TMER+ gene 73- cells (bottom right quadrant), 3) TMER- gene 73+ cells (upper left quadrant), 4) TMER^{low} gene 73^{low} cells (lower left edge of the upper right quadrant), and 5) TMER^{high} gene 73^{high} cells (upper right quadrant). The definition of TMER positive events was defined based on background fluorescent levels observed in TMER-TKO infected cultures (Fig. 6B). These 5 populations were each assigned a unique color for subsequent analysis (Fig. 6C).

We then compared the cellular phenotype and gene expression within these 5 distinct populations. Analysis of TMERs, gene 73, actin RNA, RCA protein, cell size (forward scatter), and granularity (side scatter) revealed multiple types of viral gene expression. As expected, TMER- gene 73- cells (in black) had no evidence of virus infection, with no detectable viral protein (RCA) or actin downregulation (Fig. 6D-E). Cells with low expression of either the TMERs and/or gene 73 contained viral RNAs, but

had minimal expression of either viral protein or actin downregulation (Fig. 6D-E). In stark contrast, cells that were TMER^{high} gene 73^{high} (in red, Fig. 6D-E) had multiple characteristics of progressive virus infection including a prominent fraction of cells that expressed RCA and/or had actin RNA downregulation. Further, TMER^{high} gene 73^{high} cells were consistently smaller in cell size (defined by forward scatter, FSC) and higher in granularity (defined by side scatter, SSC), a feature that was unique to this phenotype (Fig. 6D-E).

Given the heterogeneous patterns of RNA and protein expression among lytically-infected cells, we next queried TMER subcellular localization as a function of viral gene expression using imaging flow cytometry. While the majority of TMER+ cells had a primarily nuclear TMER localization (defined by DAPI co-localization, as in [27]), the frequency of cells with nuclear TMER localization was highest among TMER+ gene 73- cells and lowest among TMER+ gene 73+ RCA+ cells (Fig. 7A-B). These data suggest that the TMERs can be localized in either the nucleus or cytoplasm during γ HV68 lytic replication, and that this localization is not strictly a function of magnitude of gene expression.

Finally, we used tSNE, a dimensionality reduction algorithm, to better delineate the relationship between TMER, gene 73, RCA protein and actin downregulation across populations defined by variable TMER and gene 73 expression. Consistent with our histogram analysis (Fig. 6D-E), uninfected and intermediate populations that expressed either TMERs or gene 73 were relatively uniform in gene expression (Fig. 7C). In contrast, TMER^{high} gene 73^{high} cells expressed a wider array of phenotypes, including both a predominant fraction of cells that were actin RNA^{low} RCA+, as well as a distinct

group of cells that were actin RNA⁺ RCA⁻ (Fig. 7C). Notably, RCA expression and actin degradation were inversely correlated, with very few cells that expressed RCA also high for actin RNA. Actin RNA⁺ populations among TMER^{high} gene 73^{high} cells were associated with larger cell size (Supplementary Fig. 2). The diversity of phenotypes among TMER^{high} gene 73^{high} cells was confirmed by biaxial gating of actin RNA versus RCA protein expression (Fig. 7D). In total, these data indicate heterogeneous progression of lytic replication in vitro. While some cells have robust viral mRNA and protein expression, additional cell subsets are characterized by limited or divergent gene expression.

DISCUSSION

Herpesvirus gene expression has been historically analyzed in bulk cell populations. These studies have provided an essential cornerstone to understanding the transcriptional and translational capacity of the herpesviruses. Despite this, recent studies on cellular and viral transcription from other systems have emphasized a high degree of cell-to-cell variation in gene expression [6-11, 13, 14], something we have further investigated here. By applying the PrimeFlow™ methodology to measure endogenous viral gene expression across multiple gammaherpesviruses, and multiple stages of infection, we have gained critical new insights into the inter-relationships of gene expression at the single-cell level.

A primary focus of the current study has been to analyze expression of γ HV ncRNAs. Although the TMERs, EBERs and PAN RNA all represent abundant γ HV ncRNAs, these ncRNAs are transcribed by distinct mechanisms: KSHV PAN is a highly-inducible, RNA pol II-transcribed ncRNA [20], in contrast to the RNA pol III-transcribed TMERs and EBERs [15, 28]. This differential regulation was mirrored in the expression patterns we observed. Whereas TMERs and EBERs were detected in a large fraction of latently infected cells, PAN RNA was expressed in a low frequency of latently infected cells, with prominent induction following cell stimulation and the induction of reactivation. The viral ncRNAs were efficiently detected, as might be predicted due to their abundance. The viral gene 73 encodes a transcription factor that is expressed at a far lower level and are also efficiently detected, demonstrating that rare mRNAs can be measured coincidentally with abundant RNAs and with proteins, with no modifications required. A unique advantage of our current approach is the ability to measure the

frequency of ncRNA expressing cells and changes in expression within individual cells. This has been particularly insightful for the identification of rare PAN RNA⁺ cells in untreated BCBL-1 cells and a TMER^{high} subpopulation of cells in reactivating A20.γHV68 cells. Integrating this method with cell sorting will afford future opportunities to investigate unique properties of these rare cell populations.

Among the viral ncRNAs measured, in-depth analysis of TMER expression during γHV68 infection has revealed new insights into infection. In the context of latency, the TMERs are constitutively expressed in many, but not all, latently infected cells using the A20.γHV68 model. Further, stimulating these cells to undergo reactivation has a minimal effect on the frequency of cells expressing intermediate levels of TMERs (i.e. TMER^{mid} cells), instead resulting in the appearance of a minor population of TMER^{high} cells. Notably, TMER^{high} cells show additional features of lytic cycle progression, including actin RNA degradation and RCA protein expression. Why only some latently infected cells show the TMER^{high} phenotype, and what regulates the inducible expression of the RNA pol III-transcribed TMERs remain important questions raised by this analysis.

Of the γHVs studied here, only γHV68 has a robust in vitro lytic replication system. Our studies on γHV68 lytic replication revealed multiple unanticipated results. By using cultures that contained both infected and uninfected cells, our analysis identified at least four different subsets of cells, stratified by differential viral gene expression of the TMERs and gene 73. Strikingly, during lytic replication there were many cells with limited viral gene expression, expressing low levels of either the TMERs and/or gene 73, but lacking additional signs of virus gene expression (i.e. actin RNA

degradation or RCA protein expression). Conversely, only some viral RNA⁺ cells showed robust viral expression characterized by a constellation of gene expression, defined as TMER^{high} gene 73^{high} actin RNA^{low} RCA⁺. While there is precedence that reactivation from latency in KSHV infection can be asynchronous [9], this heterogeneity of viral gene expression during in vitro lytic replication was unanticipated and suggests that lytic infection under these reductionist conditions is either asynchronous, abortive, or inefficient. This heterogeneity of gene expression raises important questions regarding the universality of the prototypical cascade of immediate early, early and late gene expression that is widely accepted in the herpesvirus field and suggests additional levels of complexity that may be obscured by bulk cell analysis.

This method allows multiplexed analysis of single-cell gene expression, to both directly measure viral RNAs and downstream consequences of gene expression including viral protein production and host RNA degradation, secondary to protein translation. This approach has notable advantages to conventional analyses of gene expression: 1) it can measure endogenous viral gene expression (both mRNA and ncRNA) in the absence of recombinant viruses or marker genes, and 2) it can rapidly analyze gene and protein expression inter-relationships, across millions of cells, providing unique complementary strengths to other single-cell methodologies (e.g. single-cell RNA-seq). In future, this method can be further integrated with additional antibody-based reagents, to simultaneously query post-translational modifications (e.g. protein phosphorylation) as a function of cell cycle stage. It is also notable that through the use of imaging flow cytometry, it is possible to interrogate subcellular RNA and protein localization throughout distinct stages of virus infection.

369 In total, these studies demonstrate the power of single-cell analysis of
370 herpesvirus gene expression. Our data emphasize the heterogeneity of γ HV gene
371 expression at the single-cell level. The factors that underlie this heterogeneity are
372 currently unknown, but could reflect either asynchronous or inefficient infection in many
373 infected cells (e.g. in the context of lytic infection). Whether this variation arises from
374 viral or cellular heterogeneity is a fundamental question for future research.

