

# **1 *In vitro* and *in vivo* characterization of a recombinant**

## **2 rhesus cytomegalovirus containing a complete genome**

**4 Husam Taher<sup>1#</sup>, Eisa Mahyari<sup>1,2#</sup>, Craig Kreklywich<sup>1</sup>, Luke S. Uebelhoer<sup>1a</sup>, Matthew R.**  
**5 McArdle<sup>1b</sup>, Matilda J. Moström<sup>3</sup>, Amruta Bhusari<sup>1</sup>, Michael Nekorchuk<sup>1,2</sup>, Travis**  
**6 Whitmer<sup>1c</sup>, Elizabeth A. Scheef<sup>3</sup>, Lesli M. Sprehe<sup>3</sup>, Dawn Roberts<sup>4</sup>, Colette M. Hughes<sup>1</sup>,**  
**7 Kerianne A. Jackson<sup>1</sup>, Andrea N. Selseth<sup>1</sup>, Abigail B. Ventura<sup>1</sup>, Yujuan Yue<sup>5</sup>, Kimberli A.**  
**8 Schmidt<sup>5</sup>, Jason Shao<sup>6</sup>, Paul T. Edlefsen<sup>6</sup>, Jeremy Smedley<sup>2</sup>, Richard J. Stanton<sup>4</sup>, Michael**  
**9 K. Axthelm<sup>2</sup>, Jacob D. Estes<sup>1,2</sup>, Scott G. Hansen<sup>1</sup>, Amitinder Kaur<sup>3</sup>, Peter A. Barry<sup>5</sup>,**  
**10 Benjamin N. Bimber<sup>1,2</sup>, Louis J. Picker<sup>1</sup>, Daniel N. Streblow<sup>1</sup>, Klaus Früh<sup>1</sup>, and Daniel**

**11 Malouli<sup>1\*</sup>**

**12**  
**13** <sup>1</sup>Vaccine and Gene Therapy Institute, Oregon Health and Science University, Beaverton,  
**14** Oregon, United States of America

**15** <sup>2</sup>Oregon National Primate Research Center, Oregon Health and Science University, Beaverton,  
**16** Oregon, United States of America

**17** <sup>3</sup>Tulane National Primate Research Center, Covington, LA, United States,

**18** <sup>4</sup>Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff, United  
**19** Kingdom

**20** <sup>5</sup>Center for Comparative Medicine and Department of Medical Pathology, University of  
**21** California, Davis, California, USA

**22** <sup>6</sup>Statistical Center for HIV/AIDS Research and Prevention, Vaccine and Infectious Disease  
**23** Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA.

**24**

25 <sup>#</sup>These authors contributed equally.

26

27 Current address:

28 <sup>a</sup>Department of Pediatrics, Oregon Health & Science University, Portland, Oregon, USA.

29 <sup>b</sup>Department of Biochemistry, University of Utah, Salt Lake City, Utah, USA

30 <sup>c</sup>Merck & Co., Inc., Kenilworth, New Jersey, USA

31

32 Short Title: Construction of a clonal rhesus cytomegalovirus BAC containing a complete genome.

33

34 \*Corresponding author: Daniel Malouli

35 Vaccine and Gene Therapy

36 Institute, Oregon Health and Science University,

37 505 NW 185<sup>th</sup> Ave., Beaverton, OR 97006.

38 Phone: (503) 418-2738

39 Fax: (503) 418-2701

40 E-mail: [maloulid@ohsu.edu](mailto:maloulid@ohsu.edu)

41

42 **Abstract** (300 words)

43 Cytomegaloviruses (CMVs) are highly adapted to their host species resulting in strict species  
44 specificity. Hence, *in vivo* examination of all aspects of CMV biology employs animal models  
45 using host-specific CMVs. Infection of rhesus macaques (RM) with rhesus CMV (RhCMV) has  
46 been established as a representative model for infection of humans with HCMV due to the close  
47 evolutionary relationships of both host and virus. However, the commonly used 68-1 strain of  
48 RhCMV has been passaged in fibroblasts for decades resulting in multiple genomic changes due  
49 to tissue culture adaptation that cause reduced viremia in RhCMV-naïve animals and limited  
50 shedding compared to low passage isolates. Using sequence information from primary RhCMV  
51 isolates we constructed a full-length (FL) RhCMV by repairing all presumed mutations in the 68-  
52 1 bacterial artificial chromosome (BAC). Inoculation of adult, immunocompetent, RhCMV-naïve  
53 RM with the reconstituted virus resulted in significant replication in the blood similar to primary  
54 isolates of RhCMV and furthermore led to extensive viremia in many tissues at day 14 post  
55 infection. In contrast, viral dissemination and viremia was greatly reduced upon deletion of genes  
56 also lacking in 68-1. Transcriptome analysis of infected tissues further revealed that chemokine-  
57 like genes deleted in 68-1 are among the most highly expressed viral transcripts both *in vitro* and  
58 *in vivo* consistent with an important immunomodulatory function of the respective proteins. We  
59 conclude that FL-RhCMV displays *in vitro* and *in vivo* characteristics of a wildtype virus while  
60 being amenable to genetic modifications through BAC recombineering techniques.

61

62 **Author Summary** (150–200 word non-technical summary)

63 Human cytomegalovirus (HCMV) infections are generally asymptomatic in healthy  
64 immunocompetent individuals, but HCMV can cause serious disease after congenital infection and  
65 in individuals with immunocompromised immune systems. Since HCMV is highly species specific  
66 and cannot productively infect immunocompetent laboratory animals, experimental infection of  
67 rhesus macaques (RM) with rhesus CMV (RhCMV) has been established as a closely related  
68 animal model for HCMV. By employing the unique ability of CMV to elicit robust and lasting  
69 cellular immunity, this model has also been instrumental in developing novel CMV-based vaccines  
70 against chronic and recurring infections with pathogens such as the human immunodeficiency  
71 virus (HIV) and *Mycobacterium tuberculosis* (*Mtb*). However, most of this work was conducted  
72 with derivatives of the 68-1 strain of RhCMV which has acquired multiple genomic alterations in  
73 tissue culture. To model pathogenesis and immunology of clinical HCMV isolates we generated a  
74 full-length (FL) RhCMV clone representative of low passage isolates. Infection of RhCMV-naïve  
75 RM with FL-RhCMV demonstrated viremia and tissue dissemination that was comparable to that  
76 of non-clonal low passage isolates. We further demonstrate that FL-RhCMV is strongly attenuated  
77 upon deletion of gene regions absent in 68-1 thus demonstrating the usefulness of FL-RhCMV to  
78 study RhCMV pathogenesis.

79

80

81 **Introduction**

82 Chronic human cytomegalovirus (HCMV) infections are generally asymptomatic in healthy,  
83 immunocompetent individuals and seroprevalence ranges from approximately 45% in developed  
84 countries to almost 100% of the population in the developing world (1). However, the virus can  
85 cause significant disease after congenital infection and in individuals with immunocompromised  
86 immune systems (2). No vaccines against HCMV exist, and treatment with antiviral drugs can  
87 limit acute infections but cannot eliminate the persistent virus (3). Cytomegaloviruses are double  
88 stranded DNA viruses belonging to the herpesvirus subfamily *Betaherpesvirinae* and have so far  
89 been exclusively found in mammals, mainly rodents and primates (4). CMVs contain the largest  
90 genomes of all herpesviruses and current annotations predict upwards of 170 open reading frames  
91 (ORFs) for most species. Ribozyme profiling data suggests that the actual number of translated  
92 viral mRNAs is likely significantly higher (5), however only a subset of these produce high levels  
93 of protein during infection of fibroblasts (6, 7). Co-evolution of these viruses with their host  
94 species over millions of years has led to a sequence relationship between CMV species that  
95 generally mirrors that of their hosts while also resulting in strict species specificity (8, 9). Hence,  
96 HCMV does not replicate and is not pathogenic in immunocompetent animals, and animal models  
97 of HCMV thus generally rely on studying infection of a given host with their respective animal  
98 CMV. The most commonly used models are mice, rats, guinea pigs and rhesus macaques (RM).  
99 The close evolutionary relationship of RM to humans (as compared to rodents) is mirrored in the  
100 evolutionary relationship of the rhesus CMV (RhCMV) genome to HCMV as the overall genomic  
101 organization is similar and most viral gene families are found in both CMV species (10).

102 Infection of RM with RhCMV has thus become a highly useful animal model for HCMV  
103 including a model for congenital infection (11). In addition, RhCMV has been used extensively to

104 explore the possibility of harnessing the unique immune biology of CMV as a novel vaccine  
105 strategy, in particular the ability to elicit and maintain high frequencies of effector memory T cells  
106 (12). This work revealed not only that RhCMV-based vectors are remarkably effective in  
107 protecting RM against challenge with simian immunodeficiency virus (SIV), *Mtb* and *Plasmodium*  
108 *knowlesi* (13-16), but also uncovered a unique and unexpected ability of RhCMV to be genetically  
109 programmed to elicit CD8<sup>+</sup> T cells that differ in their MHC restriction (17, 18). Importantly, highly  
110 attenuated RhCMV vaccine vectors that display highly reduced viremia, dissemination and  
111 shedding maintain the adaptive immune program and the ability to protect against pathogen  
112 challenge (19, 20).

