nature portfolio

Peer Review File

Human cytomegalovirus harnesses host L1 retrotransposon for efficient replication

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This research report provides evidence supporting the intriguing idea that human cytomegalovirus (HCMV) increases expression of the host L1 retrotransposon protein (L1 ORF2p) that concentrates in subnuclear viral replication compartments where it physically interacts with the viral DNA polymerase processivity subunit (UL44 protein) and augments HCMV DNA replication by resolving 'stalled' viral DNA replication forks. Circumstantial evidence also suggests the HCMV-associated increase in L1 retrotransposon gene expression is partly brought about by the binding of specific host transcription factors (YY1 and RUNX3) in the in L1 retrotransposon gene. A rigorous analysis found no convincing evidence of tell-tale signs of L1 ORF-mediated HCMV DNA mutagenesis (transposon insertion or other genetic changes from DNA damage), despite an increase of pH2AX (marker of DNA breaks) in the vicinity of viral replication compartments where L1 ORF2p molecules concentrate.

This work applies a wide variety of state-of-the-art techniques and strategies that are logically laid out. While this can be viewed as a strength, it also adds complexity for the reader that is compounded by the switching back and forth between two very different types of HCMV-infected cells and the side issues such as digging into how HCMV up-regulates L1 gene expression. The rigor with which studies were done to address the possibility of L1 ORF-mediated HCMV DNA mutagenesis/evolution is commendable and important but accounts for a substantial amount of negative data presented in the main body of the text along with a multi-panel figure.

The following concerns or questions about some aspects of methodology, controls, and limitations of the results casts a shadow of uncertainty over the fascinating conclusion that L1 ORF proteins accelerate HCMV DNA replication by resolving stalled viral DNA replication forks. The evidence behind this conclusion requires clarification and strengthening.

The discussion should also address potential limitations of the work.

Major comments:

The PCR and Sanger sequencing methods that were performed to determine the DNA cassette expressing shL1-1 or sh1-2 was inserted into the HCMV genome correctly does not rule out the possibility of adventitious genetic changes having been introduced elsewhere in the viral genome during the generation of the recombinant BACmids. The authors should provide additional evidence that validates the integrity of the recombinant virus genomes (shRNA-expressing BACmids). The foundational studies should also include information about the time course and amount of shL1-1 and sh1-2 RNAs produced by the recombinant viruses, as well as expression levels of IE1/IE2 at MOI 0.5 (this MOI was applied to HFF in subsequent figures).

The authors should explain why the exogenously expressed shIL1 greatly lowers the proportion of HFF cells expressing IE1/2 protein (Fig. 2F) but does not lower amount of IE1/2 in U373 cells (Fig. 2A). The result in Fig. 2F is inconsistent with the result shown in Fig. 6B.

A difference in amount of input virus could explain the modest decrease (2.5-fold or less) in HCMV shL1-1 and shL1-2 genome copy number compared to parent WT or shLacZ, shown in Fig. 6A. The relative amount of HCMV DNA at 24 hpi (pre-DNA replication) for the four viruses should either be shown in a figure or stated in the Fig. 6A legend.

Colocalization coefficients are reported in multiple figures of confocal imaging results. The colocalization coefficient is a key outcome measure that requires additional information about the method of analysis used to determine this coefficient (see comment below for Fig 4 as example). A validation study of the confocal image-derived 'colocalization coefficient' measure (i.e., Fig 6 - IE1/2 vs EdU and EdU vs BrdU) is encouraged, such as using FACS to measure amounts of IE1/2, EdU, or BrdU in individual cells across a population of cells.

A mechanistic understanding of the results shown in Fig. 6 is limited by absence of information about 1) whether L1 proteins are present at viral DNA replication forks, as has been published for the UL44 protein; 2) if the L1 ORF2p-UL44 interaction is actually involved (the only results showing a physical interaction between L1 ORF2p and UL44 were generated in co-transfected HeLa cells); and 3) whether an orthogonal approach will corroborate the shL1 findings.

Given the use of U373 cells for several other experiments, information should be provided as to whether shL1-1 and sh1-2 viruses have lower levels of viral DNA replication in these cells. if so, consider whether dox-inducible L1 expression changes the outcome.

Fig 6 shows that depletion of L1 proteins (via shL1 expression) in HFF decreases incorporation of BrdU in viral replication compartments following a camptothecin (CPT) pulse. The authors interpret this to mean that L1 proteins resolve stalled viral DNA replication forks. This is a reasonable possibility, but additional tests are needed to substantiate the idea. Topoisomerase I inhibitors, like CPT, inhibit herpesvirus DNA replication, cause double-strand DNA breaks, and have other effects on infected cells. The authors should determine if other classes of drugs that stop HCMV DNA replication fork progression by a different mechanism produce similar findings.

The authors should comment on the large body of literature about the role of DNA damage response in promoting herpesvirus replication that has not been linked to viral genome instability. It is possible that the L1-associated DNA damage response hastens the incorporation of BrdU post-CPT treatment in a manner that does not align with the authors' model.

Other comments:

Line 23. Betaherpesvirinae is a subfamily (Orthoherpesviridae family; previously called the Herpesviridae family). https://ictv.global/taxonomy

Fig 1A legend. Need to reference publication that generated GSE120890 dataset.

Fig 2B legend. Need to know time after infection.

Fig 2E. A multistep viral replication curve was selected at MOI 0.1 to show that shL1-1 and sh1-2 viruses produce less cell-free virus. It's not uncommon to find viral recombinants that have a growth defect at low MOI and not high MOI. What was there a growth defect at high MOI?

Line 87 & Fig 2F - Fig 2F legend needs info on MOI and days after infection. Line 87 could be construed as 9 dpi for both Figs 2E and F, but this is not the case for Fig 2F. What is the proportion of IE1/IE2+ nuclei for each of the different groups in Fig 2F?

Fig 3D legend. RE: endogenous ORF1p IP. Need to specify cell type and time after infection.

Fig 3G legend. Need to provide % decrease in L1 ORF2p. The decrease appears to be minimal.

Fig S5A. What do the 4 rows represent? Need to clarify in figure or legend.

Fig 4A. Visual inspection reveals partial colocalization of UL44 and L1 ORF2p flag in the viral replication compartment of infected U373. What is the estimated % colocalization of UL44 and L1 ORF2p flag in the viral replication compartment?

Fig 4B legend. Need to provide information about MOI and time after infection.

Fig 4D result and methods. From the images shown in Fig 4C, it appears there is less colocalization of UL44 and pHA2x than what is reflected in Fig 4D results (correlation coefficient). This may have something to do with the method of analysis to determine colocalization coefficient. The method of analysis applied requires better explanation and additional detail. According to the methods, the images were captured using a Zeiss LSM 700 confocal microscope, which is an inverted scope. Do the number of cells per sample represent the number of cells that were in an image? Is the colocalization analysis applied to the entire image? More detail should be provided in methods about the original magnification used and pixels per image size. The degree of colocalization is dependent on the threshold values selected. How were the thresholds for the two channels selected to determine colocalization within a pixel?

Fig 6B. It is difficult to evaluate this low magnification image. This does not look like an MOI of 0.5 as stated in the figure legend if the HFFs were confluent. What is the rationale behind doing this study at 31 hpi when viral replication compartment formation is minimal to non-existent and other experiments are done at ≥48 hpi when viral replication compartments have formed?
What is with the 'white' foci? An image of higher magnification should be shown in a supplementary figure that enables evaluation of the IE1/IE2 (Alexa 568) and EdU (Alexa 488) colocalization, preferably with a DNA counterstain (separate images and Alexa 488 and 568 merge).

Because this section of results asserts that the "L1 retrotransposon-UL44 interaction accelerates viral DNA replication", it would have been more desirable to assess UL44 protein and EdU colocalization, as both colocalize in viral replication compartments and UL44 protein IFA was done in Fig 4.

Line 280. MAB810R detects both IE1 and IE2 (directed to amino terminal ends of both proteins), though it's IE1 that is mostly represented in IFA.

Reviewer #2 (Remarks to the Author):

Overall, this is an outstanding paper. HCMV is a significant human pathogen. The role of host factors in viral DNA replication is an emerging area of interest that warrants further study. The hypothesis that HCMV utilizes host retrotransposons to facilitate its replication is novel and exciting. The paper is well written, and the experiments are very well-designed. With a couple of exceptions noted below, the data is convincing, beautifully presented, and supports the conclusions of the authors. The citations are appropriate, but the authors might mention that RUNX3 has been previously shown to regulate chromatin remodeling in T cells (https://doi.org/10.1016/j.immuni.2018.03.028) and that host DNA translesion repair polymerases have been shown to be important for HCMV DNA replication and genome stability. (https://doi.org/10.1073/pnas.2203203119)
Some points could use clarification:
1. The ATAC-seq data is difficult to interpret. Fig. 1F shows differences in accessibility around the L1Hs TSS region between infected and uninfected cells. However, it was not possible to visualize these differences in the tracks from GSE 217152, since the specific locations were not provided. Please add a table with specific locations of L1 elements that are differentially affected by HCMV infection, including statistical analysis.
2. Is Fig. 3C labeled correctly? It is unclear to me why pUL44 is present in the supernatant rather than the beads in the samples that were untreated with RNAse A. Please clarify why this shows an association between pUL44 and L1 ORF1p.
3. The meaning of Lines 255-257 is unclear.

Reviewer #3 (Remarks to the Author):

Summary
Hwang et al. show that HCMV activates L1 retrotransposon expression by RNAseq and Western blot for L1 ORF1 protein (Fig. 1A-D). They further analyze ATAC-seq data (Fig 1F, Supplemental figure 2) to identify RUNX3 and YY1 as candidate transcription factors that bind to the regulatory elements of L1. shRNA depletion of RUNX3 and YY1 reduce L1 expression for ORF1 (Fig. 1H). They generate a recombinant HCMV expressing shL1 or control LacZ to demonstrate that L1 knockdown reduces viral replication (Fig. 2). They use proteomics to show that L1 OR1 interacts with HCMV protein UL44 (DNA processivity factor). A hydrophobic pocket is identified in UL44 responsible for binding to L1 ORF2 protein, which also affects ORF1 binding (Fig. 3). HCMV induces gH2AX DNA damage, and this is shown to be dependent on ORF1. L1 endonuclease, but not its RT activity is shown to be responsible for gH2AX foci formation (Fig. 4). Deep sequencing and HAT-seq are used to investigate L1 integration into HCMV and generation of HCMV variants, but no significant integration and no L1-dependent mutation frequency is observed (Fig. 5). EdU and BrdU double labeling is used to show that L1 facilitates DNA replication and replication fork restart after camptothecin block (Fig. 6).

Overall- this is an impressive and compelling study demonstrating the functional role of L1 transposon in HCMV replication cycle. The methodology is multimodal and rigorous, experiments are generally well controlled and data analysis is clearly described. The findings are highly original and significant to the field of HCMV replication, host-interaction and to the broader field of virology through interaction of two virus types.