MATERIALS AND METHODS

Viruses and tissue culture. All γ HV68 viruses were derived from the γ HV68 strain WUMS (ATCC VR-1465) [29], using bacterial artificial chromosome-derived wild-type (WT) γ HV68 or γ HV68.TMER-Total KnockOut (TMER-TKO) [16]. Virus stocks were passaged, grown, and titered as previously described [16]. Mouse 3T12 fibroblasts (ATCC CCL-164) were inoculated with 5 plaque forming units/cell for one hour, followed by inoculum removal and replacement with fresh media, with analysis between 8-18 hpi. Parental, virus-negative A20 B cells, or A20. γ HV68 (HE2.1) B cells [17], were treated with vehicle (untreated) or stimulated with 12-O-tetradecanolphorbol-13-acetate (TPA) 20 ng/ml (Sigma) (in DMSO) harvested 24 hr later. BCBL-1 B cells, latently infected with KSHV (HHV-8), were cultured in RPMI containing 20% FBS, 1% Penicillin/Streptomycin with L-glutamine, 1% HEPES and 50 μ M β ME. BCBL-1 B cells were treated with vehicle (untreated) or stimulated with 20 ng/ml TPA (in DMSO) and Sodium Butyrate (NaB) 0.3 mM (Calbiochem) (in water) and then harvested 72 hr later. BL41 B cells (negative for KSHV and EBV) were cultured in RPMI with 10% FBS, 1% Penicillin/Streptomycin with L-glutamine, and 50 μ mol β ME. Mutu I cells, an EBV-infected, type I latency Burkitt's lymphoma cell line [23] were cultured in RPMI with 10% FBS, 1% Penicillin/Streptomycin and L-glutamine. Mutu I or BL41 B cells were either treated with vehicle (DMSO) or stimulated with 20 ng/ml TPA (in DMSO) and then harvested 48 hr later. BCBL-1 and BL41 cells were generously provided by Dr. Rosemary Rochford (University of Colorado), with additional BCBL-1 cells obtained from the NIH AIDS reagent program (catalog # 3233). Mutu I cells were generously provided by Dr. Shannon Kenney (University of Wisconsin).

Flow cytometric analysis. Cells were harvested at the indicated time points and processed for flow cytometry using the PrimeFlow™ RNA Assay (Thermo Fisher). Cells were incubated with an Fc receptor blocking antibody (2.4G2) for 10 min and then fixed with 2% PFA (Fisher), washed with PBS (Life Technology). Cells were stained with a rabbit antibody against the γ HV68 gene 4 protein, regulator of complement activation (RCA) [19], labeled with Zenon R-phycoerythrin rabbit IgG label reagent (Life Technologies) following manufacturer's protocol. Samples were subjected to the PrimeFlow™ RNA Assay following manufacturer's protocols, using viral and host target probes conjugated to fluorescent molecules (Table S1). DAPI (BioLegend) was used on a subset of samples following manufacturer's protocol, prior to PrimeFlow™ probe hybridization. Flow cytometric analysis was done on LSR II (BD Biosciences), Fortessa (BD Biosciences), and ZE5 (Bio-Rad) flow cytometers, with compensation values based on antibody-stained beads (BD Biosciences), and modified as needed post-collection using FlowJo.

Imaging flow cytometry. Cells were treated as described above then harvested, and split into two aliquots: one for conventional flow cytometry, and one for imaging flow cytometry, acquired on an Amnis ImageStream®^X Mark II imaging flow cytometer (MilliporeSigma) with a 60X objective and low flow rate/high sensitivity using INSPIRE® software. Brightfield (BF) and side scatter (SSC) images were illuminated by LED light and a 785nm laser respectively. Fluorescent probes were excited off 405nm, 488nm, and 642nm lasers with the power adjusted properly to avoid intensity saturation of the

camera. Single color controls for compensation were acquired by keeping the same acquisition setting for samples, with the difference of turning the BF LED light and 785nm (SSC) laser off.

The acquired data were analyzed using IDEAS[®] software (MilliporeSigma). Single cells that were in focus were defined as a population with a high “gradient RMS” value, an intermediate “Area” value, and a medium to high “Aspect ratio” value for subsequent analysis. Positive and negative events for each fluorescent marker were determined using the “Intensity” feature. TMER nuclear localization was quantified using “Similarity” feature, the log-transformed Pearson’s correlation coefficient by analyzing the pixel values of two image pairs [27]. The degree of nuclear localization of TMER was measured by correlating the pixel intensity of two images with the same spatial registry. The paired TMER and DAPI images were quantified by measuring the “Similarity Score” which cells with high similarity scores display high TMER nuclear localization with similar image pairs. By contrast cells with low similarity scores show low TMER nuclear localization with dissimilar image pairs.

RNA and qRT-PCR. RNA was isolated using Trizol (Life Technologies) per manufacturer’s protocol and re-suspended in DEPC treated water. 3 µg of RNA was treated with DNase 1 (Promega) for 2 hours at 37°C, heat inactivated for 10 min at 65°C. 500 ng of RNA was then subjected to reverse transcription using SuperScript II (Life Technologies) following manufacturer’s protocol for gene specific, oligo(dT), or random primers (Life Technologies). Quantitative PCR (qPCR) was performed using iQ SYBR Green super mix (Bio-Rad) follow manufacturer’s protocol using host and viral

primer sets (Table S2) or using QuantiTech Primer Assay (Qiagen) for 18s (Hs-RRN18S_1_SG). qPCR conditions: 3 min at 95°C, amplification cycles for 40 cycles of 15 sec at 95°C, annealing/ extension at temperature for specific primer set for 1 min ending with a melt curve which started at 50°C or 55°C to 95°C increasing 0.5°C for 0:05 sec. A standard curve for each primer set was generated by pooling a portion of each sample together and doing a 1:3 serial dilution. 75 ng of cDNA of the unknown samples was loaded per qPCR reaction/primer set, with reactions run on a Bio-Rad 384 CFX LightCycler and data analyzed using Bio-Rad CFX manager software. Data analysis was done using the 1:3 standard curve as the control Ct value to calculate the delta ct, and the Pfaffl equation was used to define the fold difference between the gene of interest and 18s (reference gene) [30]. qPCR products were analyzed by melt curve analysis, with all reactions having a prominent, uniform product. In the case of primers with an aberrant melt curve product (e.g. that arose at late cycles), products were clearly a different product as defined by melt curve analysis.

KSHV genome quantification. BCBL-1 or BL41 cells were plated at 7.5e5 cells/well in a 6 well plate with 20 ng/ml TPA and 0.3 mM NaB or vehicle only (DMSO and H₂O). Cells and supernatant were harvested at 72 hrs post-treatment, hard-spun for 30 min at 4° C and DNA was isolated using the DNeasy Blood and Tissue kit following manufacturer's protocol, except for sample digestion for 1 hour instead of 10 min. 100 ng of DNA per sample was used for qPCR analysis via SYBR green detection using KSHV ORF50 primers (5' -TCC GGC GGA TAT ACC GTC AC- 3' and 5'- GGT GCA GCT GGT ACA GTG TG-3') [31]. qPCR was analyzed using relative quantification normalized against

unit mass calculation, ratio = $E^{\Delta Ct}$ (Real-Time PCR Application Guide, Bio-Rad Laboratories Inc. 2006).

Software and Statistical analysis. All flow cytometry data were analyzed in FlowJo (version 8.8.7 or 10.5.0), with flow cytometry data shown either as histogram overlays or pseudo-color dot plots (with or without smoothing), showing outliers (low or high resolution) on log₁₀ scales. Statistical analysis and graphing were done in GraphPad Prism (Version 6.0d and 7.0d). Statistical significance was tested by unpaired t test (when comparing two conditions) or by one-way ANOVA (when comparing three or more samples) subjected to multiple corrections tests using recommended settings in Prism. X-shift analysis: For automated mapping of flow cytometry data using X-shift, data were obtained from compensated flow cytometry files, exported from FlowJo, using singlets that were live (defined by sequential gating on single cells by FSC-H vs. FSC-W and SSC-H vs. SSC- W, that were DAPI bright vs. SSC-A). These events were imported into the Java based program Vortex (<http://web.stanford.edu/~samusik/vortex/>) [26]. Four parameters [TMER (AlexaFluor (AF) 488), RCA (PE), gene 73 (AF647), and Actin (AF750)] were selected for clustering analysis using the X-shift algorithm. The following settings were used when importing the data set into Vortex: i) Numerical transformation: arcsinh(x/f), f=150, ii) noise threshold: apply noise threshold of 1.0 (automatic and recommended setting), iii) feature rescaling: none, and iv) normalization: none, v) a Euclidean noise filter was used with a Minimal Euclidean length of the profile of 1.0, and vi) an import max of 1,000 rows from each file after filtering was selected. The following settings were used when preparing the data set for clustering analysis: i) distance

measure: angular distance, ii) clustering algorithm: X-shift (gradient assignment), iii) density estimate: N nearest neighbors (fast), iv) number of neighbors for density estimate (K): from 150 to 5, with 30 steps, and v) number of neighbors for mode finding (N): determine automatically. After the cluster analysis was completed, all results were selected and the K value that corresponded with optimal clustering (the elbow point) was calculated, in this case K= 50. All clusters (seven clusters total) for the optimal K value were selected and a Force-Directed Layout was created. The maximum number of events sampled from each cluster was 20, and the number of nearest neighbors was 10. All settings used for this analysis were automated or explicitly recommended (<https://github.com/nolanlab/vortex/wiki>). Force-Directed layouts in Fig. 6A were saved as graphml files from Vortex, opened in the application Gephi v 0.9.1, and colored by different variables (Cluster ID, experimental group, Actin mRNA, RCA, Gene 73, and TMERs respectively) in Adobe Illustrator CC 2017. Full details on use of the X-shift algorithm and analysis pipeline can be found in [32]. tSNE analysis: Gated events for each of the six identified populations were exported from FlowJo, and then imported into Cytobank (www.cytobank.org) for analysis using the viSNE algorithm. Each file was used for a separate viSNE analysis (six total runs), where all available events were selected for clustering (202,669, 128,028, 29,096, 5,610, 8,956, 35,850 respectively) and four parameters were selected for clustering (Actin mRNA, RCA, Gene 73, and TMERs). The resulting tSNE plots were colored according to expression using the "rainbow" color option, with individual events shown using the stacked dot option. The channel range was user-defined for each marker according to the range in expression established in Fig. 6E.