113 However, the vast majority of these immunological and challenge studies relied on a  
114 molecular clone of RhCMV that was derived from strain 68-1 which differs significantly from  
115 circulating RhCMV strains. The RhCMV strain 68-1 was originally isolated in 1968 from the urine  
116 of a healthy RM (21) and had been extensively passaged on fibroblasts for more than 30 years  
117 before being cloned as a BAC (22). During this time, 68-1 has acquired multiple tissue culture  
118 adaptations (10) including an inversion in a genomic region homologous to the HCMV ULb'  
119 region. This inversion simultaneously deleted the genes Rh157.5 and Rh157.4 (UL128 and UL130  
120 in HCMV), two members of the viral pentameric receptor complex (PRC), as well as three of six  
121 genes encoding chemokine-like proteins homologous to the HCMV UL146 family (23). Similar  
122 to PRC-deficient HCMV, the loss of a functional PRC resulted in restricted cell tropism of 68-1  
123 RhCMV *in vitro* (24, 25). PRC-dependent infection of non-fibroblast cells, such as epithelial and  
124 endothelial cells, was increased upon the insertion of the Rh157.5 and Rh157.4 genes obtained  
125 from the unrelated RhCMV 180.92 strain (25). Furthermore, strain 68-1 showed reduced viremia  
126 and shedding compared to the low passage isolates UCD52 and UCD59 upon primary infection of

127 RhCMV-seronegative RM (26). UCD52, UCD59 and 180.92 have also been used in congenital  
128 infection studies (11, 27, 28). However, UCD52 and UCD59 (29, 30) represent non-clonal isolates  
129 that have been passaged on rhesus epithelial cells instead of fibroblasts, a culture methods that  
130 preserves the PRC but can also lead to tissue culture adaptations (31, 32). The 180.92 strain was  
131 shown to consist of a mixture of a tissue culture adapted and wildtype variants with the latter  
132 rapidly emerging as the dominant variant *in vivo* (33). Thus, there is a need for the construction of  
133 a BAC-cloned RhCMV representative of primary isolates to enable studies that reflect circulating  
134 RhCMV strains and recapitulate the pathogenesis of HCMV. In addition, such a tissue culture non-  
135 adapted, but genetically modifiable RhCMV clone would also be a useful tool to model HCMV-  
136 based vaccine development for live-attenuated candidates derived from clinical isolates (34).

137 Here, we describe the construction of such a BAC-cloned RhCMV genome in which all  
138 presumed mutations in 68-1 that result in altered ORFs were repaired thus closely reflecting a  
139 clone of the original 68-1 isolate prior to tissue culture passage. We demonstrate that the resulting  
140 viral sequence, termed FL-RhCMV, is representative of contemporary RhCMV isolates from  
141 multiple primate centers. FL-RhCMV demonstrates *in vitro* growth characteristics that are very  
142 similar to those reported for primary isolates of HCMV, including the rapid accumulation of  
143 mutations in the gene homologous to HCMV RL13. Furthermore, we show that FL-RhCMV  
144 displays wildtype-like viremia in RhCMV-seronegative RM. The availability of the first RhCMV  
145 BAC clone containing a complete genome sequence granting the derived virus all characteristics  
146 of a circulating isolate will enable the selected modulation of tissue tropism, pathogenesis and  
147 immune stimulation. This is exemplified by our demonstration that the deletion of the RhCMV  
148 homologs of HCMV UL128, UL130 and UL146 profoundly impacted viral dissemination and  
149 proliferation during acute infection. Thus, we report the generation of a RhCMV BAC that

150 represents a primary isolate and that can serve as a modifiable progenitor for studies using RhCMV

151 as model for HCMV infection or HCMV-based vaccine vectors.

152

153 **Results**

154 **Construction of a full length (FL) RhCMV BAC and *in vitro* characterization.**

155 Compared to circulating and low passage isolates, RhCMV strain 68-1 has acquired a large  
156 inversion in the region homologous to one end of the HCMV “unique long” (UL) sequence of the  
157 genome (commonly referred to as the ULb' region), flanked by deletions of multiple ORFs on  
158 either side of the inversion (23, 35, 36). When sequencing the clonal bacterial artificial  
159 chromosome (BAC) of 68-1, we additionally identified multiple viral ORFs that contained point  
160 mutations predicted to result in frameshifts or premature terminations of the annotated proteins  
161 (10). One of these point mutations is located in Rh61/Rh60 (UL36 in HCMV) and renders the  
162 encoded inhibitor of extrinsic apoptosis non-functional (37). By reversing the frameshift in  
163 Rh61/Rh60 and by inserting the missing PRC members Rh157.5 and Rh157.4 (UL128 and UL130  
164 in HCMV) from the unrelated RhCMV strain 180.92, BAC-cloned 68-1 was partially repaired  
165 resulting in clone RhCMV 68-1.2 which exhibits broader cell tropism (25). However, the 68-1.2  
166 RhCMV genome sequence still differed significantly from the sequence of low passage RhCMV  
167 isolates due to additional mutations that were likely acquired during the prolonged tissue culture  
168 of the original 68-1 isolate. The inverted segment in the UL-homologous region of 68-1 RhCMV  
169 was recently re-examined by amplifying and sequencing DNA from the original urine sample used  
170 for virus isolation in 1968 (38). This work revealed the sequence of genes deleted in the UL region  
171 upon later passage of 68-1 including the homologs of UL128 and UL130 which showed substantial  
172 sequence variation compared to the corresponding genes of 180.92 used in the repaired 68-1.2.  
173 This is likely due to significant polymorphism across strains for these genes in RhCMV (39). To  
174 create a BAC that most closely resembles a clone of the original 68-1 primary urine isolate we  
175 therefore synthesized the entire gene region containing the inverted and missing genes in the UL

176 region in two overlapping fragments that were then inserted into RhCMV 68-1.2 by homologous  
177 recombination (**Fig. 1**). Subsequently, we used *en passant* recombination to repair all point  
178 mutation resulting in truncated ORFs as well as a nonsynonymous point mutation in Rh164  
179 (UL141). Finally, we deleted a transposon from Rh167 (O14) that was inadvertently acquired  
180 during the construction of the RhCMV 68-1.2 BAC. We confirmed the correct sequence of our  
181 BAC by restriction digest and next generation sequencing (NGS) and termed the final construct  
182 full length RhCMV (FL-RhCMV).

183 To characterize the phylogenetic relationship of the 68-1 derived FL-RhCMV BAC clone  
184 to related old world NHP CMV species, we cultured and sequenced new isolates from RhCMV,  
185 cynomolgus CMV (CyCMV), Japanese macaque CMV (JaCMV) and baboon CMV (BaCMV)  
186 from two different US primate centers. We also performed next generation sequencing on viral  
187 DNA isolated from stocks of the extensively characterized RhCMV isolates UCD52 and UCD59  
188 grown on epithelial cells and included these genome sequences into our analysis. For comparison,  
189 we included all NHP CMV sequences of complete or mostly complete genomes deposited in  
190 GenBank (**Fig. 2**). As expected, FL-RhCMV clustered with all other RhCMV isolates and was  
191 more distantly related to the CMVs from other NHPs, with the evolutionary relationship of CMV  
192 species tracing the evolutionary relationship between their corresponding host species.

193 To ensure that FL-RhCMV contained the full ORFeome of all presently confirmed and  
194 predicted viral genes of circulating RhCMV strains, we compared the full annotation of FL-  
195 RhCMV with that of other old world NHP CMVs (35, 40-42). All RhCMV genomes lack an  
196 internal repeat sequence so that the genomic regions corresponding to the unique long (U<sub>L</sub>) or the  
197 unique short (U<sub>S</sub>) coding regions are fixed in a given orientation whereas HCMV and ChCMV  
198 genomes can freely switch between four isomeric forms (**Supplementary Fig. 1**). Interestingly,

199 the Us-homologous region of BaCMV and Drill CMV (DrCMV) is fixed in the opposite  
200 orientation compared to RhCMV consistent with an isomer fixation event independent from the  
201 RhCMV lineage indicating that single isomers were fixed during the evolution of old world NHP  
202 CMVs on more than one occasion. A closer analysis of the genomes revealed that all primary  
203 RhCMV isolates without obvious deletions or inversions are predicted to contain the exact same  
204 ORFs in the same order. No strain-specific ORFs were identified based on our previously  
205 established RhCMV annotation (10). FL-RhCMV contains all ORFs found in other RhCMVs  
206 indicating that the full genome content has been restored (**Fig. 3**). A closer examination of full  
207 genome alignments of all known old world NHP CMV genome sequences additionally allowed us  
208 to further refine our previously established annotations with changes largely comprising  
209 reannotations of start codons and splice donor- and acceptor sites (**Supplementary Table 1**).  
210 Comparing the viral ORFeomes across old world NHP CMV species revealed a very high degree  
211 of conservation in the entire lineage of viruses so that the entire RhCMV annotation can almost  
212 seamlessly be transferred to all related species. While our results are based on comparative  
213 genomics and hence need to be confirmed experimentally by mass spectrometry or ribozyme  
214 profiling, it is interesting to note that most ORFs that differ between NHP CMV species are due  
215 to gene duplication events that occurred in six different loci across the genome (**Supplementary**  
216 **Fig. 2-7**). Taken together we conclude that the FL-RhCMV clone we engineered is likely a  
217 representative of the genomes contained in the original 68-1 isolate.