Specific Comments for Consideration

1. Fig S2 ATAC-seq suggests that large changes occur in the body and TES of the various L1 family member (S2A). This seems inconsistent with promoter (TSS) activation through RUNX3 and YY1. The authors should consider other mechanisms of HCMV activating L1 transcription, such as deregulating heterochromatin silencing factors (HUSH complex) or possibly Pol III transcription. Are cellular mRNA genes regulated by RUNX1 and YY1 hyperactivated? How does HCMV activate RUNX1 and YY1?
2. Fig4. Line 136. It is not obvious that the L1 ORF2 is colocalizing with UL44 and CMV replication compartments. This needs to be quantified, since it is not obvious by these images, which suggests many non-colocalizing signals.
3. Fig 4. Need to show L1 ORF2 immunofluorescence pattern in uninfected cells.
4. Fig 4 and S5. It is very interesting finding that ORF2 contributes to gH2AX formation. Inhibitors of RT did not affect gH2AX, but do they have any effect on HCMV replication?
5. I appreciate the extensive effort to find L1 insertions and mutational burden in HCMV genome, despite the negative results. First question is whether the pipeline and methods were sufficient to detect integrations. Is there a positive control test, perhaps using the data set from Pox virus? The next question is if there is no evidence of integration/transposition, why doesn't L1 integrate into HCMV, and how is this restricted- is there inactivation of L1 or other resistance of HCMV to L1 transposition?
6. Fig 6. The camptothecin experiment is interesting, but may not be sufficient to conclude that L1 is overcoming replication fork blocks. This could be attributed to a separate HCMV replication activity, and L1 is only facilitating a subsequent step, such as processivity or homologous recombination. The authors should discuss alternative interpretations of this experiment.
7. The paper would benefit, in my opinion, if the authors expand the very brief Discussion section to include comments on the potential alternative possible mechanism of HCMV transcription activation of diverse transposable elements (e.g. epigenetic factors other than YY1 and RUNX3), and the mechanism of action of L1 in promoting CMV replication.

Response to Referees:

Manuscript NCOMMS-23-61315A

Human cytomegalovirus harnesses host L1 retrotransposon for efficient replication

We thank the reviewers for their thoughtful comments and constructive feedback on our manuscript, and we are pleased to see that the reviewers also recognize the significance of this work. We have revised the manuscript to address their suggestions.

Point-by-point responses to the reviewers' comments are provided below, written in blue text. We believe that we have addressed all of the concerns and have substantially strengthened the manuscript. We hope you will find it acceptable for publication.

Key revisions in the revised manuscript:

1. **New evidence for HCMV UL44-L1 ORF2p interaction at the replication fork**

   We have appended new data supporting our original hypothesis that "L1 resolves replication stress at the HCMV replication fork." To confirm the spatial context of L1 ORF2p-UL44 interaction, we conducted aniPOND, a high-resolution biochemical method that can isolate proteins at the replication fork, and found that L1 ORF2p localized to the HCMV replication fork (Fig. 6F). Notably, the interaction between L1 ORF2p and UL44 at the replication fork was required for inducing DNA breaks within HCMV replication compartments (Fig. 6G and 6H). These data support our claim that L1 retrotransposons contribute to alleviating replication stress at the viral replication fork, thereby increasing viral replication efficiency.

2. **HCMV DNA replication measurement with multifaceted approach**

   We have enhanced our analysis of HCMV DNA replication using multiple orthogonal assays: DNA quantification using qPCR (Fig. 6A), EdU incorporation assay by microscopy (Fig. 6B), and flow cytometry (Fig. S10D). All these measurements support that L1 accelerates HCMV DNA replication.

3. **Additional discussion on the mechanism of L1-mediated DNA replication**

   In the Discussion section, we have expanded our discussion on the complex interaction between L1 and HCMV, inducing DNA damage response and simultaneously enhancing DNA replication, and we have further elaborated on the potential limitations of our work.

Referee's comments:

Reviewer #1 (Remarks to the Author):

This research report provides evidence supporting the intriguing idea that human cytomegalovirus (HCMV) increases expression of the host L1 retrotransposon protein (L1 ORF2p) that concentrates in subnuclear viral replication compartments where it physically interacts with the viral DNA polymerase processivity subunit (UL44 protein) and augments HCMV DNA replication by resolving 'stalled' viral DNA replication forks. Circumstantial evidence also suggests the HCMV-associated increase in L1 retrotransposon gene expression is partly brought about by the binding of specific host transcription factors (YY1 and RUNX3) in the in L1 retrotransposon gene. A rigorous analysis found no convincing evidence of tell-tale signs of L1 ORF-mediated HCMV DNA mutagenesis (transposon insertion or other genetic changes from DNA damage), despite an increase of pH2AX (marker of DNA breaks) in the vicinity of viral replication compartments where L1 ORF2p molecules concentrate.

This work applies a wide variety of state-of-the-art techniques and strategies that are logically laid out. While this can be viewed as a strength, it also adds complexity for the reader that is compounded by the switching back and forth between two very different types of HCMV-infected cells and the side issues such as digging into how HCMV up-regulates L1 gene expression. The rigor with which studies were done to address the possibility of L1 ORF-mediated HCMV DNA mutagenesis/evolution is commendable and important but accounts for a substantial amount of negative data presented in the main body of the text along with a multi-panel figure.

The following concerns or questions about some aspects of methodology, controls, and limitations of the results casts a shadow of uncertainty over the fascinating conclusion that L1 ORF proteins accelerate HCMV DNA replication by resolving stalled viral DNA replication forks. The evidence behind this conclusion requires clarification and strengthening.

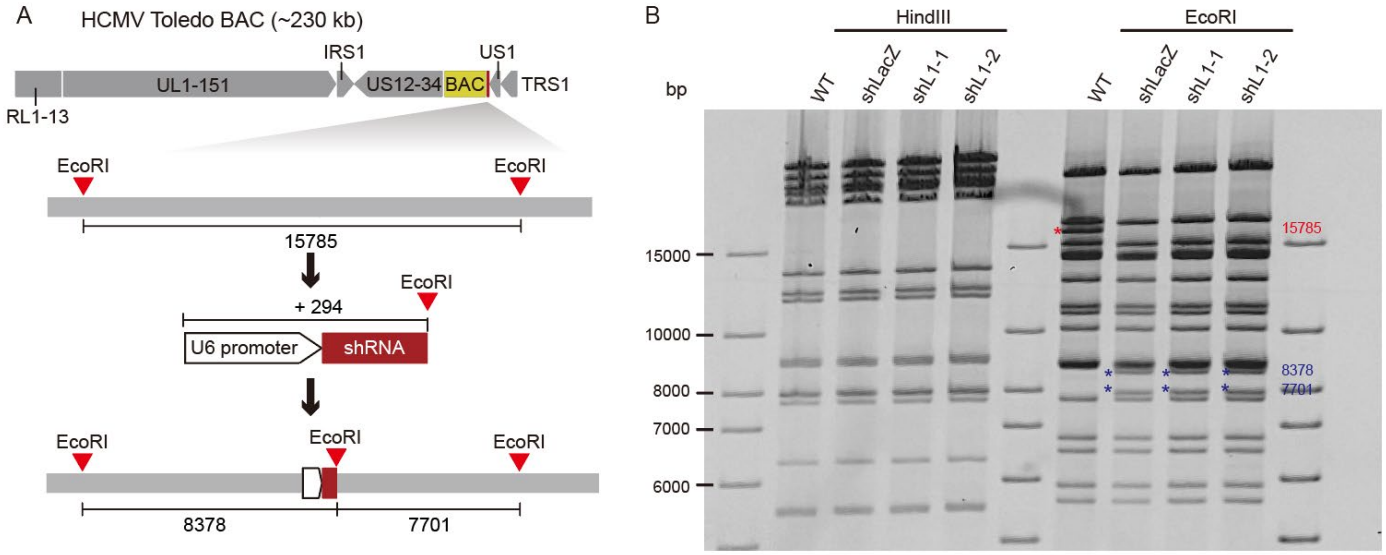
The discussion should also address potential limitations of the work.

We sincerely appreciate the reviewer's recognition of the significance of our work and suggestions to strengthen our data.

Major comments:

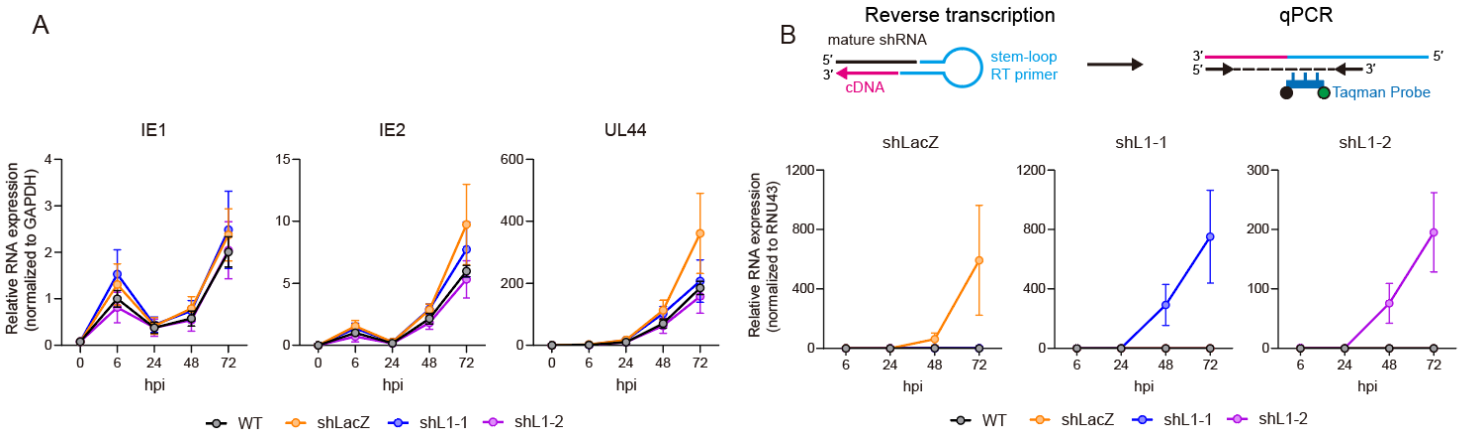
1. The PCR and Sanger sequencing methods that were performed to determine the DNA cassette expressing shL1-1 or sh1-2 was inserted into the HCMV genome correctly does not rule out the possibility of adventitious genetic changes having been introduced elsewhere in the viral genome during the generation of the recombinant BACmids. The authors should provide additional evidence that validates the integrity of the recombinant virus genomes (shRNA-expressing BACmids). The foundational studies should also include information about the time course and amount of shL1-1 and sh1-2 RNAs produced by the recombinant viruses, as well as expression levels of IE1/IE2 at MOI 0.5 (this MOI was applied to HFF in subsequent figures).