ACKNOWLEDGMENTS

The authors would like to acknowledge insightful comments made by members of the Clambey and van Dyk laboratories, support of flow cytometry services through ClinImmune, the Dept. of Immunology & Microbiology, and the University of Colorado Cancer Center, the provision of KSHV and EBV cell lines by Dr. Rosemary Rochford (University of Colorado) and Dr. Shannon Keeney (University of Wisconsin), and expert technical guidance from Matt Cato and Dr. Nori Ueno (Thermo Fisher). T.C. and B.A. are employees of EMD Millipore and have a potential conflict of interest. E.T.C. was a recipient of the 2016 North America Affymetrix Single-cell Grant Recipient, for studies unrelated to this manuscript.

FUNDING

This research was funded by National Institutes of Health grants R01CA103632 and R01CA168558 to L.F.V.D., R21AI134084 to E.T.C. and L.F.V.D., and by an American Heart Association National Scientist Development grant (#13SDG14510023), a Colorado CTSI Novel methods development grant, and funding from the University of Colorado Dept. of Anesthesiology to E.T.C.. The Colorado CTSI is supported by NIH/NCATS Colorado CTSA Grant Number UL1 TR002535. Contents are the authors' sole responsibility and do not necessarily represent official NIH views. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

536 **AUTHOR CONTRIBUTIONS**

537 Conceptualization: Eric T. Clambey, Linda F. van Dyk

538 Data curation: Lauren M. Oko, Eric T. Clambey

539 Formal analysis: Lauren M. Oko, Abigail K. Kimball, Rachael E. Kaspar, Benjamin

540 Alderete, Tim Chang, Eric T. Clambey

541 Funding acquisition: Eric T. Clambey, Linda F. van Dyk

542 Investigation: Lauren M. Oko, Ashley N. Knox

543 Methodology: Lauren M. Oko, Benjamin Alderete, Tim Chang, Linda F. van Dyk, Eric T.

544 Clambey

545 Project administration: Eric T. Clambey, Linda F. van Dyk

546 Resources: Lauren M. Oko, Benjamin Alderete, Tim Chang

547 Software: Abigail K. Kimball

548 Supervision: Eric T. Clambey, Linda F. van Dyk

549 Validation: Lauren M. Oko, Eric T. Clambey

550 Visualization: Lauren M. Oko, Abigail K. Kimball, Rachael E. Kaspar, Benjamin

551 Alderete, Tim Chang, Eric T. Clambey

552 Writing – original draft: Eric T. Clambey, Linda F. van Dyk

553 Writing – review & editing:

554

REFERENCES

1. Pellett PJ, Roizman B. *Herpesviridae*. Fields Virology. 6th ed2013.
2. Longnecker RM, Kieff, E., Cohen, J.I. Epstein-Barr Virus. Fields Virology. 6th ed2013.
3. Ganem D. KSHV infection and the pathogenesis of Kaposi's sarcoma. Annu Rev Pathol. 2006;1:273-96. doi: 10.1146/annurev.pathol.1.110304.100133. PubMed PMID: 18039116.
4. Barton E, Mandal P, Speck SH. Pathogenesis and host control of gammaherpesviruses: lessons from the mouse. Annual review of immunology. 2011;29:351-97. doi: 10.1146/annurev-immunol-072710-081639. PubMed PMID: 21219186.
5. Cesarman E. Gammaherpesviruses and lymphoproliferative disorders. Annu Rev Pathol. 2014;9:349-72. doi: 10.1146/annurev-pathol-012513-104656. PubMed PMID: 24111911.
6. Shalek AK, Satija R, Adiconis X, Gertner RS, Gaublomme JT, Raychowdhury R, et al. Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature. 2013;498(7453):236-40. doi: 10.1038/nature12172. PubMed PMID: 23685454; PubMed Central PMCID: PMC3683364.
7. Battich N, Stoeger T, Pelkmans L. Control of Transcript Variability in Single Mammalian Cells. Cell. 2015;163(7):1596-610. doi: 10.1016/j.cell.2015.11.018. PubMed PMID: 26687353.
8. Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. Science.

- 2015;348(6233):aaa6090. doi: 10.1126/science.aaa6090. PubMed PMID: 25858977;
PubMed Central PMCID: PMCPMC4662681.
9. Adang LA, Parsons CH, Kedes DH. 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*. 2006;80(20):10073-82. doi: 10.1128/JVI.01156-06. PubMed PMID: 17005685; PubMed Central PMCID: PMCPMC1617294.
10. Borah S, Nichols LA, Hassman LM, Kedes DH, Steitz JA. Tracking expression and subcellular localization of RNA and protein species using high-throughput single cell imaging flow cytometry. *RNA*. 2012;18(8):1573-9. doi: 10.1261/rna.033126.112. PubMed PMID: 22745225; PubMed Central PMCID: PMCPMC3404377.
11. Ma JZ, Russell TA, Spelman T, Carbone FR, Tschärke DC. Lytic gene expression is frequent in HSV-1 latent infection and correlates with the engagement of a cell-intrinsic transcriptional response. *PLoS Pathog*. 2014;10(7):e1004237. doi: 10.1371/journal.ppat.1004237. PubMed PMID: 25058429; PubMed Central PMCID: PMCPMC4110040.
12. Shnayder M, Nachshon A, Krishna B, Poole E, Boshkov A, Binyamin A, et al. Defining the Transcriptional Landscape during Cytomegalovirus Latency with Single-Cell RNA Sequencing. *MBio*. 2018;9(2). Epub 2018/03/15. doi: 10.1128/mBio.00013-18. PubMed PMID: 29535194; PubMed Central PMCID: PMCPMC5850328.
13. Porichis F, Hart MG, Griesbeck M, Everett HL, Hassan M, Baxter AE, et al. High-throughput detection of miRNAs and gene-specific mRNA at the single-cell level by flow

cytometry. Nat Commun. 2014;5:5641. doi: 10.1038/ncomms6641. PubMed PMID: 25472703; PubMed Central PMCID: PMCPMC4256720.

14. Baxter AE, Niessl J, Fromentin R, Richard J, Porichis F, Charlebois R, et al. Single-Cell Characterization of Viral Translation-Competent Reservoirs in HIV-Infected Individuals. Cell Host Microbe. 2016;20(3):368-80. doi: 10.1016/j.chom.2016.07.015. PubMed PMID: 27545045; PubMed Central PMCID: PMCPMC5025389.

15. Diebel KW, Claypool DJ, van Dyk LF. A conserved RNA polymerase III promoter required for gammaherpesvirus TMER transcription and microRNA processing. Gene. 2014;544(1):8-18. doi: 10.1016/j.gene.2014.04.026. PubMed PMID: 24747015.

16. Diebel KW, Oko LM, Medina EM, Niemeyer BF, Warren CJ, Claypool DJ, et al. Gammaherpesvirus small noncoding RNAs are bifunctional elements that regulate infection and contribute to virulence in vivo. MBio. 2015;6(1):e01670-14. doi: 10.1128/mBio.01670-14. PubMed PMID: 25691585; PubMed Central PMCID: PMCPMC4337559.

17. Forrest JC, Speck SH. 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. 2008;82(15):7688-99. doi: 10.1128/JVI.02689-07. PubMed PMID: 18495760; PubMed Central PMCID: PMCPMC2493333.

18. Cheng BY, Zhi J, Santana A, Khan S, Salinas E, Forrest JC, et al. Tiled microarray identification of novel viral transcript structures and distinct transcriptional profiles during two modes of productive murine gammaherpesvirus 68 infection. J Virol.

2012;86(8):4340-57. doi: 10.1128/JVI.05892-11. PubMed PMID: 22318145; PubMed Central PMCID: PMCPMC3318610.

19. Kapadia SB, Molina H, van Berkel V, Speck SH, Virgin HW. Murine gammaherpesvirus 68 encodes a functional regulator of complement activation. J Virol. 1999;73(9):7658-70. PubMed PMID: 10438856; PubMed Central PMCID: PMC104293.

20. Sun R, Lin SF, Gradoville L, Miller G. Polyadenylylated nuclear RNA encoded by Kaposi sarcoma-associated herpesvirus. Proceedings of the National Academy of Sciences of the United States of America. 1996;93(21):11883-8. Epub 1996/10/15. PubMed PMID: 8876232; PubMed Central PMCID: PMCPMC38153.