218

### 219 ***In vitro* characterization of FL-RhCMV**

220 As we have reported earlier (10), one of the ORFs frequently mutated in passaged RhCMV and  
221 other old world NHP CMV isolates is the RL11 family member Rh13.1 (**Fig. 3**). This ORF is

222 homologous to HCMV RL13 which is often lost or mutated upon tissue culture passage of HCMV  
223 (32). Loss of a functional RL13 protein was shown to result in more rapid cell to cell spread in  
224 tissue culture suggesting that RL13-deficient HCMV mutants have a substantial growth advantage  
225 *in vitro* (43). Loss of RL13 could be prevented in HCMV by conditional expression of RL13  
226 mRNA under the control of a tet operator (tetO) and growth in tet-repressor (tetR)-expressing  
227 fibroblasts (43). As we have repaired this ORF and likely restored its function during the  
228 construction of FL-RhCMV we wanted to examine whether conditional expression of Rh13.1  
229 would similarly affect the spread of FL-RhCMV in tissue culture. Hence, we inserted tandem tetO  
230 sequences 131 bp upstream of the Rh13.1 start codon and transfected the resulting FL-  
231 RhCMV/Rh13.1/tetO BAC DNA into telomerized rhesus fibroblasts (TRFs) expressing tetR (44).  
232 The cells were overlaid to prevent cell-free spread and upon recovery of virus we measured viral  
233 plaques sizes after 18 days. As a control, we included a FL-RhCMV in which the Rh13.1 ORF had  
234 been deleted (FL-RhCMVΔRh13.1). The development of plaques was severely impeded in TRFs  
235 transfected with FL-RhCMV or FL-RhCMV/Rh13.1/tetO (**Fig. 4A, B**). In contrast, FL-  
236 RhCMVΔRh13.1 spread rapidly in TRF and expression of the tetR led to a partial rescue of plaque  
237 formation by FL-RhCMV/Rh13.1/tetO (**Fig. 4A, B**).

238 As an alternative approach to conditionally express Rh13.1 we explored the use of  
239 aptazyme riboswitches mediating the tetracycline dependent degradation of mRNAs *in cis* (45).  
240 We inserted the Tc40 aptazyme sequence upstream and the Tc45 aptazyme sequence downstream  
241 of the Rh13.1 coding region in FL-RhCMV and monitored the stability of Rh13.1 and the  
242 surrounding genomic region by NGS upon recovery and propagation of virus in the presence or  
243 absence of tetracycline. FL-RhCMV/Rh13.1/apt grown in the absence of tetracycline displayed  
244 multiple mutations and deletions in this genomic region as early as passage 2 (**Fig. 4C**). In contrast,

245 by activating the aptazyme using tetracycline we were able to generate virus stocks that contained  
246 an intact Rh13.1 sequence (**Fig. 4C**). These data are consistent with Rh13.1 being selected against  
247 in FL-RhCMV similar to selection against RL13 in HCMV because these homologous proteins  
248 impede spread in tissue culture. We further conclude that mutations in the Rh13.1 homologs found  
249 in many old world NHP CMV genomes (**Fig. 3**) are due to rapid tissue culture adaptations whereas  
250 the parental isolates likely contained an intact ORF. Thus, Rh13.1 and its homologs are preserved  
251 *in vivo*, but are selected against *in vitro*.

252 It was previously shown that repair of the PRC increased the ability of RhCMV 68-1.2 to  
253 infect epithelial and endothelial cells without affecting growth characteristics in fibroblasts (25).  
254 Similarly, growth characteristics of FL-RhCMV/Rh13.1/apt were comparable to that of 68-1 and  
255 PRC-repaired 68-1.2 in rhesus fibroblasts with respect to kinetics and peak titers in a multistep  
256 growth curve (**Fig. 5A**). Since these comparable growth kinetics were observed in the absence of  
257 tetracycline induced Rh13.1 mRNA degradation, we conclude that while the presence of Rh13.1  
258 does effect virus cell to cell spread after transfection of BAC DNA (**Fig. 4A, B**), we cannot  
259 observe a phenotype in the context of a multistep-growth curve when starting with infectious  
260 virus and without overlaying the cell monolayer to prohibit cell free spread. Since the PRC is  
261 important for entry into non-fibroblast cells (25) we quantified infection levels of 68-1, 68-1.2 and  
262 FL-RhCMV upon entry into rhesus retinal epithelial (RPE) cells or primary rhesus fibroblasts  
263 using flow cytometry. When normalized to infected fibroblasts, 68-1 showed a strongly reduced  
264 ability to enter RPE compared to 68-1.2 (**Fig. 5B**) consistent with previous reports (25). In contrast,  
265 a FL-RhCMV vector carrying an SIVgag insert replacing Rh13.1 (FL-RhCMVΔRh13.1gag)  
266 displayed an increased ability to enter RPE cells compared to 68-1. However, infection rates on  
267 RPE cells with FL-RhCMV were consistently lower compared to RhCMV 68-1.2 in multiple

268 independent experiments. In HCMV, strain specific differences in tropism can arise from  
269 alterations in the levels of both the PRC and the gH/gL/gO trimeric receptor complex which can  
270 be caused by genetic sequence variations or altered mRNA expression levels of the proteins in  
271 each complex (46-48). Intriguingly, increased infection rates on epithelial cells have been reported  
272 for the PRC-repaired HCMV strain AD169 compared to PRC-intact low-passage isolates (49), a  
273 result very reminiscent of our data. This difference was determined to be due to the absence of the  
274 UL148 glycoprotein in AD169, a protein that will reduce PRC levels in favor of the trimeric  
275 gH/gL/gO complex on the virus membrane (50). Similarly, mRNA expression levels of the UL148  
276 homologue Rh159 late during infection were higher in FL-RhCMV compared to both 68-1 and  
277 68-1.2 (**Fig. 5C**). Since this gene is located within the genomic region that was inverted in 68-1, it  
278 is likely that it was put out of context of its original regulatory DNA elements, resulting in altered  
279 mRNA expression levels. Consistent with this explanation, examination of the mRNA expression  
280 levels of the late Rh137 (UL99, pp28) gene not encoded within the acquired inversion did not  
281 show any significant differences across the examined strains (**Fig. 5C**). We previously  
282 demonstrated that Rh159 is an ER-resident glycoprotein that intracellularly retains NK cell  
283 activating ligands, a function that is not shared with UL148 (51). However, these observations do  
284 not rule out a role of Rh159 for PRC expression and cell tropism. While further work will be  
285 required to establish this role, our results indicate that FL-RhCMV is remarkably similar to low  
286 passage clinical isolates of HCMV with respect to growth in tissue culture, tissue tropism and  
287 genetic stability *in vitro*.

288

289 **Kinetics and magnitude of infection by FL-RhCMV is similar to wildtype RhCMV**

290 It was reported previously that different from the low passage isolates UCD52 and UCD59, 68-1  
291 RhCMV displayed severely reduced viral genome copy numbers in plasma, saliva and urine in  
292 RhCMV-seronegative RM after experimental subcutaneous (s.q.) infection (26). Concordantly, we  
293 observed significant plasma viremia in three female RhCMV-naïve pregnant RM infected  
294 intravenously (i.v.) with  $1 \times 10^6$  pfu of RhCMV UCD52,  $1 \times 10^6$  pfu of RhCMV UCD59, and  $2 \times 10^6$   
295 TCID<sub>50</sub> of RhCMV strain 180.92 (**Fig. 6A**) consistent with previous reports (11). Viral genome  
296 copy numbers of approximately  $10^5$  copies/ml blood were detected by qPCR in all three animals  
297 between days 7 to 21, declining thereafter. Similar kinetics of infection and peak viremia were  
298 measured in three male RhCMV-naïve RM infected i.v. with Rh13.1-intact FL-  
299 RhCMV/Rh13.1/apt grown in the presence of tetracycline to maintain genome integrity during  
300 virus stock production (**Fig. 6B**). Since both experiments showed virtually the same development  
301 and progression of plasma viremia after i.v. inoculation (**Fig. 6C**), we conclude that *in vivo*  
302 replication of FL-RhCMV is comparable to that of low passage RhCMV isolates.