We agree that verifying BAC integrity is important. To address this, we first conducted restriction mapping of recombinant BACs used in this work using HindIII and EcoRI, which are a non-cutter and a cutter of the shRNA cassette, respectively. While HindIII digestion revealed no differential fragments between BACs, we found that there is one additional EcoRI site exclusively in shRNA-inserted BACs (shLacZ, shL1-1, and shL1-2), accurately corresponding to the intended insertion of shRNA cassette without considerable mutations (**Peer Review Fig. 1**). Second, we conducted full-length nanopore sequencing of our recombinant BACs and obtained 230 kb long sequence read of each BAC. Analysis of the BAC sequence did not reveal any functional genetic alterations. Taken together, restriction mapping and Nanopore BAC sequencing confirmed the integrity of our recombinant virus genomes. GenBank files of the full Nanopore sequencing reads have been provided in the "Source_data.zip" file.



Peer Review Fig.1: Restriction map of HCMV BACs (A) Schematic for our strategy of generating shRNA-expressing HCMV. shRNA cassette was inserted into the non-essential US1-2 intergenic region. (B) Restriction map of BAC DNAs.

As the reviewer suggested, we also assessed the time-course expression levels of shRNAs and HCMV immediate early RNAs. To achieve this, we infected each recombinant HCMV to HFF cells at a 0.5 MOI and harvested cells at 6, 24, 48, and 72 hours post-infection (hpi). To quantify mature and fully processed shRNA, we adapted a method commonly used for miRNA analysis[1]. Briefly, shRNAs were reverse-transcribed using a pool of stem-loop primers specific for shRNAs (shLacZ, shL1-1, and shL1-2) and a house-keeping small RNA (RNU43). cDNAs were subjected to quantitative PCR using stem-loop targeting probes. Quantitative PCR demonstrated that each virus expressed its own specific shRNA with expression levels increasing throughout the time course of infection (**Peer Review Fig. 2**). Additionally, we confirmed that expression kinetics of shRNAs showed the kinetics of increase after 24 hpi like a viral early gene UL44. All of these experiments suggest that our recombinant viruses work as intended and their growth defect does not stem from the deterioration of the original BAC.



Peer Review Fig. 2: Expression of HCMV mRNAs and shRNAs. (A-B) HCMV mRNAs (A: mRNAs encoding IE1, IE2, and UL44), and shRNAs (B: shLacZ, shL1-1, and shL1-2) expressed by recombinant viruses were quantified using quantitative PCR at the indicated time point.

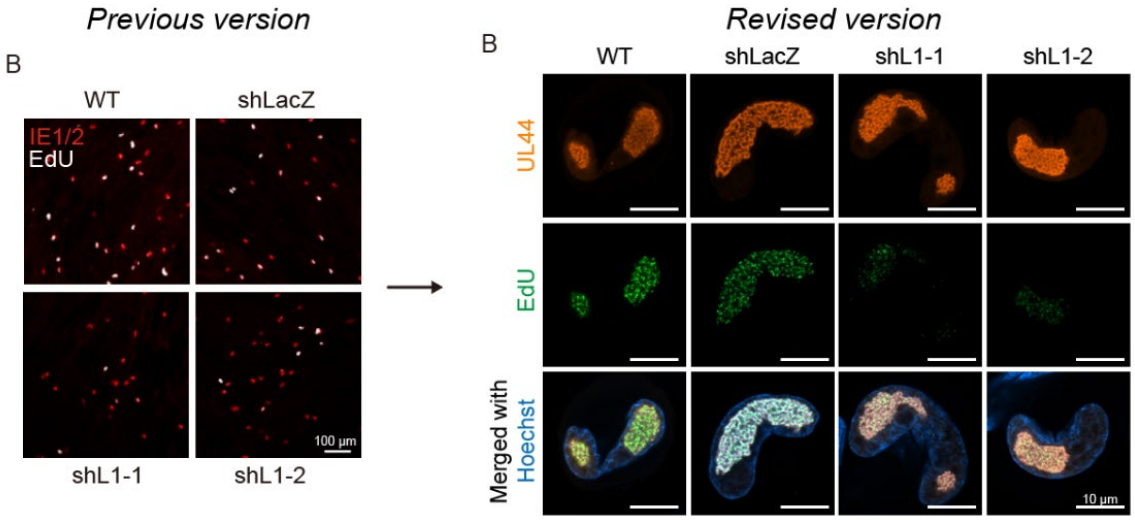
2. The authors should explain why the exogenously expressed shIL1 greatly lowers the proportion of HFF cells expressing IE1/2 protein (Fig. 2F) but does not lower amount of IE1/2 in U373 cells (Fig. 2A). The result in Fig. 2F is inconsistent with the result shown in Fig. 6B.

We apologize for this confusion from our previous descriptions lacking clear annotations. We would like to clarify the experimental design and indication of IE1/2 in Fig. 2F, 2B, and 6B.

In Fig. 2F (the right panel of Fig 2E in the revised manuscript), IE1/2 and DAPI images show the quantity of progeny virus at 9 dpi. We infected HFF with different recombinant HCMV and harvested cell-free progeny viruses at the indicated time points. Then, we quantified the difference in the number of viral progenies produced by respective recombinant viruses by infecting HFF with these cell-free viruses and counting the number of IE1/2 positive cells. Therefore, the number of IE1/2-positive nuclei in Fig. 2F indicates the number of progeny viruses produced by each recombinant virus, so this result shows that reduced viral progenies produced by shL1 HCMVs compared to WT or shLacZ, owing to the replication defect in shL1 virus.

In contrast, for Fig. 2B, we focused on the immediate impact of shL1 on viral protein expression within the initially infected U373MG cells rather than examining the production of infectious progeny as in Fig. 2F. We infected U373MG cells with the same amount of recombinant HCMV to assess expression levels of viral proteins and L1 protein. Therefore, this result shows that the expression of IE1/2 proteins is not affected by L1.

In Fig. 6B of the previous manuscript, we aimed to assess DNA replication in HCMV-infected cells. To this end, we infected HFF cells with the same amount of recombinant virus and used IE1/2 stain to discriminate HCMV-infected cells. Therefore, IE1/2 signals in Fig. 6B indicate the nuclei of HCMV-infected cells. We previously measured the EdU incorporation in these IE1/2-positive cells, but we have revised this data according to the reviewer's suggestion in the specific comments (#4, #20, #21, and #22 as numbered in this document)



Peer Review Fig. 3 The previous version (left) and the revised version (right) of Fig. 6B.

To clarify the difference in these experiments, we have revised the corresponding figure legends and clarified our experiment design more clearly in the manuscript (Lines 84-87).

3. A difference in amount of input virus could explain the modest decrease (2.5-fold or less) in HCMV shL1-1 and shL1-2 genome copy number compared to parent WT or shLacZ, shown in Fig. 6A. The relative amount of HCMV DNA at 24 hpi (pre-DNA replication) for the four viruses should either be shown in a figure or stated in the Fig. 6A legend.

We agree with your concerns. We have added HCMV genome copy number across the four experimental conditions (WT, shLacZ, shL1-1, and shL1-2) at 24 hpi in Fig. S10B. This data shows no significant differences in the amount of input virus at the initial time point, 24 hpi.

4. Colocalization coefficients are reported in multiple figures of confocal imaging results. The colocalization coefficient is a key outcome measure that requires additional information about the method of analysis used to determine this coefficient (see comment below for Fig 4 as example). A validation study of the confocal image-derived 'colocalization coefficient' measure (i.e., Fig 6 - IE1/2 vs EdU and EdU vs BrdU) is encouraged, such as using FACS to measure amounts of IE1/2, EdU, or BrdU in individual cells across a population of cells.

We thank the reviewer for a constructive comment regarding the use of colocalization coefficients in our image analysis.

'Colocalization coefficients' indicates the proportion of overlapping pixels between two different signals within a single confocal image. As described in our Method section, we calculated colocalization coefficients as follows:

$$colocalization\ coefficients\ = \frac{\#\ of\ pixels\ of\ channel\ 1\ located\ on\ channel\ 2}{Total\ \#\ of\ pixels\ of\ channel\ 2}$$

For pH2AX and UL44 images in Fig. 4C, 4E, and 6G of the revised manuscript, we calculated how many UL44 pixels were also positive for pH2AX. Using the Zen Blue software (ZEISS), we first selected ROI specifying the viral replication compartments (VRCs) in a single virus-infected cell. To exclude the background signal and observe the event in viral replication compartments, we adjusted the threshold of UL44 (≥60) and pH2AX (≥60) and calculated the colocalization coefficients using those pixels with the intensity over the threshold. We obtained each value by analyzing a single cell, with each dot representing a value from a single virus-infected cell (scatter dot plot of Fig 4D, 4F, 6H, S5D, and S6B). These measurements served as a simple but robust metric to show the spatial relationship between γH2AX and UL44, indicating the high degree of overlap between the marker of DNA damage and VRCs. To clarify this approach in the main text, we have added a description of our method of analysis to determine colocalization coefficients in the Methods section of the revised manuscript (Lines 322-327).

For BrdU and EdU assays, we previously calculated how many EdU pixels were also positive for BrdU. These measurements also served as a metric to show the high degree of overlap between the VRC and incorporating nucleotides. However, by carefully revising our original manuscript according to the reviewer's comment, we recognized our interpretation of EdU or BrdU assays could be advanced by improving our calculation method. Therefore, in the revised manuscript, we have improved our calculation method to directly measure the intensity of incorporated EdU or BrdU, not just the overlapping area, by utilizing the 'mean pixel intensity' (total signal intensity divided by the number of pixels). Briefly, using imageJ, we first generated a mask of the total VRC area (based on UL44 signals in Fig. 6B), overlaid the UL44 mask on top of the fluorescent nucleotide signals (EdU or BrdU) to isolate the signal originating from VRC, and measured the mean pixel intensity of EdU within the UL44 area. This method of quantification excludes noise coming from host DNA replication and most directly measures the degree of DNA replication within viral replication compartments. This analysis clearly showed that the extent of EdU incorporation in the UL44 area is reduced in shL1-1 and shL1-2 samples (Fig. 6B and 6C).

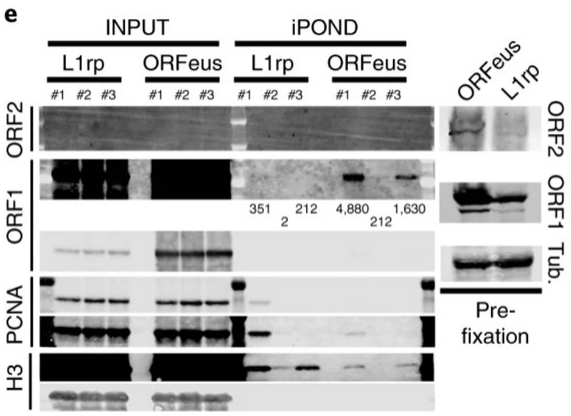
As suggested by the reviewer, we conducted flow cytometry analysis for EdU and UL44. After conventional gating to isolate singlet live cells, we selected a population positive for UL44 and EdU sequentially, followed by comparing the intensity of EdU in these cells. WT and shLacZ HCMV-infected cells showed higher levels of EdU intensity than shL1 virus-infected cells. This data further supports our model that L1 accelerates viral DNA replication. We have incorporated the flow cytometry data in the supplementary information (Fig. S10D) and revised the manuscript accordingly (Lines 205-210).