21. Marchini A, Longnecker R, Kieff E. Epstein-Barr virus (EBV)-negative B-lymphoma cell lines for clonal isolation and replication of EBV recombinants. J Virol. 1992;66(8):4972-81. Epub 1992/08/01. PubMed PMID: 1321281; PubMed Central PMCID: PMCPMC241347.

22. Cao S, Strong MJ, Wang X, Moss WN, Concha M, Lin Z, et al. High-throughput RNA sequencing-based virome analysis of 50 lymphoma cell lines from the Cancer Cell Line Encyclopedia project. J Virol. 2015;89(1):713-29. doi: 10.1128/JVI.02570-14. PubMed PMID: 25355872; PubMed Central PMCID: PMC4301145.

23. Gregory CD, Rowe M, Rickinson AB. Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line. The Journal of general virology. 1990;71 (Pt 7):1481-95. Epub 1990/07/01. doi: 10.1099/0022-1317-71-7-1481. PubMed PMID: 2165133.

24. Covarrubias S, Richner JM, Clyde K, Lee YJ, Glaunsinger BA. Host shutoff is a conserved phenotype of gammaherpesvirus infection and is orchestrated exclusively

- from the cytoplasm. J Virol. 2009;83(18):9554-66. doi: 10.1128/JVI.01051-09. PubMed PMID: 19587049; PubMed Central PMCID: PMC2738246.
25. Rowe M, Glaunsinger B, van Leeuwen D, Zuo J, Sweetman D, Ganem D, et al. Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(9):3366-71. doi: 10.1073/pnas.0611128104. PubMed PMID: 17360652; PubMed Central PMCID: PMC1805610.
26. Samusik N, Good Z, Spitzer MH, Davis KL, Nolan GP. Automated mapping of phenotype space with single-cell data. Nature methods. 2016;13(6):493-6. doi: 10.1038/nmeth.3863. PubMed PMID: 27183440; PubMed Central PMCID: PMCPMC4896314.
27. Maguire O, Collins C, O'Loughlin K, Miecznikowski J, Minderman H. Quantifying nuclear p65 as a parameter for NF-kappaB activation: Correlation between ImageStream cytometry, microscopy, and Western blot. Cytometry A. 2011;79(6):461-9. doi: 10.1002/cyto.a.21068. PubMed PMID: 21520400; PubMed Central PMCID: PMCPMC3140714.
28. Rosa MD, Gottlieb E, Lerner MR, Steitz JA. Striking similarities are exhibited by two small Epstein-Barr virus-encoded ribonucleic acids and the adenovirus-associated ribonucleic acids VAI and VAII. Mol Cell Biol. 1981;1(9):785-96. Epub 1981/09/01. PubMed PMID: 9279391; PubMed Central PMCID: PMCPMC369362.
29. Virgin HWt, Latreille P, Wamsley P, Hallsworth K, Weck KE, Dal Canto AJ, et al. Complete sequence and genomic analysis of murine gammaherpesvirus 68. J Virol. 1997;71(8):5894-904. PubMed PMID: 9223479; PubMed Central PMCID: PMC191845.

30. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29(9):e45. PubMed PMID: 11328886; PubMed Central PMCID: PMCPMC55695.
31. Li Q, He M, Zhou F, Ye F, Gao SJ. Activation of Kaposi's sarcoma-associated herpesvirus (KSHV) by inhibitors of class III histone deacetylases: identification of sirtuin 1 as a regulator of the KSHV life cycle. J Virol. 2014;88(11):6355-67. Epub 2014/03/29. doi: 10.1128/JVI.00219-14. PubMed PMID: 24672028; PubMed Central PMCID: PMCPMC4093851.
32. Kimball AK, Oko LM, Bullock BL, Nemenoff RA, van Dyk LF, Clambey ET. A Beginner's Guide to Analyzing and Visualizing Mass Cytometry Data. J Immunol. 2018;200(1):3-22. Epub 2017/12/20. doi: 10.4049/jimmunol.1701494. PubMed PMID: 29255085; PubMed Central PMCID: PMCPMC5765874.

FIGURE LEGENDS

Figure 1. RNA-Flow cytometry using the PrimeFlow™ method affords robust and sensitive analysis of endogenous γ HV genes at the single-cell level. Viral RNA analysis in γ HV68-infected fibroblasts by qRT-PCR (A, C) or by flow cytometric analysis using PrimeFlow™ at 16 hpi (B, D, E, F). Samples were either mock, TMER-TKO, or WT γ HV68-infected, with infections done using 5 plaque forming units/cell. qRT-PCR standardized to 18s RNA, at the indicated times. All flow cytometric events gated on a generous FSC x SSC gate, followed by singlet discrimination. PrimeFlow™ analysis quantified probe fluorescence for (B) TMERs, (D) gene 73, or (E) gene 18, relative to side-scatter area (SSC-A). Probe fluorescence is indicated, with all probes detected using either AlexaFluor (AF) 647 or 488 conjugates. Data representative of 2 independent experiments, each done with biological replicates.

Figure 2. Heterogeneous gene expression in a γ HV68 latently infected B cell line. Viral, host RNA analysis during γ HV68 latency and reactivation in A20. γ HV68 (HE2.1) cells by qRT-PCR (A) and flow cytometric analysis using PrimeFlow™ (B-G), comparing untreated or TPA-stimulated samples at 24 hrs post-treatment. Analysis includes A20, virus-negative cells and A20. γ HV68 cells. (A) qRT-PCR analysis of TMER-5 expression relative to 18s RNA in A20 and A20. γ HV68 (HE2.1) cells, untreated or stimulated with TPA for 24 hrs. (B) PrimeFlow™ detection of TMER expression in A20. γ HV68 (HE2.1) cells, comparing either samples that were unstained (solid gray) or stained for the TMERs (open black line). (C) Analysis of TMER expression in multiple conditions,

comparing untreated and stimulated A20 and A20.γHV68 cells, with gates defining the frequency of events that expressed either intermediate (mid) or high levels of TMERs. Data depict lymphocytes that were singlets, defined by sequential removal of doublets. (D) Quantification of the frequency of TMER^{high} cells in stimulated A20.γHV68 cells. (E) Histogram overlays of HygroGFP and RCA protein expression in A20.γHV68 cells comparing TMER^{mid} cells from untreated cultures (top, gray), TMER^{mid} cells from TPA-stimulated cultures (middle, blue), with TMER^{high} cells from stimulated cultures (bottom, red). (F) Quantification of the frequencies of HygroGFP+ (left) and RCA protein+ (right) cells as a function of TMER expression and treatment condition. (G) Flow cytometric analysis on an imaging flow cytometer, with each row showing an individual cell and representative images of brightfield (BF), RCA protein (RCA), DAPI, and TMER localization in TMER^{mid} cells from untreated cultures (left) or TMER^{high} RCA+ cells from stimulated (right) A20.γHV68 cells. Data are from two independent experiments, with biological replicates within each experiment for all A20.γHV68 cultures. Graphs depict the mean ± SEM, with each symbol identifying data from a single replicate. Statistical analysis was done using an unpaired t test (D) or one-way ANOVA, subjected to Tukey's multiple comparison test (A, F), with statistically significant differences as indicated, * p<0.05, **** p<0.0001.

Figure 3. Single-cell analysis of KSHV PAN RNA expression in the BCBL-1 B cell lymphoma cell line. (A) Flow cytometric analysis of PAN RNA expression in multiple conditions, from cells incubated with no probe (left), or cells subjected to hybridization using a probe for PAN RNA, comparing virus-negative BL41 cells (second from left) with

KSHV+ BCBL-1 cells that were either untreated or stimulated (with TPA and sodium butyrate (NaB)) for 72 hours. Data depict lymphocytes that were singlets, defined by sequential removal of doublets. Representative images were defined as samples that were closest to the median frequency. Quantification of (B) the frequency of PAN RNA+ cells and (C) PAN RNA median fluorescence within PAN RNA+ cells, comparing untreated or stimulated BCBL-1 cells. D, E) Flow cytometric analysis of untreated BCBL-1 cells, using histogram overlays, to compare cell size (forward scatter, FSC), granularity (side scatter, SSC) and gene 73 expression in cells that were either PAN RNA negative [-] (blue line) or PAN RNA positive [+] (red line). Data show (D) histogram overlays of these populations with fluorescence quantification provided in panel E. Gene 73 analysis includes samples in which there was no gene 73 probe (i.e. “No Probe”, in solid gray), to define background fluorescence. (F, G) Flow cytometric analysis of stimulated BCBL-1 cells, using histogram overlays, to compare cell size (forward scatter, FSC), granularity (side scatter, SSC) and gene 73 expression in cells that are either PAN RNA negative [-] (blue) or PAN RNA positive [+] (red). Data show (F) histogram overlays of these populations with fluorescence quantification provided in panel G, with gene 73 analysis including a “No Probe” sample (gray) to define background fluorescence. Due to variable baseline fluorescence values for SSC and gene 73 between experiments, values were internally standardized to fluorescent intensities within the PAN RNA negative population for each experiment, with data depicting mean \pm SEM. Symbols in panels B and C indicate values from individual samples. Data are from two independent experiments, with biological replicates within each experiment, with total number of biological replicates as follows: No Probe (n = 2),

BL41 control (n = 3), BCBL-1 untreated (n = 6), BCBL-1 stimulated (n = 6). Statistical analysis was done using an unpaired t test with statistically significant differences as indicated, * p<0.05, ** p<0.01, **** p<0.0001.