303

#### 304 ***In vivo* dissemination of FL-RhCMV-derived viral vectors**

305 A central goal of our research is to use the RhCMV animal model for the development of CMV-  
306 based vaccine vectors (12). We recently reported that deletion of the pp71-encoding RhCMV gene  
307 Rh110 resulted in reduced dissemination and lack of shedding of 68-1-derived vaccine vectors  
308 (20). Nevertheless, SIV-antigen expressing vaccines based on these live-attenuated vector  
309 backbones maintained the ability to control highly virulent SIV<sub>mac239</sub> upon challenge (19).  
310 However, since 68-1 lost its homologs of the PRC subunits UL128 and UL130 as well as homologs  
311 of the viral UL146 family of CXC chemokines it was conceivable that these deletions contributed  
312 to viral attenuation. To determine the impact of these gene deletions on viral dissemination we

313 generated viral vectors based on either FL-RhCMV or FL-RhCMV lacking the homologs of  
314 UL128, UL130 and all members of the UL146 family. As PCR- and immunological marker we  
315 selected a fusion protein of six *M. tuberculosis* antigens that was recently used to demonstrate  
316 protection against *Mtb* challenge with 68-1-based vaccine vectors (14). As antigen-insertion site  
317 we replaced the Rh13.1 gene, thus increasing vector stability while using the Rh13.1 promoter to  
318 drive antigen expression.

319 As we have shown previously (52), *in vivo* dissemination of 68-1 RhCMV is observed in  
320 RhCMV-seronegative animals. Hence we assigned six CMV-naïve RM and inoculated three with  
321 FL-RhCMVΔRh13.1/TB6Ag and three with FL-RhCMVΔRh13.1/TB6AgΔRh157.4-5ΔRh158-  
322 161. Subsequently, we took the animals to necropsy at 14 dpi to systematically measure viral  
323 genome copy numbers in tissue samples using nested PCR as described previously (20, 52). While  
324 both recombinants resulted in significant viral accumulation at the injection sites and the nearest  
325 draining lymph node, FL-RhCMV genomic DNA was highly abundant in many of the tissues  
326 examined (**Fig. 7A**). In contrast, FL-RhCMV lacking the UL128, UL130 and UL146 homologs  
327 displayed significantly reduced spreading beyond the initial site of replication (**Fig. 7A**). Solely  
328 tissue samples from the spleen retained notable viral copy numbers for the deletion mutant,  
329 although at significantly reduced levels compared to FL-RhCMV (**Fig. 7B, C**), whereas  
330 dissemination to and replication in most other tissues was almost completely abrogated. The results  
331 obtained with FL-RhCMVΔRh13.1/TB6AgΔRh157.4-5ΔRh158-161 and FL-  
332 RhCMVΔRh13.1/TB6Ag are consistent with previous observations for 68-1 RhCMV (20, 52) and  
333 UCD52 and UCD59 (26), respectively. These data also suggest that RhCMV vectors with 68-1  
334 like configuration are attenuated *in vivo*.

335 To determine the cell types infected *in vivo* by FL-RhCMV during primary lytic  
336 replication, we performed RNAscope *in situ* hybridization (ISH) in combination with  
337 immunohistochemistry for cellular markers on spleen tissue obtained from the same animals.  
338 Consistent with the high genome copy numbers observed, FL-RhCMVΔRh13.1TB6Ag was  
339 rapidly detected in tissue sections of the spleen, the large clusters of infected cells primarily  
340 localized within the white pulp with fewer individual infected cells within the red pulp (**Fig. 7B,**  
341 **C**). In contrast, the deletion mutant could only be detected very sparsely in a few infected cells  
342 across the examined tissue, localized primarily within the white pulp with sporadic rare viral  
343 RNA+ cells found within the red pulp. Co-staining with cellular markers identified the infected  
344 cells as vimentin-positive mesenchymal cells such as fibroblasts, whereas we did not find RhCMV  
345 RNA in CD34<sup>+</sup> hematopoietic stem cells or CD68/CD163-positive macrophages commonly  
346 associated with CMV latent infection. While this does not exclude the possibility that FL-RhCMV  
347 can infect other cell types, it indicates that the vast majority of cells infected in the spleen during  
348 the initial acute viremia after infection of naïve RM are in the connective tissue. Nevertheless,  
349 while the viral loads of the two different vectors differ substantially across naïve RM, they both  
350 elicited similar frequencies of TB6Ag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in all examined  
351 tissues (**Fig. 7D**). This observation is consistent with previously reported findings that, above a  
352 given threshold, T cell responses are largely independent of viral replication *in vivo* and with the  
353 reported immunogenicity of 68-1-based vectors (14, 15, 20, 53).

354

355 **RNAseq analysis of *in vivo* tissue samples identifies multiple viral transcript that are highly**  
356 **expressed across tissues.**

357 The high genome copy numbers measured in several tissues of FL-RhCMV $\Delta$ Rh13.1/TB6Ag-  
358 inoculated RM at 14dpi provided an opportunity to monitor viral gene expression from a fully  
359 characterized viral genome by RNAseq analysis *in vivo*. Total RNA was isolated from the lung,  
360 the axillary lymph node (ALN), the parotid salivary gland (PSG) and the submandibular salivary  
361 gland (SSG) as these samples showed high viral genome copy numbers (**Fig. 7A**). For comparison,  
362 we infected primary rhesus fibroblast at an MOI of 5 with FL-RhCMV $\Delta$ Rh13.1/TB6Ag and  
363 harvested total mRNA at 8, 24 and 72 hpi representing immediate early, early and late times post  
364 infection. While the average number of reads/sample were comparable between the *in vitro*  
365 (average of 86,013,721) and *in vivo* (average of 107,502,852) RNA samples, the ratio of viral/host  
366 reads was much higher *in vitro*, particularly at late times of infection, an entirely expected result  
367 as a much lower number of cells are infected in our *in vivo* samples (**Supplementary Fig. 8**). The  
368 absolute number of reads aligning to the annotated RhCMV ORFs for all *in vitro* and *in vivo*  
369 samples can be found in **Supplementary Table 2**. Analysis of *in vitro* samples across the entire  
370 FL-RhCMV genome indicated that mRNA expression of all re-introduced genes in the repaired  
371 UL-region was detectable at all time points, indicating successful restoration of a WT like gene  
372 expression cascade (**Supplementary Fig. 9**). Principal component analysis (PCA) on the  
373 normalized count matrix of RhCMV transcripts revealed that while the early *in vitro* samples  
374 clustered together, the late samples showed an mRNA expression pattern closer to expression  
375 profiles obtained from lung and ALN samples (**Fig. 8A**). This is consistent with active viral  
376 replication in these tissues at this time point. In contrast, PCA revealed that gene expression  
377 profiles of PSG and SSG samples were distinct from the other tissue samples and one another.  
378 Importantly, although generated from different outbred animals, viral gene expression patterns  
379 from the same tissue source were more closely related across all three RM than across different

380 tissue samples within the same animals. This indicates that viral mRNA expression varies within  
381 infected animals depending on the examined tissue.

382 Since expression patterns of the same tissues across animals were comparable, we  
383 combined these samples to compare the expression levels of each ORF between tissues and *in vitro*  
384 results. Surprisingly, this analysis revealed that some of the most highly expressed ORFs found  
385 both at late times post-infection *in vitro* and in all tissues examined *in vivo*, were the soluble  
386 chemokine binding protein Rh38.1 (UL22A) as well as two CXC chemokine-like genes of the  
387 UL146 family, Rh158.2 (UL146B) and Rh158.3 (UL146C) (**Fig. 8B**). Normalized to the ORF  
388 size, of the ten ORFs with the most sequence coverage in each sample (**Fig. 8C**), 80.74% mapped  
389 to these three ORFs in rhesus fibroblasts at 72 hpi, 45.94% in Lung, 31.47% in ALN, 34.38% in  
390 PSG and 32.76% in SSG. While UL22A is known to be one of the most highly expressed ORFs  
391 in HCMV (54), this dominant expression of two UL146 family members had not been observed  
392 previously. Interestingly, both UL146-homologs are deleted in 68-1 and were re-inserted during  
393 our construction of FL-RhCMV (**Fig. 1**). The abundance of viral transcripts encoding chemokine-  
394 binding and chemokine-like proteins suggests that RhCMV interference with the host's chemokine  
395 network is a major immune modulatory strategy during the acute phase of infection.

396

397 **Discussion**

398 To generate a RhCMV clone that is representative of a low passage isolate we chose to repair an  
399 existing BAC clone instead of cloning a new primary isolate since this would allow us to better  
400 compare results obtained with FL-RhCMV to historic data obtained with 68-1 BAC clone-derived  
401 recombinants and thus facilitate the identification of viral determinants of tissue tropism,  
402 pathogenesis and immune response programming. Using sequence information from the original  
403 primary 68-1 isolate (38, 55) and next generation sequencing of multiple primary RhCMV isolates  
404 we identified and reverted all mutations that resulted in frameshifts or premature termination  
405 codons in predicted ORFs. While we cannot rule out that additional mutations, particularly in non-  
406 coding regions, occurred during tissue culture as has been observed in HCMV (46), these cannot  
407 be unequivocally distinguished from strain-specific nucleotide polymorphisms and therefore  
408 remained unchanged. Since the original isolate likely contained a multitude of molecular clones,  
409 our FL-RhCMV is representative of a sub-population present in the 68-1 isolate. Full genome  
410 alignments of the old world NHP sequences generated in this study together with sequences  
411 previously submitted to GenBank allowed us to refine the genome annotation, enabling more  
412 precise genetic engineering of FL-RhCMV derived constructs in the future. Comparative genomics  
413 revealed a close conservation of the overall ORFeome across NHP CMV species (**Fig. 3**) while  
414 also allowing us to identify differences acquired by individual species during co-evolution with  
415 their respective host. These distinct disparities largely consist of gene duplications in only six  
416 different loci across the genome and they are reminiscent of a poxvirus adaptation strategy  
417 deployed to adapt to antiviral pressure by the immune system known as a genomic accordion (56),  
418 albeit on a significantly longer evolutionary timescale. Hence, these gene duplications could be  
419 the results of the ongoing arms race between the virus and the host immune defenses. At a

420 minimum, these data enable us to estimate when different CMV gene families entered the NHP  
421 CMV lineage and how they adapted over millions of years of co-evolution with their primate host  
422 (**Fig. 9**).