In summary, we evaluated the HCMV DNA replication rates using multiple readouts: genome copy number (Fig. 6A), EdU intensity in the UL44 area (Fig. 6B), and EdU intensity using flow cytometry readout (Fig. S10D). We believe that these analyses better align with the objectives of the experiments conducted in Figure 6, supporting the conclusion that L1 accelerates HCMV DNA replication.

5. A mechanistic understanding of the results shown in Fig. 6 is limited by absence of information about 1) whether L1 proteins are present at viral DNA replication forks, as has been published for the UL44 protein; 2) if the L1 ORF2p-UL44 interaction is actually involved (the only results showing a physical interaction between L1 ORF2p and UL44 were generated in co-transfected HeLa cells); and 3) whether an orthogonal approach will corroborate the shL1 findings.

We appreciate the reviewer's constructive comment.

First, we would like to refer to previous studies showing that L1 RNP complex interacts with multiple host proteins located at the replication forks[2,3], in which Mita *et al.* (2020) specifically showed that the L1 RNP localizes at the replication fork using iPOND, a method for the isolation of replication fork proteins[4] (**Peer Review Fig. 4**).



Peer Review Fig. 4 L1 RNP localized at the replication fork. Fig. 2e from Mita *et al.*, *Nature Structural and Molecular Biology* (2020). Immunoblot of iPOND inputs and immunoprecipitations of replication forks from HeLa-M2 cells expressing either ORFeus (codon-optimized L1 construct) or L1rp (original sequence). #1: BrdU pulse and click reaction sample; #2: Sample without click reaction; #3: BrdU pulse and thymidine chase sample, indicating maturing chromatin. Two panels represent different exposures of the membrane. This result shows that L1RNP localizes at the replication fork.

Based on these studies as well as the known function of UL44 during replication, we hypothesized that UL44-L1 ORF2p interactions exist "at the viral replication forks" in HCMV-infected cells. To test our hypothesis, we adopted the accelerated native isolation of protein on nascent DNA (aniPOND)[5] method to capture proteins at the replication forks in a native state. Briefly, after pBL1$^{ORF2p\text{-}3\times FLAG}$ U373MG cells were infected, nascent DNA was labeled with a short pulse of EdU, EdU-labeled DNA was conjugated with biotin using click chemistry, and biotinylated DNA-protein complexes were captured using streptavidin. In our experimental condition for aniPOND, we found that isolated nascent DNAs were predominantly of viral origin (Fig. S10G) and we also confirmed that both PCNA and UL44 were pulled down with EdU-labeled DNA (Fig. 6F), which is expected given their role at the replication fork. Notably, we detected L1 ORF2p in the pulldown samples, suggesting that L1 ORF2p is present at viral replication forks. A notable point is that pulldown efficiency was reduced for all of these proteins following a thymidine chase after EdU pulse, in which EdU-DNA indicates maturing chromatin, further demonstrating the fork-specific interaction of L1 ORF2p and UL44. These results showed that L1 ORF2p binds to and interacts with sliding clamp proteins, which can regulate DNA replication at the replication forks.

To address the reviewer's second point, we sought to investigate the functional consequences of UL44-L1 ORF2p interaction at the replication forks upon introduction of L1 ORF2p mutations in UL44 binding. For this experiment, we used L1 ORF2p Y414-415A mutant which has a defect in UL44 binding as we have shown in this work (Fig. 3E and Fig. S10H). Using this L1 ORF2p Y414-415A-expressing cell line (U373MG$^{pBL1ORF2p\text{-}3\times FLAG}$), we tested whether DNA damage induced in viral replication compartments is ascribed to this interaction. We found that pH2AX foci are dramatically reduced in Y414-415A mutation of L1 ORF2p, and its colocalization with UL44 is likewise decreased significantly (Fig. 6G and 6H). This result indicates that L1 RNP is recruited to UL44 specifically at the HCMV replication fork, inducing DNA damage response which contributes to the acceleration of HCMV DNA replication.

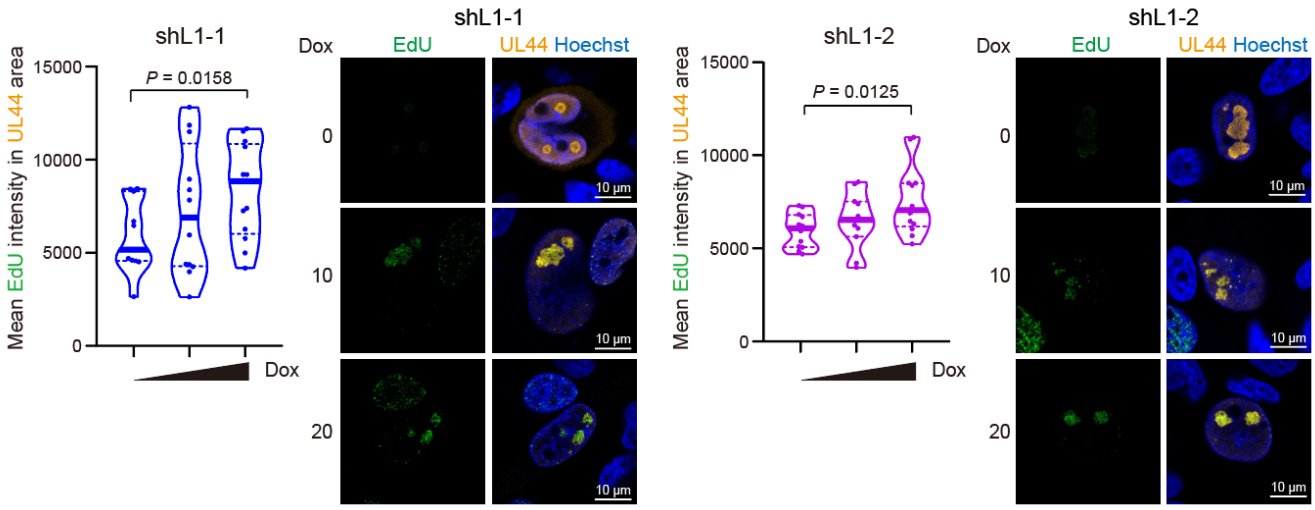
Together, we improved Figure 6 by demonstrating that the L1 ORF2p-UL44 localizes at the viral replication fork with aniPOND (Fig. 6G) and by showing that the interaction between L1 ORF2p and UL44 functionally correlates with the localization of L1 ORF2p and pH2AX signal at the viral replication complex (Fig. 6H). We have incorporated all of these new data into Figure 6 and have revised the manuscript accordingly. We believe that these additional data support our claim sufficiently and address the reviewer's points.

6. Given the use of U373 cells for several other experiments, information should be provided as to whether shL1-1 and sh1-2 viruses have lower levels of viral DNA replication in these cells. if so, consider whether dox-inducible L1 expression changes the outcome.

We agree with the reviewer's point regarding the differences between cell lines and the rescue experiment.

As the reviewer suggested, we tested whether shL1-1 and shL1-2 HCMVs have lower levels of viral DNA replication in U373MG. Consistent with HFF experiments, DNA replication of shL1-1 and shL1-2 was less productive than WT and shLacZ HCMV (Fig. S10A). This result further supports that L1 activation is required for efficient HCMV DNA replication in multiple HCMV permissive cell lines.

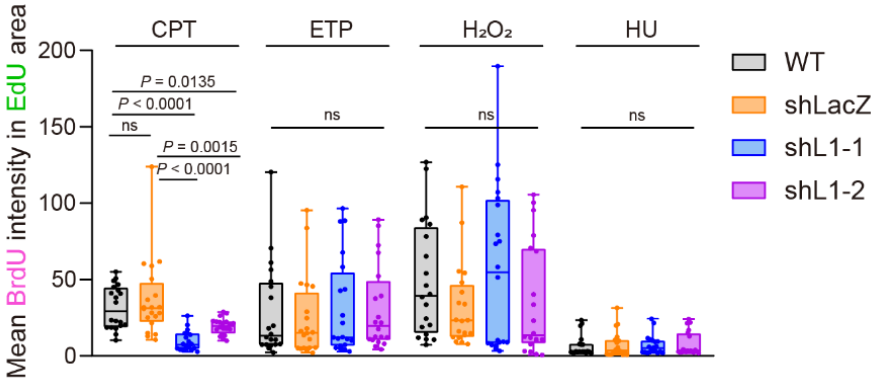
Using our dox-inducible L1 expression model (pBL1 U373MG), we tested whether ectopic overexpression of L1 could enhance the DNA replication of shL1 HCMVs. This model is suitable in that its codon-optimized L1 ORFs are not targeted by shL1, enabling retrieval of L1 expression regardless of virus type. We analyzed the EdU incorporation into VRC in a single cell level, using the same method as Fig. 6B. pBL1 U373MG cells were infected with 10 MOI of shL1 HCMVs and treated with dox for 6 h, followed by incubation with a pulse of EdU for 30 min. We assessed the direct incorporation of EdU into VRCs and observed that EdU incorporation increased with rising concentrations of dox (**Peer Review Fig. 5**). This result showed that ectopic expression of L1 could accelerate HCMV DNA replication. We believe these data support our claim of the pivotal role of L1 during HCMV DNA replication and address the reviewer's point.



Peer Review Fig. 5 Ectopic expression of L1 enhanced DNA replication of shL1 HCMVs. Cells were treated with 0, 10, or 20 ng/mL dox for 6 h, followed by incubation with EdU for 30 min.

7. Fig 6 shows that depletion of L1 proteins (via shL1 expression) in HFF decreases incorporation of BrdU in viral replication compartments following a camptothecin (CPT) pulse. The authors interpret this to mean that L1 proteins resolve stalled viral DNA replication forks. This is a reasonable possibility, but additional tests are needed to substantiate the idea. Topoisomerase I inhibitors, like CPT, inhibit herpesvirus DNA replication, cause double-strand DNA breaks, and have other effects on infected cells. The authors should determine if other classes of drugs that stop HCMV DNA replication fork progression by a different mechanism produce similar findings.

We agree with the reviewer's comment. As suggested, usage of other classes of drugs inducing replication stalling would be helpful to substantiate the specific involvement of L1 in resolving fork stalling. Therefore, we additionally tested the effect of Etoposide (ETP), hydrogen peroxide (H_2O_2), and hydroxyurea (HU) that could induce fork stalling with different mechanism[6]. First, we titrated the concentration of drugs that induce similar levels of DNA damage responses to CPT (Fig. S10E). Then we tested if the different recombinant HCMV overcome replication disruption by these drugs using the same method as Fig. 6D and 6E. However, the levels of newly synthesized DNA (BrdU) showed no differences between shL1s and the control viruses in any of the drug treatment experiments except for the CPT (**Peer Review Fig. 6**).