Figure 4. Single-cell analysis of EBV EBER expression in the Mutu I B cell

lymphoma cell line. (A) Flow cytometric analysis using the PrimeFlow™ method, to quantify EBER expression in multiple conditions, from cells incubated with no probe (left), or cells subjected to hybridization using a probe for the EBERs, comparing virus-negative BL41 cells (second from left) with Mutu I EBV+ cells that were either untreated (with DMSO) or stimulated (with TPA in DMSO) for 48 hours. Data depict lymphocytes that were singlets, defined by sequential removal of doublets. Representative images were defined as samples that were closest to the median frequency, with data depicting mean ± SEM. (B) Quantitation of median EBER fluorescence within EBER+ Mutu I cells in untreated and stimulated cultures. Horizontal dashed line indicates the background fluorescent signal from BL41 controls. Data are from two independent experiments, with biological replicates within each experiment, with total biological replicates as follows: No Probe (n = 2), BL41 Control untreated (n = 6), Mutu I untreated (n = 6), Mutu I stimulated (n = 6). Statistical analysis was done using an unpaired t test with statistically significant differences as indicated, * p<0.05.

Figure 5. Actin mRNA degradation identifies virally-infected cells experiencing

virus-induced host shutoff. Actin mRNA analysis by qRT-PCR (A,D) or by flow cytometric analysis using PrimeFlow™ (B,C,E-G), comparing cells with variable

infection status. (A) qRT-PCR analysis of beta-actin (Actb) mRNA expression relative to 18s RNA in mock, WT or TMER-TKO infected 3T12 fibroblasts at 18 hpi. (B) PrimeFlow™ analysis of actin mRNA in 3T12 fibroblasts, either unstained (“No probe”), mock-infected or infected with WT γ HV68 or TMER-TKO at 18 hpi. (C) PrimeFlow™ analysis of actin mRNA and TMER expression in WT γ HV68 infected fibroblasts at 18 hpi. (D) qRT-PCR analysis of beta-actin (Actb) mRNA expression relative to 18s RNA in A20, virus-negative cells and A20. γ HV68 (HE2.1) cells, untreated or stimulated with TPA for 24 hrs. (E) PrimeFlow™ analysis of actin mRNA and TMER expression in untreated and stimulated A20. γ HV68 cells, with the frequency of TMER^{high} actin mRNA^{low} cells indicated, based on the gated events. (F,G) Actin mRNA analysis by PrimeFlow™ using either (F) histogram overlays or (G) quantifying frequencies, comparing A20. γ HV68 cells that were either untreated or stimulated, further stratified by whether the cells were TMER^{mid} or TMER^{high} (using the gating strategy defined in Fig. 2C). All flow cytometry data depict single cells, defined by sequential removal of doublets. Data are from two-three independent experiments, with biological replicates within each experiment. Graphs depict the mean \pm SEM, with each symbol identifying data from a single replicate. Statistical analysis was done using one-way ANOVA, subjected to Tukey’s multiple comparison test (A, D, G), with statistically significant differences as indicated, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6. Heterogeneous viral gene expression at the single-cell level during lytic replication. Viral, host RNA flow cytometric analysis in γ HV68-infected fibroblasts at 16 hpi defined by the PrimeFlow™ method, comparing (A) X-shift clustering analysis and

(B-E) biaxial gating analysis for the indicated features. (A) Automated, clustering analysis using the X-Shift algorithm on 10,000 events total, compiled from mock- and γ HV68-infected fibroblasts at 16 hrs pi (1,000 events randomly imported per sample, mock infected n=4, γ HV68-infected n=6) identifies multiple clusters of cells with differential gene expression (7 clusters, colored distinctly, “Cluster ID”), with these clusters then depicted for expression of TMERs, gene 73, Actin mRNA, and RCA. Range of expression is identified for each parameter. (B) Analysis of TMER and gene 73 co-expression in mock (left), WT γ HV68-infected (middle), and TMER-TKO-infected (right) samples, with gates depicting populations with different gene expression profiles, defined relative to mock and TMER-TKO infected samples. (C) Color-coded populations from WT-infected sample in panel B, with each color indicating a different gene expression profile. (D) Histogram overlays of the five populations identified in panel C for the indicated parameters. (E) Quantitation of gene expression among the five populations identified in panel C, using the same color-coding strategy. Data are from three independent experiments, with each experiment containing biological replicates. Flow cytometry data shows single cells that are DNA+ (DAPI+). Statistical significance tested by one-way ANOVA, comparing the mean of TMER^{high} gene 73^{high} cells to all other means, followed by Dunnett’s multiple testing correction. Significance identified as *** p<0.001, **** p<0.0001.

Figure 7. Lytic replication is characterized by heterogeneous TMER localization and variable penetrance of actin RNA degradation. Viral, host RNA flow cytometric analysis in γ HV68-infected fibroblasts at 16 hpi defined by the PrimeFlow™ method. (A)

The frequency of γ HV68-infected fibroblasts with TMERs primarily in the nucleus was quantified by ImageStream, with data showing the frequency of cells in which TMER:DAPI colocalization (i.e. similarity score) was >1 . (B) Images showing brightfield (BF), TMER, RCA protein (RCA), DAPI, gene 73 and actin mRNA localization, comparing cells with nuclear TMER localization (left) versus cytoplasmic TMER localization (right). (C) Analysis of cell subpopulations stratified by TMER and gene 73 expression (defined in Fig. 6C), subjected to the tSNE dimensionality reduction algorithm. Data and cell populations are derived from the dataset presented in Fig. 6, showing all DNA+ (DAPI+) single cells (FSC-A, SSC-A) subjected to the tSNE algorithm. The tSNE algorithm provides each cell with a unique coordinate according to its expression of Actin mRNA, RCA, Gene 73, and TMERs, displayed on a two-dimensional plot (tSNE1 versus tSNE2). Visualization grid of tSNE plots, with plots arranged according to marker expression (rows) relative to phenotype of the cellular population examined (columns). (D) Biaxial analysis of actin RNA versus RCA protein, among the five populations identified in Fig. 6. Flow cytometry data shows single cells that are DNA+ (DAPI+). Data in panels A-B from two independent experiments, in panel C-D from three independent experiments.