423 Another advantage of our chosen repair strategy was that it allowed us to recreate a  
424 complete genome at the BAC stage in the absence of the selective forces of *in vitro* tissue culture.  
425 Indeed, upon culture of BAC derived Rh13.1-intact FL RCMV in fibroblasts we observed the rapid  
426 accumulation of mutations in the gene and the region surrounding the gene. This is strikingly  
427 similar to the clinical HCMV isolate Merlin which displayed the same instability in the  
428 homologous gene RL13 when an RL13-intact BAC was used for transfection (43). Similar  
429 observations have been reported for additional HCMV isolates (32), although a small number of  
430 passaged strains appear capable of maintaining an intact RL13 ORF (57). RL13 seems to limit  
431 viral spread, particularly in fibroblasts (57) but the exact mechanism of this inhibition is not clear.  
432 Rh13.1 belongs to the RL11 family of single transmembrane glycoproteins present in all old world  
433 NHP CMVs, as well as great ape and HCMV, but not in CMVs of new world primates (**Fig. 9**).  
434 The functional conservation of Rh13.1 and RL13 is surprising since the RL11 family is highly  
435 diverse both within a given CMV species and especially when comparing family members between  
436 great ape and old world monkey CMVs (10, 40, 58). It thus seems likely that while Rh13.1 and  
437 RL13 are selected against *in vitro*, there is a strong selection for their presence *in vivo*. RL13 has  
438 been shown to bind to antibody Fc portions (59) and it is thus possible that it serves as an immune  
439 evasion protein in the host. Whether this function is conserved in NHP CMVs is currently  
440 unknown. To enable the study of Rh13.1-intact vectors, we therefore generated two different  
441 tetracycline-regulated systems that allow for the conditional expression of Rh13.1 so that the virus  
442 can be grown *in vitro* without selection against Rh13.1 whereas mRNA would be expressed *in vivo*

443 in the absence of tetracycline. Indeed, we observed *in vivo* viremia of Rh13.1-intact FL-RhCMV  
444 that was comparable to low passage isolates.

445 However, we also observed that FL-RhCMV lacking Rh13.1 displayed substantial *in vivo*  
446 spread that was significantly more pronounced than a mutant that lacked the UL128 and UL130-  
447 homologous subunits of the pentameric complex together with all genes homologous to HCMV  
448 UL146 and UL147. These data suggest that Rh13.1-deleted viruses might maintain most of the  
449 wildtype characteristics in primary infection of immunocompetent adult animals. We also know  
450 that Rh13.1 is not required for the establishment and maintenance of persistent infections since  
451 strain 68-1 lacks a functional Rh13.1, yet persists as shown by long-term immune responses and  
452 shedding (53). Given a possible function of Rh13.1 in evasion of antibody responses, it would be  
453 interesting to compare spreading of Rh13.1 intact and deleted viruses in the presence of anti-CMV  
454 antibodies. While inactivation of RL13 generally represents the first tissue culture adaptation step  
455 observed in HCMV, this is often followed by the loss of one or multiple members of the PRC (43).  
456 Although we did not observed loss of PRC members in FL-RhCMV, this might be due to the  
457 limited numbers of passages we examined. Since PRC mutations occurred in 68-1 RhCMV during  
458 prolonged tissue culture it is likely that further passaging of FL-RhCMV would result in  
459 adaptations akin to HCMV indicating that the overall genome stability and the sequence of  
460 adaptation are likely similar across primate CMV species.

461 The strong attenuation of a “68-1 like” FL-RhCMV lacking homologs of UL128, UL130  
462 and UL146 observed in primary infection suggests that gene deletions in these regions are the  
463 likely reason for the previously reported lack of measurable viremia and shedding of 68-1 RhCMV  
464 (26). Further studies will be required to determine the individual contribution of pentamer subunits  
465 and UL146-related chemokine homologs to viral dissemination and spread. However, it was

466 unexpected that two of the most highly expressed genes, both *in vitro* and *in vivo*, belong to the  
467 UL146 family and that these two genes, Rh158.2 (UL146B) and Rh158.3 (UL146C), are deleted  
468 in strain 68-1. The UL146 gene family of chemokine like proteins is only found in primate CMVs  
469 (**Fig. 9**). Given their homology to chemokines such as interleukin 8 they were likely acquired from  
470 the host. HCMV contains two family members: UL146 and UL147. While a single UL147  
471 homologous gene can be found across all primate CMV species with a moderate level of  
472 conservation, the UL146-homologs are highly diverse within a CMV species. Moreover, while  
473 HCMVs only contain a single UL146 member, the number of genes can vary greatly in other  
474 primate CMVs (**Supplementary Fig. 4**). CCMV contains two genes almost equally related to the  
475 HCMV UL146 member. However, new world NHP CMVs contain a single UL146 family member  
476 that is highly divergent. Conversely, old world NHP CMVs encode five to seven UL146 homologs.  
477 Since 68-1 lacks these highly expressed genes, they are not required for the establishment and  
478 maintenance of persistent infection but it is possible that these chemokine homologs support  
479 viremia and dissemination during primary infection or upon re-infection, a possibility reinforced  
480 by the recent observation that inserting the HCMV UL146 protein into MCMV significantly  
481 enhances virus dissemination kinetics in infected mice (60).

482 The *in vitro* and *in vivo* characteristics of FL-RhCMV described here are consistent with  
483 this virus being representative of wildtype RhCMV. Based on these observations we anticipate  
484 that this recombinant will be useful for RM models of CMV pathogenesis, such as the fetal  
485 inoculation model as well as a model of congenital infection (61, 62). In addition, FL-RhCMV can  
486 serve as a translational model for the development of live-attenuated vectors derived from clinical  
487 isolates of HCMV. As recently reported, our strategy for HCMV-based vectors is to start with a  
488 clinical isolate to ensure persistence and then introduce genetic modifications that increase vector

489 safety while maintaining desired immunological features (34). The availability of a complete  
490 RhCMV genome will allow us to recapitulate HCMV-vector design strategies and test these  
491 designs in RM challenge models for AIDS, tuberculosis and malaria. FL-RhCMV-based vectors  
492 will thus be highly useful for both basic and translational aspects of CMV research.

493

494 **Materials and Methods**

495 **Cells and viruses.**

496 Telomerized rhesus fibroblasts (TRFs) have been described before (63). Primary embryonal rhesus  
497 fibroblasts were generated at the ONPRC. Both cell lines were maintained in DMEM complete,  
498 Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and antibiotics (1×  
499 Pen/Strep, Gibco), and grown at 37°C in humidified air with 5% CO<sub>2</sub>. Rhesus retinal pigment  
500 epithelial (RPE) cells were a kind gift from Dr. Thomas Shenk (Princeton University, USA) and  
501 were propagated in a 1:1 mixture of DMEM and Ham's F12 nutrient mixture with 5% FBS, 1 mM  
502 sodium pyruvate, and nonessential amino acids. Monkey kidney epithelial (MKE) (64) cells were  
503 maintained in DMEM-F-12 medium (DMEM-F12) (Invitrogen) supplemented with epithelial cell  
504 growth supplement (ScienCell), 1 mM sodium pyruvate, 25 mM HEPES, 100 U/ml penicillin,  
505 100g/ml streptomycin, 2 mM L-glutamine (Invitrogen), and 2% fetal bovine serum/SuperSerum  
506 (Gemini Bio-Products). The RhCMV 68-1 BAC (22) has been characterized extensively. The BAC  
507 for RhCMV 68-1.2 (25) which was based on the 68-1 BAC was provided by Dr. Thomas Shenk  
508 (Princeton University, USA). Both 68-1 and 68-1.2 were derived via electroporation (250V,  
509 950μF) of BAC-DNA into primary rhesus fibroblasts. Full cytopathic effect (CPE) was observed  
510 after 7-10 days and the supernatants were used to generate viral stocks. RhCMV UCD52 and  
511 RhCMV UCD59 have been continuously passaged on MKE cells to maintain their PRC and to  
512 minimize tissue culture adaptations. To generate enough viral DNA for a full genome analysis,  
513 monolayers of MKE at 90 – 100% confluence were inoculated with RhCMV (MOI: 0.01).  
514 Infections progressed to ~90% CPE at which time supernatant (SN) and cells were collected and  
515 centrifuged at 6000 x g for 15 minutes at 4°C. SN was passed through a 0.45μm filter. SN was  
516 then centrifuged at 26,000 x g for 2 hours at 4°C. The SN was decanted and the virus pellet was

517 resuspended and washed in ~ 20ml of cold 1X PBS. Virus was pelleted by ultracentrifugation at  
518 72,000 x g (Rotor SW41Ti at 21,000 rpm) for 2 hours at 4°C. This was repeated once more. SN  
519 was decanted and the remaining viral pellet was thoroughly resuspended in ~1-2 ml of cold 1X  
520 PBS. Viral stock was stored in 50µl aliquots. Viral DNA was isolated from these viral stocks using  
521 the QIAamp DNA Mini Kit (Qiagen).