Peer Review Fig. 6 BrdU incorporation after drug treatment.

We believe this is due to the difference in each drug's effect on replication. Since HU blocks nucleotide biosynthesis by targeting the ribonucleotide reductase, it is reasonable that HU treatments interrupt BrdU incorporation all together, which was observed in our assay as a generally damped BrdU signal regardless of L1. Oxidative stress by H_2O_2 induces global DNA damage at various distances from replication forks[7] and could result in heterogenous BrdU incorporation due to varying degrees of stress among cells. Based on our phenotype with CPT which targets topoisomerase I during replication, we tested whether ETP (topoisomerase II inhibitor)-induced replication stress would also depend on L1 for its recovery. However, we did not observe any effect and this may be because ETP causes the dispersal of replication proteins, such as human processivity factor PCNA[8] from the replication fork. Indeed, it was shown previously that newly synthesized DNA colocalizes with DNA damage foci of CPT-treated cells, but much less into those of ETP-treated cells[9].

Despite our efforts to mimic endogenous replication stress using various drugs, in our experiment, CPT has proven to be the most suitable drug for observing fork stalling in replicating HCMV DNA. Nevertheless, we agree with the reviewer's point that there should be other possible mechanisms we could not investigate in this work, so we have rephrased our previous descriptions to ensure the objective interpretation of our data (Line 229, "*fork stalling caused by **topoisomerase I inhibition**"*), and have added a discussion on the alternative mechanisms of action in the Discussion section (4th paragraph).

8. The authors should comment on the large body of literature about the role of DNA damage response in promoting herpesvirus replication that has not been linked to viral genome instability. It is possible that the L1-associated DNA damage response hastens the incorporation of BrdU post-CPT treatment in a manner that does not align with the authors' model.

We appreciate the reviewer's suggestion and we have added a new paragraph (4th paragraph in the Discussion) to reflect the reviewer's comment. Our HCMV-hijacking of L1 retrotransposons model proposes 1) a putative source of DNA damage response in viral replication compartments during HCMV infection and explains 2) how HCMV can produce a large amount of its DNA with minimal error during lytic replication. Especially, we think the second point is well-aligned with the previous findings that DNA damage response is required for efficient herpesvirus replication[10-14], as the reviewer noted. We believe that future studies are needed to fully understand the complex interplay between virus and host DNA damage response machinery.

Other comments:

9. Line 23. Betaherpesvirinae is a subfamily (Orthoherpesviridae family; previously called the Herpesviridae family). https://ictv.global/taxonomy

Thank you for pointing this out. We have revised it as follows: "*Human cytomegalovirus (HCMV) is a member of the Betaherpesvirinae* ***subfamily*** *with global seroprevalence of up to 90%.*"

10. Fig 1A legend. Need to reference publication that generated GSE120890 dataset.

We have updated the reference in Fig 1A legend.

11. Fig 2B legend. Need to know time after infection.

We have updated this in Fig 2B legend as follows: *"Western blot analysis showing L1 knockdown effect of shL1 RNA-inserted HCMV in U373MG at* ***5 dpi****."*

12. Fig 2E. A multistep viral replication curve was selected at MOI 0.1 to show that shL1-1 and sh1-2 viruses produce less cell-free virus. It's not uncommon to find viral recombinants that have a growth defect at low MOI and not high MOI. What was there a growth defect at high MOI?

We thank the reviewer for this comment. It also confirmed that shL1 HCMV shows growth defect at high MOI infection. We performed a one-step growth curve assay by infecting HFF with 2 MOI viruses. Production of progeny viruses saturated after 4 dpi, indicating that most cells are infected at 2 MOI infection. Measurement of the infectious unit per mL showed that progeny production of shL1-1 and shL1-2 viruses was significantly reduced. This data supports that L1 promotes HCMV replication regardless of MOI. We have incorporated this data in the revised version of Fig. 2F.

13. Line 87 & Fig 2F - Fig 2F legend needs info on MOI and days after infection. Line 87 could be construed as 9 dpi for both Figs 2E and F, but this is not the case for Fig 2F. What is the proportion of IE1/IE2+ nuclei for each of the different groups in Fig 2F?

Thank you for noting this. We apologize for the lack of clarity in the figure legend. The right panel of Fig. 2E and 2F (Fig 2F in the previous manuscript) are the representative images of HFFs that were infected with cell-free viruses and used to count the number of infectious units per mL (IFU) shown in the line plot. To assess IFU, we counted all the IE1/IE2-positive nuclei in those HFF cultures. To clarify the indication of these images, we have revised the corresponding figure legend as follows*: "HFF cells were infected with the cell-free viruses collected at 9 dpi from 0.1 MOI infection (E, right panel) and at 7 dpi from 2 MOI infection (F, right panel).*

14. Fig 3D legend. RE: endogenous ORF1p IP. Need to specify cell type and time after infection.

We have updated this in the Fig 3D legend as follows: *"Endogenous L1 ORF1p Co-IP assay of **U373MG cells at 4 dpi** showing interactions between L1 RNP and UL44. Given differences in detection efficiencies, long and short exposures of L1 ORF1p and L1 ORF2p blots are presented. Flow-through was saved before IP wash."*

15. Fig 3G legend. Need to provide % decrease in L1 ORF2p. The decrease appears to be minimal.

We appreciate the reviewer's detailed review. We have now incorporated the measurement of band intensity in Fig. 3G, showing an approximate 20-60 % decrease in co-immunoprecipitated L1 ORF2p with mutant UL44. Note that the small visual difference may be due to low sensitivity in detecting L1 ORF2p and the resulting background signal as noted in multiple studies so far[3,15,16]. Also considering that L1 ORF1p and ORF2p preferentially form a ribonucleoprotein complex with each other, large decreases in L1 ORF1p support our findings. The levels of L1 ORF1p band intensity have also been added to the revised Fig. 3G for clarity. We hope this revised analysis addresses the reviewer's concern.

16. Fig S5A. What do the 4 rows represent? Need to clarify in figure or legend.

We apologize for the lack of clarity in the figure legend. The four rows in Fig. S5A represented four different representative images of UL44 and L1 ORF2p-3×FLAG. We have replaced them with high-resolution images and have updated the figure legend accordingly.

17. Fig 4A. Visual inspection reveals partial colocalization of UL44 and L1 ORF2p flag in the viral replication compartment of infected U373. What is the estimated % colocalization of UL44 and L1 ORF2p flag in the viral replication compartment?

We thank the reviewer for pointing this out. To estimate the % colocalization of UL44 and L1 ORF2p-3×FLAG (number of FLAG pixels in UL44 area / total number of FLAG pixels), we acquired high-resolution images using Airyscan SR mode of Zeiss LSM980, which can resolve points below the diffraction limit (~120nm in XY) and analyzed these images using ImageJ. We extracted FLAG pixels with top 1% intensity and calculated how many FLAG pixels were on the UL44 area. The average % colocalization was calculated to be 54.45 % in 10 representative images. We have incorporated the % colocalization plot and the representative images in Fig. 4A and Fig. S5A including images of mock-infected cells.

18. Fig 4B legend. Need to provide information about MOI and time after infection.

We have updated this in the Figure 4B figure legend as follows: *"Immunoblot assay using **1 MOI** of WT, shLacZ, shL1-1, and shL1-2 HCMV-infected HFFs at **4 dpi** (left)."*

19. Fig 4D result and methods. From the images shown in Fig 4C, it appears there is less colocalization of UL44 and pHA2x than what is reflected in Fig 4D results (correlation coefficient). This may have something to do with the method of analysis to determine colocalization coefficient. The method of analysis applied requires better explanation and additional detail. According to the methods, the images were captured using a Zeiss LSM 700 confocal microscope, which is an inverted scope. Do the number of cells per sample represent the number of cells that were in an image? Is the colocalization analysis applied to the entire image? More detail should be provided in methods about the original magnification used and pixels per image size. The degree of colocalization is dependent on the threshold values selected. How were the thresholds for the two channels selected to determine colocalization within a pixel?

As mentioned in the previous response to Comment #4, all of our confocal image data and corresponding measurements were analyzed in a cell-by-cell manner. Therefore, each value in the scatter plot (Fig. 4D, 4F, and S5D) was obtained from the ROI of a single virus-infected cell.

As the reviewer suggested, we have incorporated the details for our methods of analysis using colocalization coefficients including pixels per image size and the threshold setting to calculate the

colocalization coefficients in the Methods section as follows: "*Images were obtained by LSM700 confocal laser-scanning microscope (ZEISS) with C-Apochromat 40×/1.20NA water objective at a resolution of 512×512 pixels with a pixel size of 0.31 µm. The colocalization coefficients values were determined on a per-cell basis by counting the number of pH2AX pixels with an intensity ≥60 and overlapping with UL44 pixels having an intensity ≥60 using Zen Blue (ZEISS).*"

We believe this additional information adequately addresses the reviewer's questions regarding the methodology for colocalization analysis.

20. Fig 6B. It is difficult to evaluate this low magnification image. This does not look like an MOI of 0.5 as stated in the figure legend if the HFFs were confluent. What is the rationale behind doing this study at 31 hpi when viral replication compartment formation is minimal to non-existent and other experiments are done at ≥48 hpi when viral replication compartments have formed?

We agree with your concern. In the revised manuscript, we have replaced the Fig. 6B image with the higher resolution and higher magnification images of UL44 and EdU at 72 hpi, a time point when active HCMV replication is more evident. Additionally, we have quantified EdU intensities in the UL44 colocalized area from individual cells and have presented these results in Fig 6C to provide a more comprehensive analysis.

21. What is with the 'white' foci? An image of higher magnification should be shown in a supplementary figure that enables evaluation of the IE1/IE2 (Alexa 568) and EdU (Alexa 488) colocalization, preferably with a DNA counterstain (separate images and Alexa 488 and 568 merge).

We apologize for the confusing data presentation. To visualize how much HCMV-infected cells are replicating, we presented infected cells in red and EdU-positive infected cells in white. We have now included the higher magnification images (Fig. 6B), showing UL44, EdU, and Hoechst staining.

22. Because this section of results asserts that the "L1 retrotransposon-UL44 interaction accelerates viral DNA replication", it would have been more desirable to assess UL44 protein and EdU colocalization, as both colocalize in viral replication compartments and UL44 protein IFA was done in Fig 4.

We agree. We have revised the Fig 6B data to show EdU and UL44 colocalization (Fig. 6B) and have measured EdU intensity within UL44 areas (Fig. 6C) as already discussed in our response to previous comments (Comments #4, #20, and #21).

23. Line 280. MAB810R detects both IE1 and IE2 (directed to amino terminal ends of both proteins), though it's IE1 that is mostly represented in IFA.