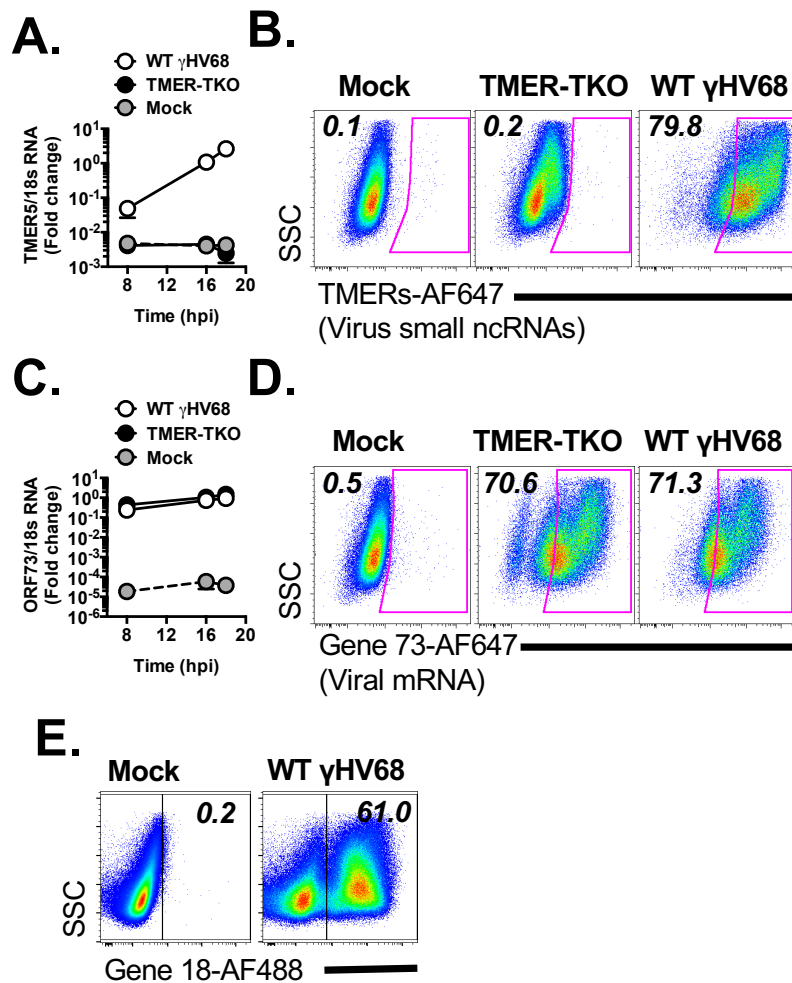


Figure 1
Okó et al

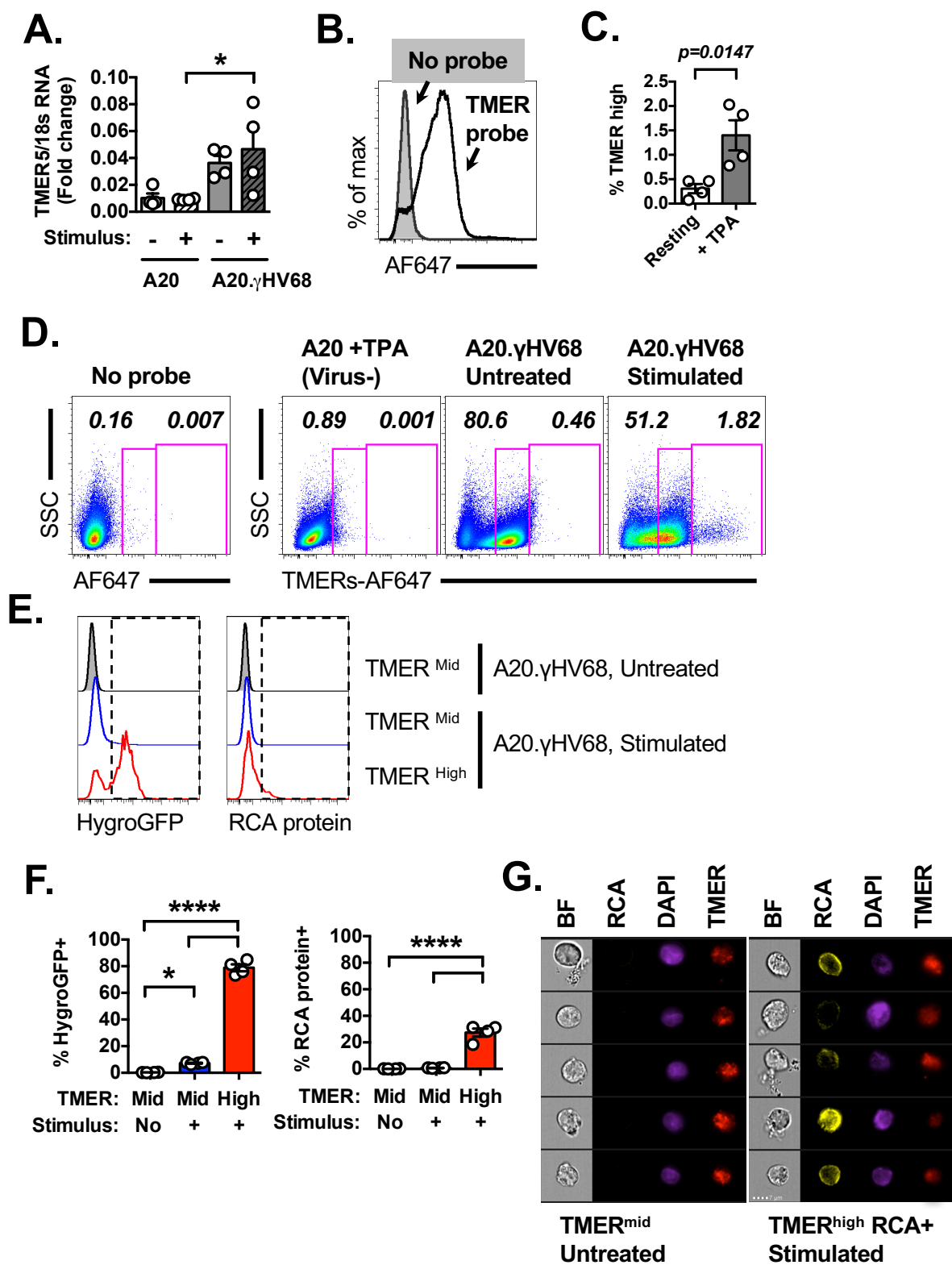


Figure 2
Okó et al

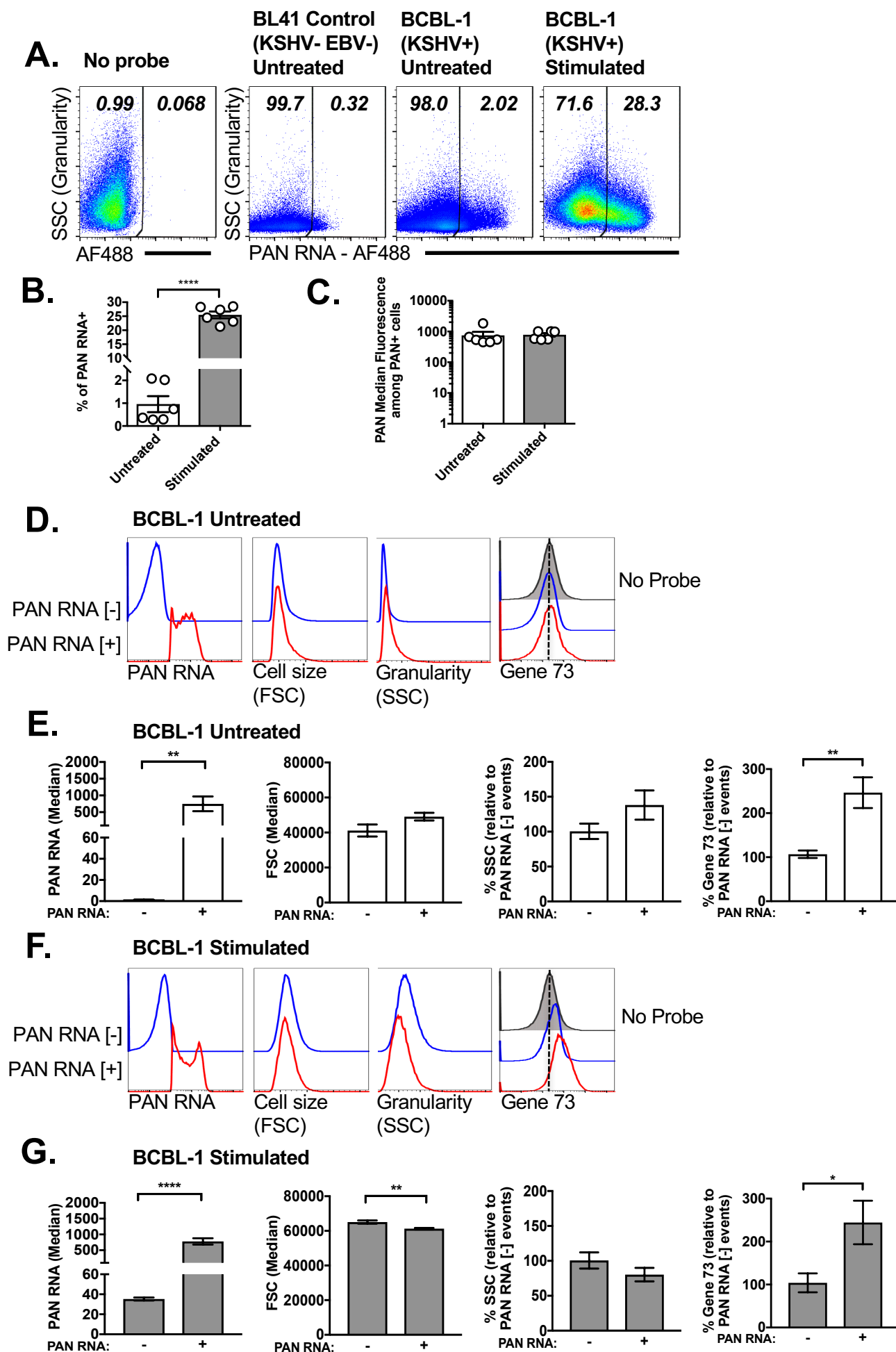


Figure 3
Okó et al

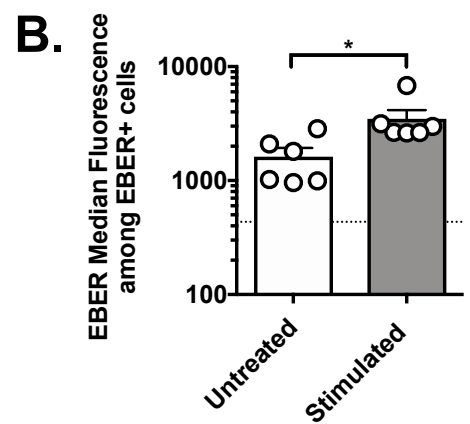
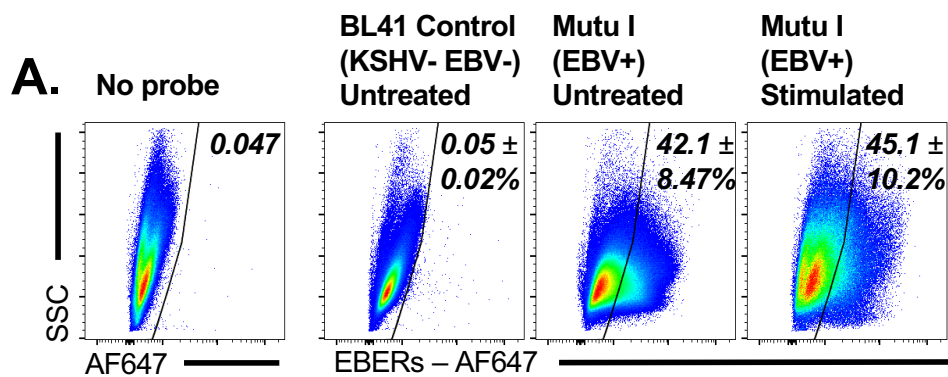