522

523 **BAC recombineering using en passant homologous recombination.**

524 Recombinant RhCMV clones were generated by *en passant* mutagenesis, as previously described  
525 for HCMV (65), and adapted by us for RhCMV (66). This technique allows the generation of  
526 “scarless” viral recombinants, i.e. without containing residual heterologous DNA sequences in the  
527 final constructs. The homologous recombination technique is based on amplifying an I-SceI  
528 homing endonuclease recognition site followed by an aminoglycoside 3-phosphotransferase gene  
529 conferring kanamycin resistance (KanR) with primers simultaneously introducing a homology  
530 region upstream and downstream of the selection marker into the intermediate BAC cloning  
531 product. As en passant recombinations are performed in the GS1783 *E-coli* strain that can be used  
532 to conditionally express the I-SceI homing endonuclease upon arabinose induction (65),  
533 expression of the endonuclease with simultaneous heat shock induction of the lambda ( $\lambda$ ) phage  
534 derived Red recombination genes will lead to selective DNA double strand breaks with subsequent  
535 scarless deletion of the selection marker. The immunologically traceable markers used in the study,  
536 namely the SIVmac239 GAG protein as well as the *M.tuberculosis* Erdman strain derived Tb6Ag  
537 fusion protein, have been described before (14, 53). To introduce these genes into the FL-RhCMV  
538 backbone, we first introduced a homology region flanking an I-SceI site and a KanR selection  
539 marker into the selected inserts. We then amplified the transgenes by PCR and recombined the

540 entire insert into the desired location in the FL-RhCMV BAC. The KanR cassette was  
541 subsequently removed scarlessly as described above. All recombinants were initially characterized  
542 by XmaI restriction digests and Sanger sequencing across the modified genomic locus. Lastly all  
543 vectors were fully analyzed by next generation sequencing to exclude off-target mutations and to  
544 confirm full accordance of the generated with the predicted full genome sequence.

545

#### 546 **Isolation of the old world non-human primate CMV species from urine samples.**

547 Virus isolation was performed as previously described (39). Briefly, urine samples were obtained  
548 through collection from cage pans, by cystocentesis or following euthanasia. From samples  
549 collected at the Oregon National Primate Research Center (ONPRC) we isolated BaCMV 31282  
550 from a male olive baboon (*Papio anubis*), BaCMV 34826 from a female hamadryas baboon (*Papio*  
551 *hamadryas*), CyCMV 31709 from a female cynomolgus macaques (*Macaca fascicularis*) of  
552 Cambodian origin, JaCMV 24655 from a male Japanese macaque (*Macaca fuscata*) and RhCMV  
553 34844 from a male rhesus macaque (*Macaca mulatta*) of Indian origin. Additionally, we  
554 successfully isolated RhCMV KF03 from a cage pan collected urine sample from a male rhesus  
555 macaque (*Macaca mulatta*) of Indian origin housed at the Tulane National Primate Research  
556 Center (TNPRC). All urine samples were first clarified from solid contaminants by centrifugation  
557 at 2,000 x g for 10 minutes at 4°C and then filtered through a 0.45 µm filter (Millipore) to clear  
558 the urine of any bacterial or fungal contamination. Next, we spin-inoculated 0.5 ml – 2 ml of  
559 clarified urine onto primary rhesus fibroblasts in a 6 well plate at 700 x g for 30 minutes at 25°C.  
560 The cells were placed on a rocker for 2 hours at 37°C and, after removing the inoculum, washed  
561 once with PBS. The infected cells were cultured in DMEM plus 10% fetal bovine serum for 2-3  
562 days, trypsinized and seeded in a T-175 cell culture flask. All samples were monitored weekly for

563 CPE for up to six weeks or until plaque formation was visible. Every two weeks or after the  
564 appearance of plaques, cells were trypsinized and re-seeded to facilitate viral spread through the  
565 entire monolayer. Virus propagation was kept to an absolute minimum and viral stocks were  
566 prepared with the minimum number of passages required to be able to infect eight T-175 flasks for  
567 stock production (typically 1-3 passages).

568

#### 569 **Isolation and purification of viral DNA for next generation sequencing (NGS).**

570 The modified Hirt extraction (67) protocol used for the preparation of CMV viral DNA has been  
571 described (39). Briefly, supernatants from cells that were spin-inoculated with the original urine  
572 sample were collected at full CPE and used to infect three T-175 flasks of primary rhesus  
573 fibroblasts. After 7–10 days, the supernatant was harvested and clarified by centrifugation, first at  
574 2,000 x g for 10 minutes at 4°C and subsequently at 7,500 x g for 15 minutes. Virus was pelleted  
575 through a sorbitol cushion (20% D-sorbitol, 50 mM Tris [pH 7.4], 1 mM MgCl<sub>2</sub>) by centrifugation  
576 at 64,000 x g for 1 hour at 4°C in a Beckman SW28 rotor. The pelleted virus was resuspended in  
577 500 µl 10.1 TE Buffer (10 mM Tris, pH 8.0; 0.1 mM EDTA, pH 8.0) and 500 µl 2x lysis buffer  
578 (20 mM Tris-Cl, pH 8.0; 50 mM EDTA, pH 8.0; 200 mM NaCl; 1.2% w/v SDS) was added. To  
579 digest the purified virion and to release the viral DNA, 250 µg Proteinase K was added and the  
580 solution was incubated for 2 h at 37°C. This was followed by two rounds of phenol/chloroform  
581 extractions and the viral DNA was precipitated overnight with absolute ethanol at -80°C. The  
582 DNA was pelleted for 20 minutes at 13,200 x g at 4°C, washed once with 70% ethanol, and  
583 subsequently resuspended in autoclaved double deionized water. DNA concentrations were  
584 determined using a ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.).

585

586 **Generation of Next Generation Sequencing libraries and Next Generation sequencing.**

587 Illumina sequencing libraries were generated by first fragmenting the viral DNA using an S2  
588 Sonicator and by subsequently converting the fragments into libraries using the standard TruSeq  
589 protocol. All libraries were quality controlled on a Bioanalyzer (Agilent) and library concentration  
590 was determined by real time PCR and SYBR Green fluorescence. Finally, the next generation  
591 sequencing was performed on either an iSeq- or a MiSeq Next-Generation Sequencing platform  
592 (Illumina). The libraries were multiplexed at equal concentrations and loaded into a reagent  
593 cartridge at 9 pM and single read sequencing was performed for 300 cycles with 6 additional cycles  
594 of index reads. The Geneious Prime software was used for all NGS data analysis. To minimize  
595 sequencing errors, the sequencing reads were trimmed of all regions exceeding the error  
596 probability limit of 0.1%. All reads with a total length of less than 50 bp after trimming were  
597 eliminated from further analysis to decrease the background due to unspecific alignment of reads  
598 during *de novo* and reference guided assemblies. All full genome sequences were first *de novo*  
599 assembled using the processed sequencing data, and subsequently all reads were aligned to the  
600 generated consensus sequence in a reference-guided assembly. All detected single nucleotide  
601 polymorphisms (SNPs) that showed a frequency of more than 50% in a location with a depth of at  
602 least 10% of the mean depth were manually curated. All nucleotide changes that were considered  
603 to be likely the results of actual changes opposed to sequencing errors or software alignment issues,  
604 were changed in the consensus sequences and the referenced guided assemblies were repeated  
605 until no SNP showed a frequency of 50% or more. The resulting final sequence was considered  
606 the representative consensus sequences of all clones contained in the primary viral isolates.