Thank you for noting this. We have corrected the text to accurately reflect that MAB810R detects both IE1 and IE2 as follows: *"Cells were then permeabilized with 0.1% Triton X-100, incubated with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and stained with HCMV IE1/2 antibody (MAB810R; Millipore) and FITC-conjugated anti-mouse antibody (115-095-146; Jackson Laboratories)."*

Reviewer #2 (Remarks to the Author):

Overall, this is an outstanding paper. HCMV is a significant human pathogen. The role of host factors in viral DNA replication is an emerging area of interest that warrants further study. The hypothesis that HCMV utilizes host retrotransposons to facilitate its replication is novel and exciting. The paper is well written, and the experiments are very well-designed. With a couple of exceptions noted below, the data is convincing, beautifully presented, and supports the conclusions of the authors.

The citations are appropriate, but the authors might mention that RUNX3 has been previously shown to regulate chromatin remodeling in T cells (https://doi.org/10.1016/j.immuni.2018.03.028) and that host DNA translesion repair polymerases have been shown to be important for HCMV DNA replication and genome stability. (https://doi.org/10.1073/pnas.2203203119)

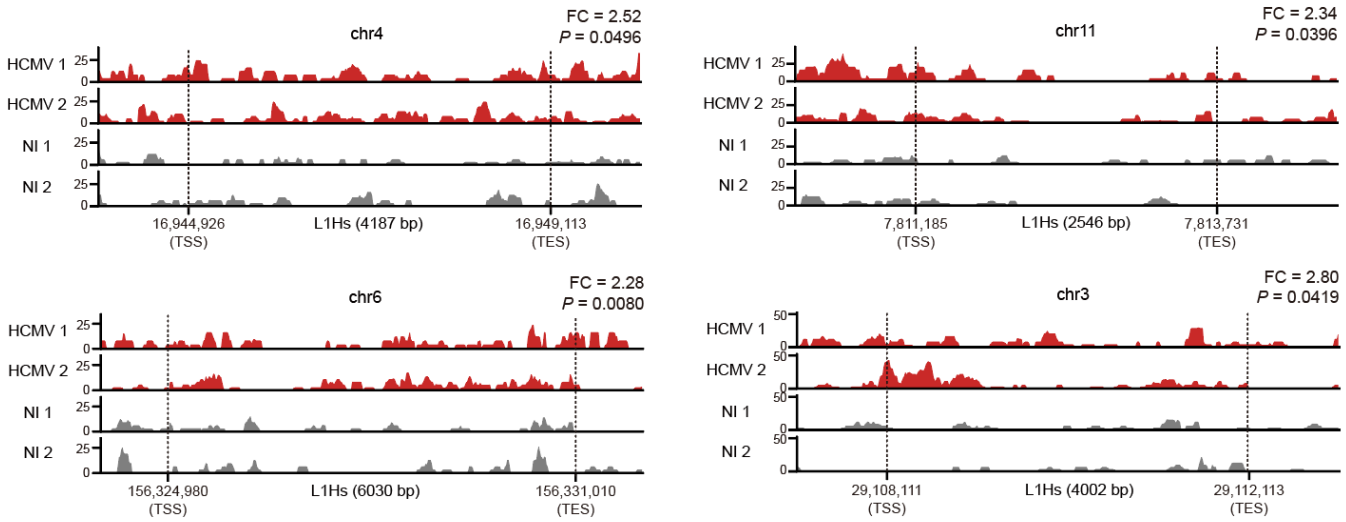
We appreciate the reviewer's positive feedback and helpful comments. According to your suggestion, we amended the 2nd paragraph of the Discussion section to mention the multi-regulatory role of RUNX3 as well as YY1. While we acknowledge the importance of DNA translesion polymerase in HCMV replication and genome stability, our focus in this paper is specifically on HCMV-induced DNA damage responses. Therefore, we feel it is beyond the scope of this work.

Some points could use clarification:

1. The ATAC-seq data is difficult to interpret. Fig. 1F shows differences in accessibility around the L1Hs TSS region between infected and uninfected cells. However, it was not possible to visualize these differences in the tracks from GSE 217152, since the specific locations were not provided. Please add a table with specific locations of L1 elements that are differentially affected by HCMV infection, including statistical analysis.

We thank the reviewer for this comment for a clearer interpretation of ATAC-seq data.

As suggested, we have added a supplementary table providing details about ATAC-seq signal, including the L1 lineage, specific location, length, fold changes, and the statistical analysis (p-value, n=2; **Table S1**). Here, we have included the example tracks for the increased read pileup of individual L1Hs element upon HCMV infection (**Peer Review Fig. 7**). Fig. 1F presents a summarized plot for ATAC-seq data, focusing on the TSS region as it specifically represents the intact L1Hs, while the TES region may include the exaggerated signal from duplicated mapping. We hope this additional data and explanation clarifies the interpretation of the ATAC-seq results.



Peer Review Fig. 7 Representative map of ATAC-seq signals increased by HCMV infection.

2. Is Fig. 3C labeled correctly? It is unclear to me why pUL44 is present in the supernatant rather than the beads in the samples that were untreated with RNAse A. Please clarify why this shows an association between pUL44 and L1 ORF1p.

We would like to clarify that the experiment in Fig. 3C involves the immunoprecipitation of L1 ORF1p-**FLAG** to purify L1 ribonucleoprotein complex (L1 RNP), and the "RNase A-" indicates the elution performed using excessive FLAG peptides.

Following a previously reported method[17], we investigated the interaction between UL44 and L1 RNP by using two different elution methods: 1) FLAG peptides competitive elution (labeled as RNase A- in the previous manuscript) and 2) RNase A treatment elution. FLAG peptides competitive elution is an antigen competitive elution by adding excessive amounts of Flag peptides, which causes the dissociation of FLAG-tagged L1 RNP from antibody-bead complexes. In this condition, UL44 was detected with L1 RNP which means UL44 interacts with L1 RNP. RNase A elution, otherwise, promotes the dissociation of RNA-dependent interaction, and notably, RNase A elution disrupts the L1 RNP interaction with UL44, which indicates that RNA is required for this interaction. However, RNase A treatment was not able to affect the interaction between L1 ORF1p and L1 ORF2p. It may be due to the tight association of L1 ORF1p with L1 ORF2p and its parental mRNA[18,19], which may shield the accessibility of RNase A.

We agree that the RNase A- labeling can be misinterpreted. Regarding Fig. 3C, we have corrected the RNase A- labeling to "FLAG peptides" and have added additional explanations in the main section as well as a schematic representation in the Fig 3C left panel. We believe this revision enhances the clarity of our experimental aim and indication.

3. The meaning of Lines 255-257 is unclear.

We thank the reviewer for suggesting to clarify this section. We had intended to highlight UL44 role as a molecular bridge for L1 ORF2p and HCMV genomic DNA but realized that it could be unclear. We have completely revised this (4th paragraph in the Discussion section; Lines 276-281) to clarify our argument.

Reviewer #3 (Remarks to the Author):

Summary

Hwang et al. show that HCMV activates L1 retrotransposon expression by RNAseq and Western blot for L1 ORF1 protein (Fig. 1A-D). They further analyze ATAC-seq data (Fig 1F, Supplemental figure 2) to identify RUNX3 and YY1 as candidate transcription factors that bind to the regulatory elements of L1. shRNA depletion of RUNX3 and YY1 reduce L1 expression for ORF1 (Fig. 1H). They generate a recombinant HCMV expressing shL1 or control LacZ to demonstrate that L1 knockdown reduces viral replication (Fig. 2). They use proteomics to show that L1 OR1 interacts with HCMV protein UL44 (DNA processivity factor). A hydrophobic pocket is identified in UL44 responsible for binding to L1 ORF2 protein, which also affects ORF1 binding (Fig. 3). HCMV induces gH2AX DNA damage, and this is shown to be dependent on ORF1. L1 endonuclease, but not its RT activity is shown to be responsible for gH2AX foci formation (Fig. 4). Deep sequencing and HAT-seq are used to investigate L1 integration into HCMV and generation of HCMV variants, but no significant integration and no L1-dependent mutation frequency is observed (Fig. 5). EdU and BrdU double labeling is used to show that L1 facilitates DNA replication and replication fork restart after camptothecin block (Fig. 6).

Overall- this is an impressive and compelling study demonstrating the functional role of L1 transposon in HCMV replication cycle. The methodology is multimodal and rigorous, experiments are generally well controlled and data analysis is clearly described. The findings are highly original and significant to the field of HCMV replication, host-interaction and to the broader field of virology through interaction of two virus types.

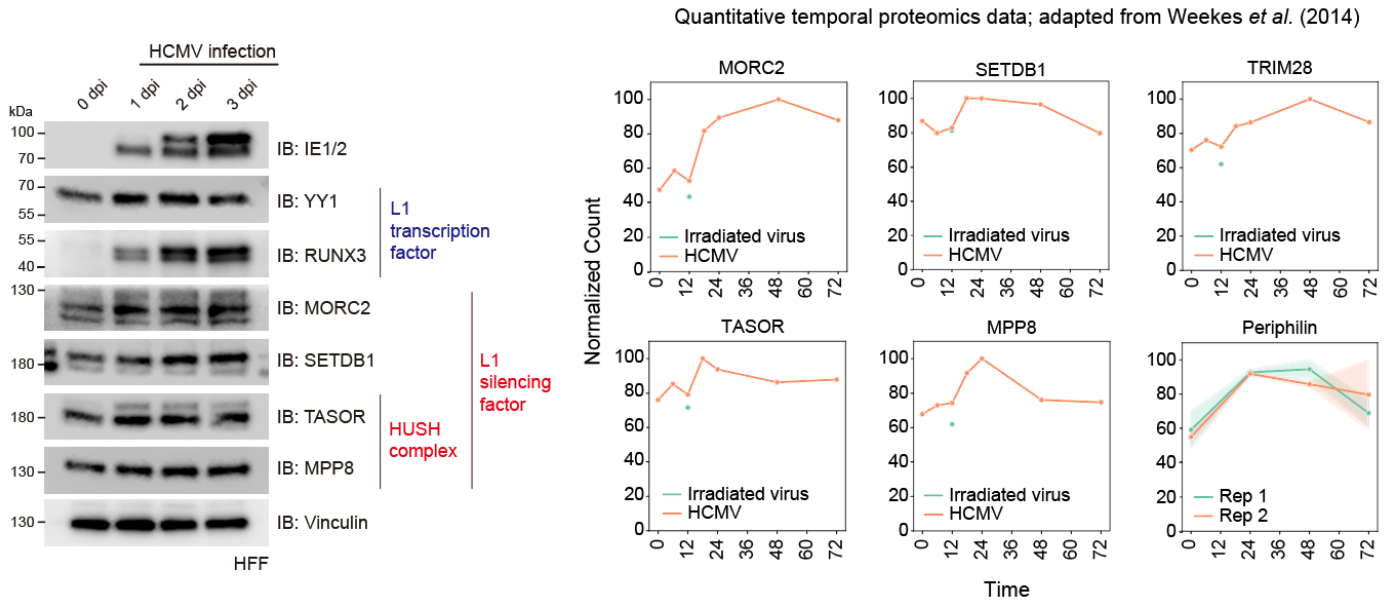
We sincerely appreciate the reviewer’s recognition of the significance of our work and insightful comments.