Figure 4
Okoko et al

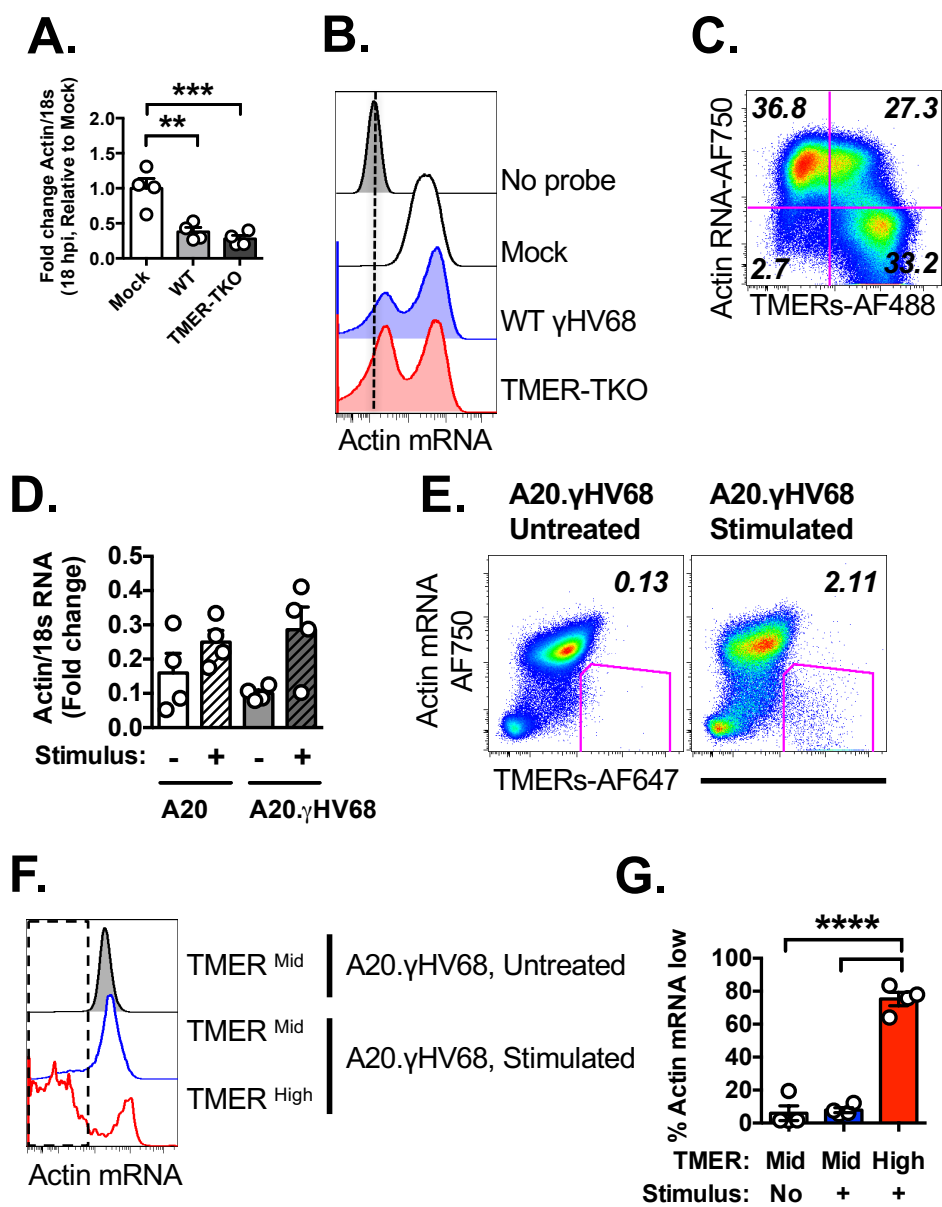


Figure 5
Okoko et al

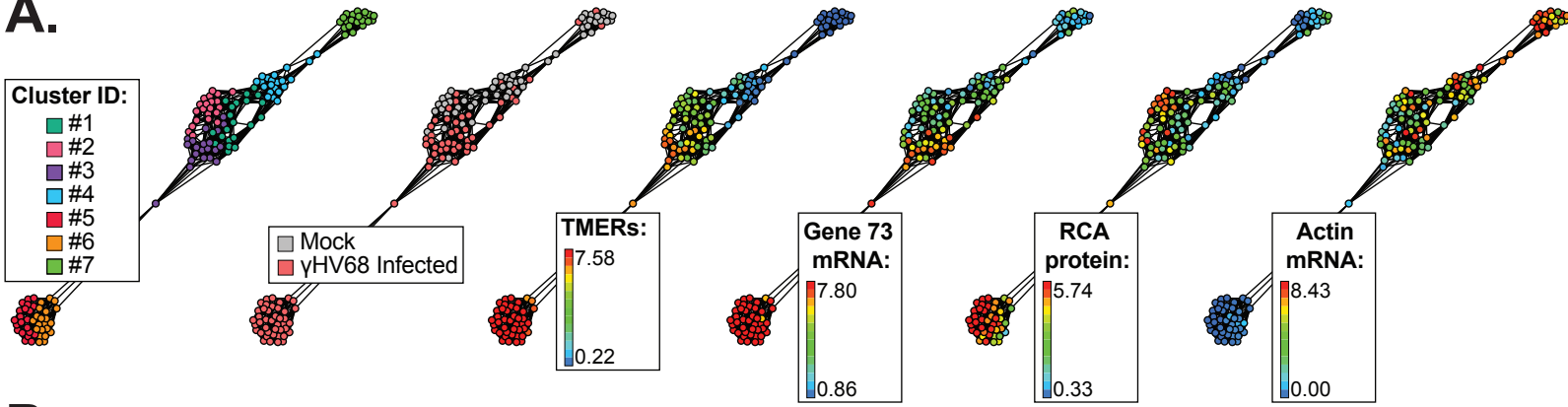
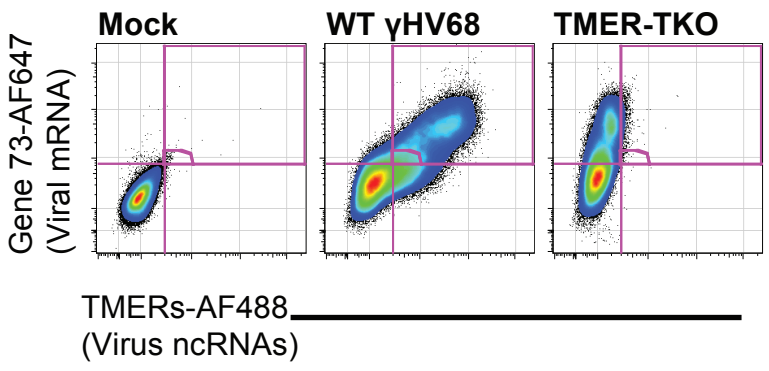
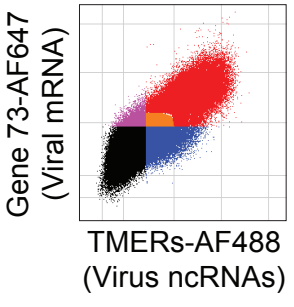
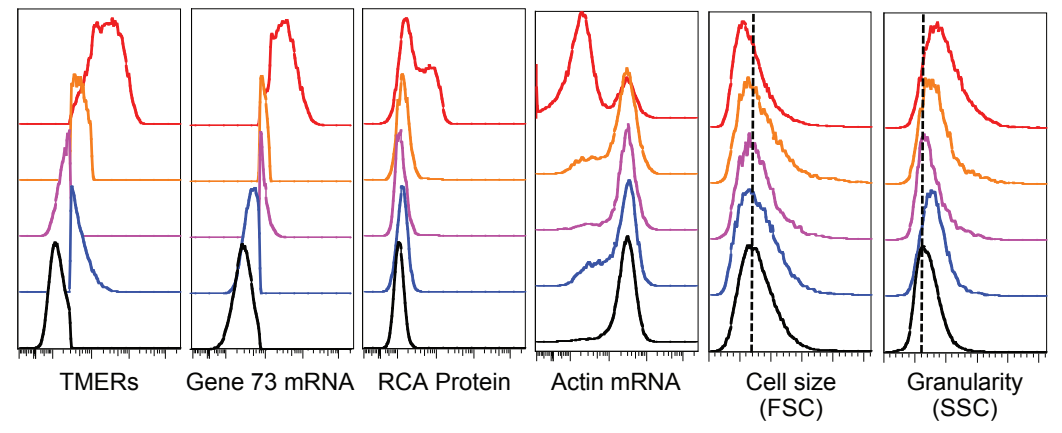
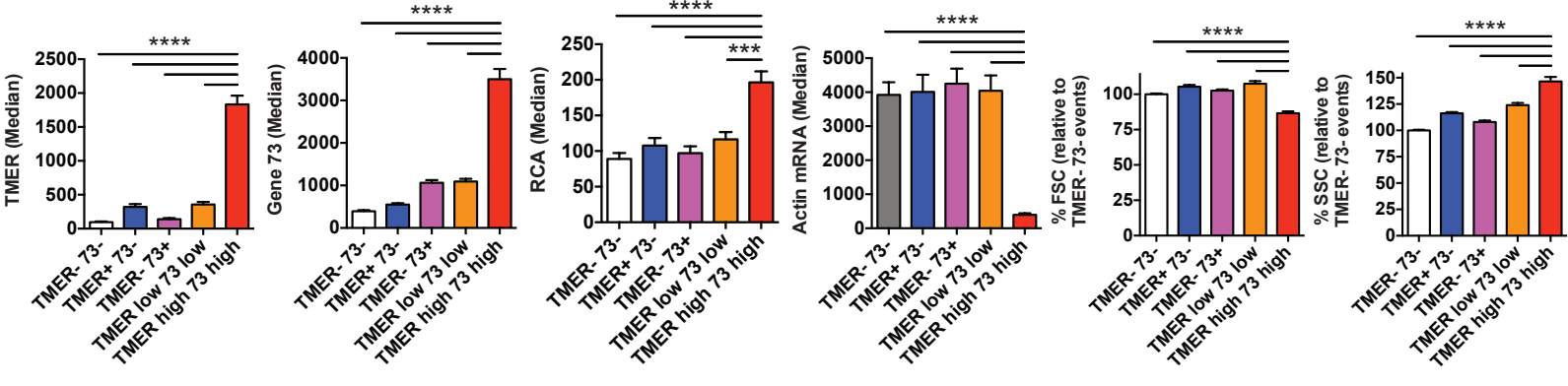
A.**B.****C.****D.****E.**