607

608 **Nucleotide sequences used and generated in this study.**

609 All full genome old world NHP CMV sequences generated in this study were submitted to  
610 GenBank. The accession numbers for the submitted isolates are as follows: BaCMV 31282  
611 (MT157321), BaCMV 34826 (MT157322), CyCMV 31709 (MT157323), JaCMV 24655  
612 (MT157324), RhCMV 34844 (MT157328), RhCMV KF03 (MT157329), RhCMV UCD52  
613 (MT157330) and RhCMV UCD59 (MT157331). Furthermore, we submitted an updated  
614 annotation for the RhCMV 68-1 BAC (MT157325), a full annotation for the partially repaired  
615 RhCMV 68-1.2 BAC (MT157326) as well as a full annotation for the FL-RhCMV BAC described  
616 here (MT157327) which was based on 68-1 and 68-1.2. Genome annotations were fine-tuned  
617 utilizing these and other NHP CMV sequences that had been previously submitted to GenBank,  
618 either by us: CyCMV 31906 (KX689263), CyCMV 31907 (KX689264), CyCMV 31908  
619 (KX689265), CyCMV 31909 (KX689266), RhCMV 180.92 (DQ120516, AAZ80589), RhCMV  
620 19262 (KX689267), RhCMV 19936 (KX689268) and RhCMV 24514 (KX689269) or by others:  
621 CyCMV Ottawa (JN227533, AEQ32165), CyCMV Mauritius (KP796148, AKT72642), SCMV  
622 Colburn (FJ483969, AEV80601), SCMV GR2715 (FJ483968, AEV80365), SCMV Stealth virus  
623 1 strain ATCC VR-2343 (U27469, U27238, U27770, U27627, U27883, U27471), BaCMV  
624 OCOM4-37 (AC090446), BaCMV OCOM4-52 (KR351281, AKG51610.1), DrCMV OCOM6-2  
625 (KR297253, AKI29779). Lastly, we also used full genome sequences of CMV species from  
626 outside the old world NHPs in our phylogenetic analysis to classify the evolutionary relationship  
627 of FL-RhCMV to other CMVs. These additional sequences include: MCMV Smith (GU305914,  
628 P27172), RCMV Maastricht (NC\_002512), RCMV England (JX867617), RCMV Berlin  
629 (KP202868), GPCMV 22122 (KC503762, AGE11533), AoHV-1 S34E (FJ483970, AEV80760),  
630 SaHV-4 SqSHV (FJ483967, AEV80915), CCMV Heberling (AF480884, AAM00704), and  
631 HCMV TR3 (MN075802).

632

633 **Phylogenetic analysis of isolated NHP CMV species.**

634 Full nucleotide sequences of rodent and primate CMV genomes were aligned using ClustalW2  
635 (68). The multiple sequence alignment was imported into Geneious Prime and a Neighbor-joining  
636 phylogenetic tree was build using the Geneious Tree Builder application and selecting the Jukes-  
637 Cantor genetic distance model using the MCMV Smith strain as an outgroup. The validity of the  
638 tree topology obtained was tested by using bootstrap analysis with 100 resamplings from the  
639 aligned sequences, followed by distance matrix calculations and calculation of the most probable  
640 consensus tree with a support threshold of 50%.

641

642 **Conditional expression of the Rh13.1 (RL13) encoded mRNA using the Tet-Off system.**

643 TRF were engineered to express the tetracycline-repressor (tetR) as previously described (69, 70).  
644 Briefly, a retrovirus was generated from vector pMXS-IP expressing the tetR ORF. Retronectin  
645 was then used to transduce TRFs with high efficiency, before selection in puromycin (1 µg/ml).  
646 Successful expression of the tetR was validated using replication deficient recombinant adenovirus  
647 vectors expressing GFP from a tetO-regulated promoter (70).

648 To create a vectors conditionally expressing Rh13.1, we inserted dual tetO sequences  
649 131bp upstream of its start codon. Next, eGFP was inserted as a marker of infection. eGFP was  
650 linked to Rh60/61 (homologous to HCMV UL36) via a P2A linker. We have previously shown  
651 for HCMV that this provides early expression of GFP, without affecting UL36 function (6). As a  
652 control, we created a virus in which we deleted the entire Rh13.1 ORF. All vectors were created  
653 using *en passant* mutagenesis.

654 To analyze the effects of Rh13.1 on plaque formation, BAC DNA was transfected into  
655 TRFs or tetR-expressing TRFs using an Amaxa Nucleofector (basic fibroblast kit, program T-16).  
656 The formation of plaques was then monitored by imaging for eGFP fluorescence at various  
657 timepoints, using a Zeiss Axio Observer Z1.

658

659 **Rh13.1 (RL13) mRNA regulation using riboswitches.**

660 To generate a FL-RhCMV vectors carrying riboswitches, we inserted the published Tc45 aptazyme  
661 sequence 19bp upstream and the Tc40 aptazyme sequence 17bp downstream of Rh13.1 (45) into  
662 the BAC by incorporating them into the homologous recombination primers. The entire 122bp  
663 sequences were introduced into the 5' and the 3' flanking regions of Rh13.1 in two independent  
664 recombination steps. The resulting BAC construct was analyzed by XmaI endonuclease restriction  
665 digest, Sanger sequencing and next generation sequencing.

666 To reconstitute the virus, we transfected BAC DNA into primary rhesus fibroblast using  
667 Lipofectamine 3000 (ThermoFischer) in the presence of 100  $\mu$ M tetracycline which was  
668 replenished every other day. For comparison, we reconstituted virus in the absence of tetracycline.  
669 After minimal passaging in the presence or absence of tetracycline virus stocks were generated,  
670 viral DNA was isolated and NGS was performed.

671

672 **Multi-step growth curves of RhCMV on primary rhesus fibroblasts.**

673 Primary rhesus fibroblast were seeded in 24 well plates and infected in duplicate with RhCMV  
674 constructs at an MOI of 0.01. The inoculum was removed after 2h and 1ml DMEM complete was  
675 added. Supernatants were collected every third day for 24 days, cells and cell debris were removed  
676 by centrifugation for 2 min at 13,000 rpm and the samples were stored at -80°C. Viral titers were

677 determined using a fifty-percent tissue culture infective dose (TCID50) assay in two technical  
678 repeats. Final titers were calculated using the arithmetic mean of the technical and biological  
679 repeats.

680

681 **RhCMV entry assays into primary rhesus fibroblast and rhesus retinal pigment epithelial  
682 cell.**

683 Stocks for RhCMV strains 68-1, 68-1.2 and FL-RhCMV were generated and viral titers were  
684 determined by TCID50. Infection levels in fibroblasts were experimentally equalized across  
685 stocks. Primary rhesus fibroblast were infected at an MOI of 0.3 and RPEs at an MOI of 10 as  
686 these MOIs were experimentally determined to result in optimal infection levels. The cells were  
687 fixed at 48 hpi and the overall percentage of RhCMV positive cells were determined by flow  
688 cytometry using a RhCMV specific antibody (52). To be able to compare infection levels between  
689 the two cell types, we set the mean infection level in fibroblasts determined in triplicate repeats to  
690 100% and expressed the mean infection level of triplicate repeats in RPEs in relation to the  
691 infection level in fibroblasts.

692

693 **Quantitative PCR (qPCR) analysis to assess mRNA expression levels.**

694 Primary rhesus fibroblasts were seeded in 6-well plates and infected either with FL-, 68-1, or 68-  
695 1.2 RhCMV at a MOI of 1. Total RNA was then isolated at 48 hours post infection (hpi).  
696 Uninfected rhesus fibroblasts were used as a negative control. After cDNA synthesis, the  
697 quantitative PCR (q-PCR) assay was performed using primers and probes specific to each gene of  
698 interest. Rh159\_forward\_primer: 5' TCAGAAATGAAGGGCAATTGTG 3'.  
699 Rh159\_reverse\_primer: 5' GCGAGCTGGCGACGTT 3'. Rh159\_probe:

700 6FAMTATCACTCGGCTATTATCMGBNFQ. Rh137\_forward\_primer: 5'  
701 GCGCAACATACTACCCAGAA 3'. Rh137\_reverse\_primer: 5'  
702 GTAGCCATCCCCATCTTCCA 3'. Rh137\_probe:  
703 6FAMCACAACTAACCTGGCCTTMBNFQ. GAPDH\_forward\_primer: 5'  
704 TTCAACAGCGACACCCACTCT 3'. GAPDH\_reverse\_primer: 5'  
705 GTGGTCGTTGAGGGCAATG 3'. GAPDH\_probe:  
706 6FAMCCACCTTCGACGCTGGMGBNFQ. The mRNA copy numbers for Rh159 (UL148) and  
707 Rh137 (UL99) were calculated and graphed as relative mRNA copy numbers normalized to the  
708 housekeeping gene (GAPDH).

709

710 **Ethics statement.**

711 All RMs housed at the Oregon National Primate Research Center (ONPRC) and the Tulane  
712 National Primate Research Center (TNPRC) were handled in accordance with good animal  
713 practice, as defined by relevant national and/or local animal welfare bodies. The RMs were housed  
714 in Animal Biosafety level (ABSL)-2 rooms. The rooms had autonomously controlled temperature,  
715 humidity, and lighting. Study RM were both pair and single cage-housed. Regardless of their  
716 pairing, all animals had visual, auditory and olfactory contact with other animals within the room  
717 in which they were housed. Single cage-housed RMs received an enhanced enrichment plan and  
718 were overseen by nonhuman primate behavior specialists. Animals were only paired with one  
719 another if they were from the same vaccination group. RMs were fed commercially prepared  
720 primate chow twice daily and received supplemental fresh fruit or vegetables daily. Fresh, potable  
721 water was provided via automatic water systems. The use of nonhuman primates was approved by  
722 the ONPRC and the TNPCR Institutional Animal Care and Use Committees (IACUC). Both

723 institutions are Category I facilities and they are fully accredited by the Assessment and  
724 Accreditation of Laboratory Animal Care International and have an approved Assurance (#A3304-  
725 01) for the care and use of animals on file with the NIH Office for Protection from Research Risks.  
726 The IACUCs adhere to national guidelines established in the Animal Welfare Act (7 U.S.C.  
727 Sections 2131–2159) and the Guide for the Care and Use of Laboratory Animals (8th Edition) as  
728 mandated by the U.S. Public Health Service Policy. For BAL procedures, monkeys were restrained  
729 and sedated by intramuscular injection of ketamine (~7 mg/kg) with dexmedetomidine (~15  
730 µg/kg). Following the procedure (and blood collection), atipamezole (5 mg/mL; the dose volume  
731 was matched to that of dexmedetomidine) was administered by intramuscular injection to reverse  
732 the effects of the dexmedetomidine sedation. To prepare RMs for blood collection alone, monkeys  
733 were administered ketamine only as described above. Monkeys were bled by venipuncture (from  
734 the femoral or saphenous veins) and blood was collected using Vacutainers. Monkeys were  
735 humanely euthanized by the veterinary staff at ONPRC and TNPCR in accordance with end point  
736 policies. Euthanasia was conducted under anesthesia with ketamine, followed by overdose with  
737 sodium pentobarbital. This method is consistent with the recommendation of the American  
738 Veterinary Medical Association.