Specific Comments for Consideration

1. Fig S2 ATAC-seq suggests that large changes occur in the body and TES of the various L1 family member (S2A). This seems inconsistent with promoter (TSS) activation through RUNX3 and YY1. The authors should consider other mechanisms of HCMV activating L1 transcription, such as deregulating heterochromatin silencing factors (HUSH complex) or possibly Pol III transcription. Are cellular mRNA genes regulated by RUNX3 and YY1 hyperactivated? How does HCMV activate RUNX3 and YY1?

We thank the reviewer for raising this point. Despite large changes in chromatin accessibility of the body and TES region, there are two reasons why we focused our attention on TSS activation. First, binding of initial transcription factors triggers changes to nucleosome formation[20]. Therefore YY1- or RUNX3-mediated TSS activation can cause increased ATAC-seq reads on the body and TES. The second reason is only few full-length L1s exist since most L1s are 5′ truncated during retrotransposition[21,22]. Given that the ATAC-seq method relies on short read sequencing, we could not reliably conclude that the reads in the gene body or TES region represent full-length intact L1s. Thus, we limited our investigation to the 5′ end region of L1 including TSS.

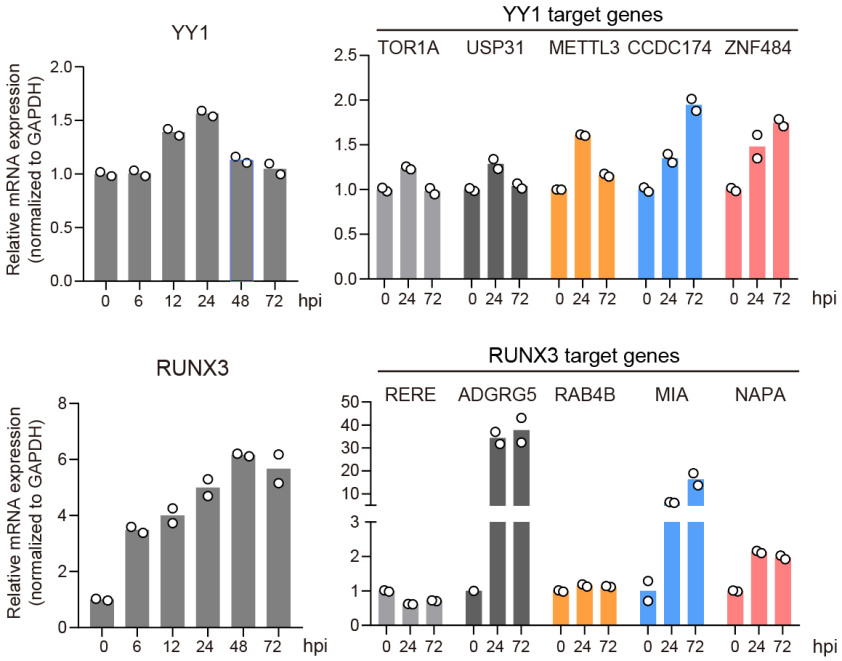
We also explored alternative mechanisms for L1 activation during HCMV infection throughout this project. One such endeavor was checking the HUSH complex, the major L1 silencing factor that recruits effector proteins, SETDB1 and MORC2. To test whether HCMV infection dysregulates L1 silencing factors by protein degradation, we examined the protein level of SETDB1, MORC2, TASOR, and MPP8 throughout the time course of HCMV infection, and found that their expression levels were either constant or even increased during HCMV infection (**Peer Review Fig. 8, left**). We were able to validate this unexpected result by reanalyzing the previously published proteomics dataset looking at protein expression levels throughout HCMV infection and found that the level of L1 silencing factors also increased or remained constant in this dataset as well[23]. (**Peer Review Fig. 8, right**).



Peer Review Fig. 8 Protein expression of L1 regulators during HCMV infection. Immunoblot (left) and normalized peptide count from mass spectrometry database [right; adapted from Weekes *et al.*, (2014)]

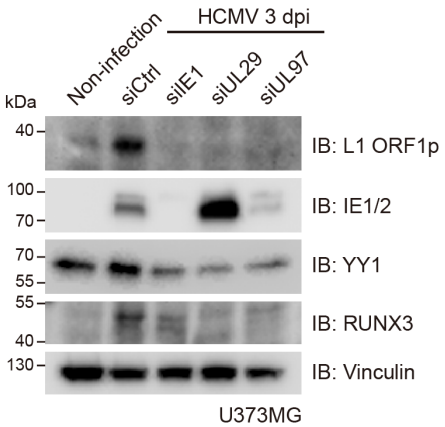
We acknowledge that this preliminary data/analysis does not preclude the possibility of L1 silencing factors being involved in L1 activation during HCMV infection. It is also conceivable that L1 disinhibition by HUSH complex and recruitment of its effector proteins precede YY1 and RUNX3-mediated transcriptional activation. However, we believe that deeper investigation of functional changes in HUSH complexes extends beyond the scope of this paper so we did not pursue this avenue further.

To address the reviewer's last inquiry, we also checked if other host transcripts activated by YY1 and RUNX3 were upregulated during HCMV infection. From the published ChIP database[24] of YY1 and RUNX3, we selected five genes top-enriched genes, and found that their expressions were indeed increased upon HCMV infection, showing almost identical patterns with their respective transcription factors (**Peer Review Fig. 9**). This result aligns our finding that HCMV infection upregulates YY1 and RUNX3 and their downstream transcripts including L1 are also upregulated as a consequence.



Peer Review Fig. 9 RNA expression of YY1, RUNX3, and their target genes.

We are also interested in how HCMV initially activates YY1 and RUNX3. Since we observed that UV-HCMV does not activate L1 (Fig. 1D), we hypothesized that viral genes expressed after infection activate L1. After a comprehensive literature search and a preliminary selection screen not shown here, we narrowed down our list to three viral candidates: IE1, UL29, and UL97, which have been shown to be involved in epigenetic regulation, especially histone deacetylase inhibition[25-27]. We transfected siRNA targeting these viral genes to U373MG cells, infected cells with HCMV, and tested expression levels of L1 ORF1p, YY1, and RUNX3. Notably, each of these three knockdowns reduced the expression levels of YY1, RUNX3, and L1 ORF1p (**Peer Review Fig. 10**). Excluding candidates that clearly affected HCMV lifecycle (i.e. IE1 and UL97), we believe UL29 is a promising viral factor that could upregulate YY1/RUNX3. This small candidate siRNA screen shows that HCMV viral genes can indeed activate YY1 and RUNX3 expression. In the current manuscript, we focused on the transcriptional regulation of L1 by YY1 and RUNX3. We hope that the mechanism through which genes like UL29 upregulates YY1/RUNX3 will be addressed in future works.



Peer Review Fig. 10 UL29 and UL97 are involved in HCMV-upregulated L1 expression.

2. Fig4. Line 136. It is not obvious that the L1 ORF2 is colocalizing with UL44 and CMV replication compartments. This needs to be quantified, since it is not obvious by these images, which suggests many non-colocalizing signals.

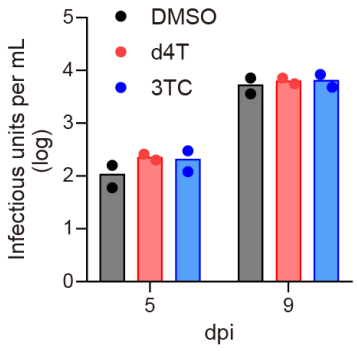
We appreciate the reviewer's suggestion for enhancing this analysis. To quantify the interaction between L1 ORF2p and UL44, we have obtained high-resolution images of L1 ORF2p and UL44 using HCMV-infected U373MG$^{pBL1ORF2p\text{-}3\times FLAG}$ cells (Fig. 4A and S5A). Then we quantified the % colocalization of UL44 and L1 ORF2p-3×FLAG (number of FLAG pixels in UL44 area / total number of FLAG pixels) using ImageJ. We extracted FLAG pixels with the top 1% intensity and calculated how many FLAG pixels were within the UL44 area. The average % colocalization was calculated to be 54.45 % in 10 representative images. We have incorporated the % colocalization plot and the representative images in Fig 4A and Fig S5A including images of mock-infected cells.

3. Fig 4. Need to show L1 ORF2 immunofluorescence pattern in uninfected cells.

We agree with the reviewer's suggestion. In line with previous observations for L1 RNP complexes[16], we found that L1 ORF2p showed cytoplasmic foci in pBL1$^{ORF2p\text{-}3\times FLAG}$ U373MG cells. We believe that L1 ORF2p localization in uninfected cells underscores the specific recruitment to VRC by UL44 in HCMV-infected cells. We have incorporated the uninfected cell images (shown as Mock) with HCMV-infected cells in Fig S5A.

4. Fig 4 and S5. It is very interesting finding that ORF2 contributes to gH2AX formation. Inhibitors of RT did not affect gH2AX, but do they have any effect on HCMV replication?

We appreciate the reviewer's suggestion to this important aspect. We tested whether RT inhibitors could have any effects on HCMV replication. We infected HFF with HCMV 0.1 MOI, treated two different RT inhibitors, stavudine (d4T) 5 μM and lamivudine 50 μM (3TC) after HCMV infection, and harvested cell-free viruses at indicated time points. Then we titrated infectious units per ml to assess viral replication. We found no differences in progeny virus production depending on RT inhibitors (**Peer Review Fig. 11**). We thought that the L1 RT process during HCMV infection did not significantly affect the HCMV replication, unlike the effect of L1 endonuclease.



Peer Review Fig. 11 Effects of RT inhibitors on HCMV growth.

5. I appreciate the extensive effort to find L1 insertions and mutational burden in HCMV genome, despite the negative results. First question is whether the pipeline and methods were sufficient to detect integrations. Is there a positive control test, perhaps using the data set from Pox virus? The next question is if there is no evidence of integration/transposition, why doesn't L1 integrate into HCMV, and how is this restricted- is there inactivation of L1 or other resistance of HCMV to L1 transposition?

We sincerely appreciate the reviewer's enthusiastic assessment of our work.

We'd like to clarify that our investigation focused specifically on clonal L1 insertions within HCMV genomes. Our hypothesis was that fitness-enhancing L1 insertions would persist and be carried by progeny viruses, leading to clonal expansion within infected populations (e.g., reaching 5% prevalence).

Our chosen methodology and sequencing throughput are well-suited for detecting clonal L1 insertions. We employed Human Active Transposon-seq (HAT-seq), a targeted capture approach proven to robustly identify low-level L1Hs integrations from bulk gDNA[28]. We achieved high sequencing throughput (avg. = 87.9 M reads/sample) for our HAT-seq libraries, exceeding benchmarks for detecting low-level mosaicisms (reported as 60 M reads/sample in Zhao *et al.*, 2019)[28].