Figure 6
Okó et al

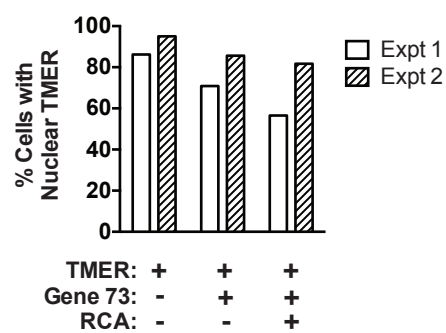
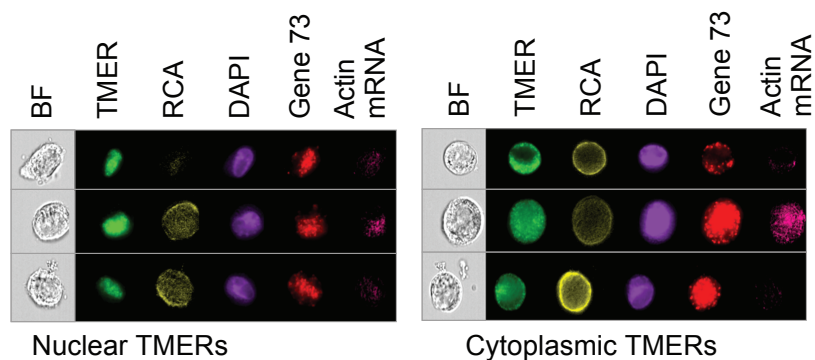
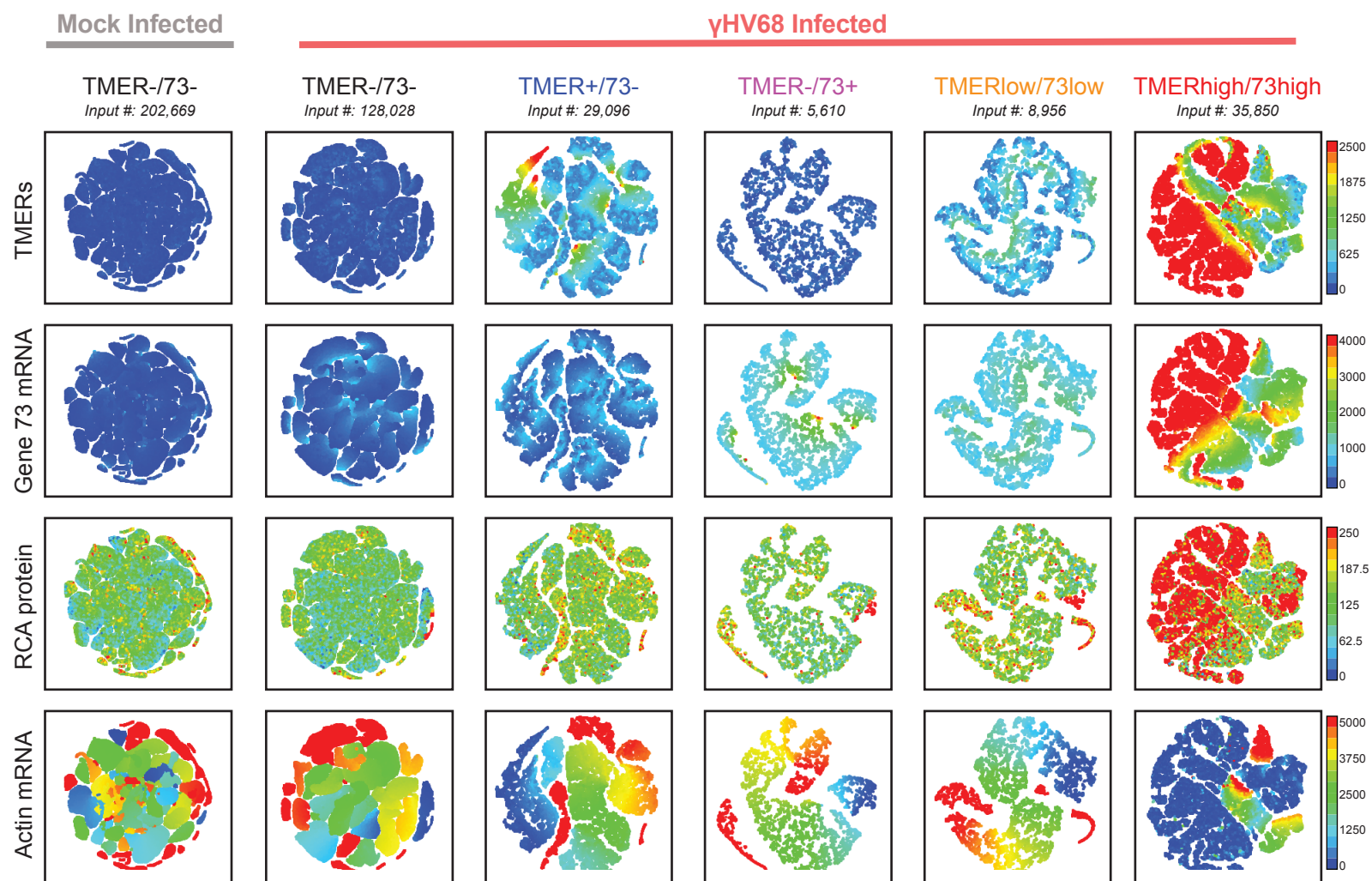
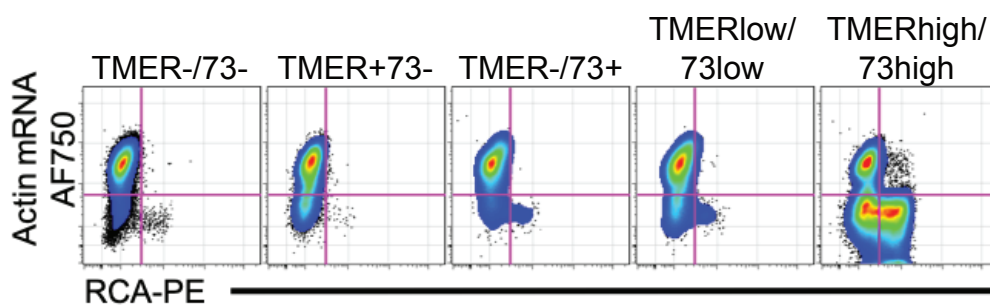
A.**B.****C.****D.**

Figure 7
Okó et al