Regarding the positive control: As a byproduct of the centrifugation-based enrichment method to harvest virus gDNA from the host cell culture, we have already included low-level positive controls (*e.g.*, <5% "spike-ins" of known L1 insertion in human gDNA) within the HCMV gDNA samples themselves. HAT-seq successfully identified numerous reference (avg. = 656 insertions/library) and non-reference L1Hs (avg. = 119/library) insertions in the human gDNA carryover, confirming the sensitivity of our method and analytical pipeline. Applying the same pipeline to the HCMV genome revealed only a few putative L1 insertions, none of which were confirmed by experimental validation. These results suggest that L1 retrotransposition may not be a primary mechanism for HCMV genetic adaptation. However, we cannot definitively rule out the possibility of L1 integration into HCMV; identifying and validating unique private insertions within individual HCMV genome remains technically challenging.

Finally, in the **Discussion** session, the cited poxvirus studies focus on L1-mediated mRNA (host gene) integration using a specific *in vitro* mCherry-host gene experimental system, which differs from our approach. We've revised our corresponding statement in the Discussion for enhanced clarity, as follows: *"L1-**mediated** retrotransposition occurs in poxviruses, facilitating horizontal gene transfer from the host to the virus."*

We thank the reviewer for this valuable insight and have updated **Supplementary Note 1. Increased L1-induced mutagenesis is not observed in HCMV for genetic adaptation**, **Table S5. Statistics on HCMV HAT-seq libraries**, and the corresponding **Methods** section for a detailed description of our methodology and rigorous data analysis.

6. Fig 6. The camptothecin experiment is interesting, but may not be sufficient to conclude that L1 is overcoming replication fork blocks. This could be attributed to a separate HCMV replication activity, and L1 is only facilitating a subsequent step, such as processivity or homologous recombination. The authors should discuss alternative interpretations of this experiment.

We appreciated the reviewer's constructive feedback. To address the review's concern, we conducted several new assays and have now added these data to the revised manuscript. All of these data support our hypothesis: 1) L1 proteins are localized at the viral replication fork (Fig. 6F), 2) L1 ORF2p and UL44 interaction is necessary for inducing DNA damage on viral replication compartments (Fig. 6G and 6H), 3) an orthogonal approach to quantify the CPT phenotype (Fig. S10F). While our new experimental findings support our hypothesis that the L1 ORF2p-UL44 interaction at the HCMV replication fork alleviates replication stress, we agree with the reviewer's point. Thus, we have revised the Results section to acknowledge the potential for alternative explanations. And, in the **Discussion** section, we have incorporated our interpretations of how L1 accelerates HCMV DNA replication (Please see 4th paragraph in the Discussion).

7. The paper would benefit, in my opinion, if the authors expand the very brief Discussion section to include comments on the potential alternative possible mechanism of HCMV transcription activation of diverse transposable elements (e.g. epigenetic factors other than YY1 and RUNX3), and the mechanism of action of L1 in promoting CMV replication.

We appreciate the reviewer's valuable insight. For the mechanism of transposable element activation, we have added a discussion on HCMV-mediated histone modifications, specifically the possibility of HCMV increasing histone acetylation and the possibility of unfolding the heterochromatin regions of TEs. We have added these new paragraphs in the Discussion section (2nd paragraph). Also, we speculate that L1-induced DNA damage may recruit other DNA repair proteins to the stalled fork region thereby contributing to reducing replication stress and/or DNA repair rate, which we have also added to the 4th paragraph in the Discussion section.

References

1 Czimmerer, Z. *et al.* A versatile method to design stem-loop primer-based quantitative PCR assays for detecting small regulatory RNA molecules. *PLoS One* **8**, e55168 (2013). https://doi.org:10.1371/journal.pone.0055168

2 Ardeljan, D. *et al.* Cell fitness screens reveal a conflict between LINE-1 retrotransposition and DNA replication. *Nature Structural & Molecular Biology* **27**, 168-178 (2020). https://doi.org:10.1038/s41594-020-0372-1

3 Mita, P. *et al.* BRCA1 and S phase DNA repair pathways restrict LINE-1 retrotransposition in human cells. *Nat Struct Mol Biol* **27**, 179-191 (2020). https://doi.org:10.1038/s41594-020-0374-z

4 Sirbu, B. M. *et al.* Analysis of protein dynamics at active, stalled, and collapsed replication forks. *Genes Dev* **25**, 1320-1327 (2011). https://doi.org:10.1101/gad.2053211

5 Leung, K. H., Abou El Hassan, M. & Bremner, R. A rapid and efficient method to purify proteins at replication forks under native conditions. *Biotechniques* **55**, 204-206 (2013). https://doi.org:10.2144/000114089

6 Zellweger, R. *et al.* Rad51-mediated replication fork reversal is a global response to genotoxic treatments in human cells. *J Cell Biol* **208**, 563-579 (2015). https://doi.org:10.1083/jcb.201406099

7 Berniak, K. *et al.* Relationship between DNA damage response, initiated by camptothecin or oxidative stress, and DNA replication, analyzed by quantitative 3D image analysis. *Cytometry A* **83**, 913-924 (2013). https://doi.org:10.1002/cyto.a.22327

8 Montecucco, A. *et al.* Etoposide induces the dispersal of DNA ligase I from replication factories. *Mol Biol Cell* **12**, 2109-2118 (2001). https://doi.org:10.1091/mbc.12.7.2109

9 Zhao, H., Rybak, P., Dobrucki, J., Traganos, F. & Darzynkiewicz, Z. Relationship of DNA damage signaling to DNA replication following treatment with DNA topoisomerase inhibitors camptothecin/topotecan, mitoxantrone, or etoposide. *Cytometry A* **81**, 45-51 (2012). https://doi.org:10.1002/cyto.a.21172

10 Merchut-Maya, J. M. *et al.* Human cytomegalovirus hijacks host stress response fueling replication stress and genome instability. *Cell Death Differ* **29**, 1639-1653 (2022). https://doi.org:10.1038/s41418-022-00953-w

11 E, X. *et al.* An E2F1-mediated DNA damage response contributes to the replication of human cytomegalovirus. *PLoS Pathog* **7**, e1001342 (2011). https://doi.org:10.1371/journal.ppat.1001342

12 Casavant, N. C. *et al.* Potential role for p53 in the permissive life cycle of human cytomegalovirus. *Journal of Virology* **80**, 8390-8401 (2006). https://doi.org:10.1128/Jvi.00505-06

13 Castillo, J. P. *et al.* Human cytomegalovirus IE1-72 activates ataxia telangiectasia mutated kinase and a p53/p21-mediated growth arrest response. *Journal of Virology* **79**, 11467-11475 (2005). https://doi.org:10.1128/Jvi.79.17.11467-11475.2005

14 Xiaofei, E. & Kowalik, T. F. The DNA damage response induced by infection with human cytomegalovirus and other viruses. *Viruses* **6**, 2155-2185 (2014). https://doi.org:10.3390/v6052155

15 Ardeljan, D. *et al.* LINE-1 ORF2p expression is nearly imperceptible in human cancers. *Mob DNA* **11**, 1 (2020). https://doi.org:10.1186/s13100-019-0191-2

16 Doucet, A. J. *et al.* Characterization of LINE-1 ribonucleoprotein particles. *PLoS Genet* **6** (2010). https://doi.org:10.1371/journal.pgen.1001150

17 Taylor, Martin S. *et al.* Affinity Proteomics Reveals Human Host Factors Implicated in Discrete Stages of LINE-1 Retrotransposition. *Cell* **155**, 1034-1048 (2013). https://doi.org:https://doi.org/10.1016/j.cell.2013.10.021

18 Basame, S. *et al.* Spatial assembly and RNA binding stoichiometry of a LINE-1 protein essential for retrotransposition. *J Mol Biol* **357**, 351-357 (2006). https://doi.org:10.1016/j.jmb.2005.12.063

19 Kulpa, D. A. & Moran, J. V. Ribonucleoprotein particle formation is necessary but not sufficient for LINE-1 retrotransposition. *Hum Mol Genet* **14**, 3237-3248 (2005). https://doi.org:10.1093/hmg/ddi354

20 Zaret, K. S. Pioneer Transcription Factors Initiating Gene Network Changes. *Annu Rev Genet* **54**, 367-385 (2020). https://doi.org:10.1146/annurev-genet-030220-015007

21 Szak, S. T. *et al.* Molecular archeology of L1 insertions in the human genome. *Genome Biol* **3**, research0052 (2002). https://doi.org:10.1186/gb-2002-3-10-research0052

22 Zingler, N. *et al.* Analysis of 5' junctions of human LINE-1 and Alu retrotransposons suggests an alternative model for 5'-end attachment requiring microhomology-mediated end-joining. *Genome Res* **15**, 780-789 (2005). https://doi.org:10.1101/gr.3421505

23 Weekes, Michael P. *et al.* Quantitative Temporal Viromics: An Approach to Investigate Host-Pathogen Interaction. *Cell* **157**, 1460-1472 (2014). https://doi.org:https://doi.org/10.1016/j.cell.2014.04.028

24 Oki, S. *et al.* ChIP-Atlas: a data-mining suite powered by full integration of public ChIP-seq data. *EMBO Rep* **19** (2018). https://doi.org:10.15252/embr.201846255

25 Nevels, M., Paulus, C. & Shenk, T. Human cytomegalovirus immediate-early 1 protein facilitates viral replication by antagonizing histone deacetylation. *Proc Natl Acad Sci U S A* **101**, 17234-17239 (2004). https://doi.org:10.1073/pnas.0407933101

26 Terhune, S. S. *et al.* Human cytomegalovirus UL29/28 protein interacts with components of the NuRD complex which promote accumulation of immediate-early RNA. *PLoS Pathog* **6**, e1000965 (2010). https://doi.org:10.1371/journal.ppat.1000965

27 Bigley, T. M., Reitsma, J. M., Mirza, S. P. & Terhune, S. S. Human cytomegalovirus pUL97 regulates the viral major immediate early promoter by phosphorylation-mediated disruption of histone deacetylase 1 binding. *J Virol* **87**, 7393-7408 (2013). https://doi.org:10.1128/JVI.02825-12

28 Zhao, B. *et al.* Somatic LINE-1 retrotransposition in cortical neurons and non-brain tissues of Rett patients and healthy individuals. *PLoS Genet* **15**, e1008043 (2019). https://doi.org:10.1371/journal.pgen.1008043

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have done a commendable job adequately addressing prior questions/concerns and adding findings/information that strengthen their conclusion

Reviewer #2 (Remarks to the Author):

In this revised manuscript, the authors have added to new data to support their novel conclusion that L1 retrotransposons induced by HCMV infection facilitate viral DNA replication by alleviating stress at the viral replication fork.
The authors have thoroughly addressed all the issues raised by the reviewers, and in so doing, they have significantly improved the quality of the manuscript. I thank the authors for the additional ATAC-seq data, which provides specific data on the location of changes in chromatin accessibility induced by HCMV infection. Regarding Fig. 3, the authors have clarified their rationale, and the additional illustration is helpful. It would be even more clear if the lanes labeled “Sup” were labeled as Eluate.
I congratulate the authors on an impressive study that significantly advances the field.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed my previous concerns. The revised manuscript is significantly improved.