739

#### 740 **Intravenous (i.v.) inoculation of CMV naïve RM with FL-RhCMV**

741 Three immunocompetent male RhCMV-seronegative Indian-origin rhesus macaques from the  
742 expanded SPF colony at the TNPRC were intravenously inoculated with  $1.79 \times 10^6$  pfu of Rh13.1-  
743 intact FL-RhCMV/Rh13.1/apt. Blood, saliva samples collected by oral sterile PBS wash, and urine  
744 from cage pans were collected at biweekly to weekly intervals until necropsy 9 weeks post  
745 RhCMV inoculation. Plasma samples were shipped to UC Davis on dry ice and stored at -80°C

746 until use. For DNA isolation, they were quickly thawed for up to 1 minute in a 37°C dry bath. Each  
747 plasma sample was vortexed for 15 seconds and then 350 $\mu$ l of the plasma was transferred to a  
748 sterile, 2.0 ml polypropylene screw cap tube (Sarstedt, 72.693.005). Plasma tubes were loaded into  
749 the QIAasympo SP (Qiagen, Germantown, MD) and purified DNA was obtained using the DNA  
750 Blood 200 $\mu$ l protocol and the QIAasympo DNA Mini kit reagent cartridge (Qiagen,  
751 931236). Quantitative PCR was performed on the plasma derived DNA using the 7900HT Real-  
752 Time PCR System (Applied Biosystems, Inc. and Thermo Fisher, Grand Island, NY), and a  
753 previously published protocol for qPCR using primers specific to RhCMV gB (71). Results were  
754 analyzed using the Sequence Detection Systems software (vs. 2.4). Fluorescence intensity in each  
755 well was measured, and a result was considered positive when the intensity exceeded 10 times the  
756 baseline fluorescence. The limit of sensitivity was reproducibly between 1 and 10 copies of  
757 template DNA. Plasma DNA viral loads were calculated as copy number per milliliter of plasma.  
758 Plasma viral load data in the FL-RhCMV inoculated RM was compared with historical control  
759 pregnant female macaques inoculated with RhCMV strains UCD52, UCD59, and 180.92 as  
760 previously reported (11). RhCMV DNA copy numbers in these animals were quantitated by real  
761 time PCR using primers and probe targeting exon 1 of the immediate early gene of RhCMV using  
762 previously published methodology (72).

763

764 **Nested real-time PCR.**

765 To examine the differences in dissemination between FL-RhCMV $\Delta$ Rh13.1/TB6Ag and FL-  
766 RhCMV $\Delta$ Rh13.1/TB6Ag $\Delta$ Rh157.4-5 $\Delta$ Rh158-161, we infected three CMV naïve RM per vector  
767 s.c. with 10<sup>7</sup> PFU. At 14 days post infection, we took the animals to necropsy and harvested tissues  
768 from which the DNA was isolated by the ONPRC Molecular Virology Support Core (MVSC)

769 using the FastPrep (MP Biomedicals) in 1 ml TriReagent (Molecular Research Center Inc.) for  
770 tissue samples under 100 mg. Additionally, 100  $\mu$ l bromochloropropane (MRC Inc.) was added to  
771 each homogenized tissue sample to enhance phase separation. 0.5 ml DNA back-extraction buffer  
772 (4 M guanidine thiocyanate, 50 mM sodium citrate, and 1 M Tris) was added to the organic phase  
773 and interphase materials, which was then mixed by vortexing. The samples were centrifuged at  
774 14,000 g for 15 minutes, and the aqueous phase was transferred to a clean microfuge tube  
775 containing 240  $\mu$ g glycogen and 0.4 ml isopropanol and centrifuged for 15 minutes at 14,000 g.  
776 The DNA precipitate was washed twice with 70% ethanol and resuspended in 100 to 500  $\mu$ l double  
777 deionized water. Nested real-time PCR was performed with primer and probe sets for the inserted  
778 *Mtb* Tb6Ag transgene (first round: for-CAGCCGCTACAGATGGAGAG and rev-  
779 CGCGCTAGGAGCAAATTCAC; second round: for-CAGCCGCTACAGATGGAGAG, rev-  
780 CGCGCTAGGAGCAAATTCAC, and probe-TGGCGGCTTGCAAT-FAM). For each DNA  
781 sample, 10 individual replicates (5  $\mu$ g each) were amplified by first-round PCR synthesis (12  
782 cycles of 95°C for 30 seconds and 60°C for 1 minute) using Platinum Taq in 50  $\mu$ l reactions. Then,  
783 5  $\mu$ l of each replicate was analyzed by nested quantitative PCR (45 cycles of 95°C for 15 seconds  
784 and 60°C for 1 minute) using Fast Advanced Master Mix (ABI Life Technologies) in an ABI  
785 StepOnePlus Real-Time PCR machine. The results for all 10 replicates were analyzed by Poisson  
786 distribution and expressed as copies per cell equivalents (73).

787

788 **Histopathology and in situ hybridization (ISH).**

789 RNA scope was performed on formaldehyde fixed, paraffin-embedded tissue sections (5  $\mu$ m)  
790 according to our previously published protocol (74) with the following minor modifications: heat-  
791 induced epitope retrieval was performed by boiling slides (95-98°C) in 1x target retrieval (ACD;

792 Cat. No. 322000) for 30 min., followed by incubation at 40°C with a 1:10 dilution of protease III  
793 (ACD; Cat. No. 322337;) in 1x PBS for 20 min. Slides were incubated with the target probe  
794 RhCMV (ACD; Cat. No. 435291) for 2 hours at 40°C and amplification was performed with  
795 RNAscope 2.5 HD Brown Detection kits (ACD; Cat. No. 322310) according to manufacturer's  
796 instructions, with 0.5X wash buffer (310091; ACD) used between steps, and developed with  
797 Alexa-fluor647 conjugated tyramide. The RhCMV probe consisted of 50zz pairs targeting the  
798 RhCMV rh38 (13zz pairs), rh39 (10zz pairs all shared with Rh39) and rh55 (37zz pairs) ORFs  
799 originally annotated in RhCMV 68-1 (55). These ORFs correspond to Rh38.1 (UL22A) and Rh55  
800 (UL33) in the here presented annotation of the RhCMV genome. To remove/inactivate the *in situ*  
801 amplification tree/HRP complexes, slides were microwaved at full power for 1 minute, followed  
802 immediately for 15 minutes at 20% power in 0.1% citraconic anhydride with 0.05% Tween-20  
803 buffer. Slides were cooled for 15 minutes in the buffer, then rinsed in ddH<sub>2</sub>O. Slides were  
804 subsequently stained for CD34 (Sigma, Cat. No. HPA036723), at a 1:200 dilution in antibody  
805 diluent (1x TBS containing 0.25% casein and 0.05% Tween-20) overnight at 4°C. Detection was  
806 performed using an anti-rabbit polymer HRP conjugated system (GBI Labs; Cat. No. D39-110),  
807 and developed with Alexa-fluor488 conjugated tyramide at a 1:500 dilution for 10 minutes. To  
808 remove the antibody/HRP complexes, a second round of microwaving was performed as described  
809 above. Slides were subsequently stained for myeloid lineage cells using a combination of mouse  
810 anti-CD68 (Biocare Medical; Cat. No. CM033C; clone KP1) and mouse anti-CD163  
811 (ThermoFisher; Cat. No. MA5-1145B; clone 10D6), at a 1:400 dilution (each) in antibody diluent  
812 for one hour at room temperature. Detection was performed using an anti-mouse polymer HRP  
813 conjugated system (GBI Labs; Cat. No. D37-110), and developed with Alexa-fluor350 conjugated  
814 tyramide at a 1:50 dilution for 15 minutes. A third round of microwaving was performed to remove

815 the antibody/HRP complexes as described above. Slides were subsequently stained for  
816 mesenchymal lineage cells using a mouse anti-vimentin (Sigma; Cat. No. HPA001762), at a  
817 1:1000 dilution in antibody diluent overnight at 4°C. Detection was performed using an anti-mouse  
818 polymer HRP conjugated system (GBI Labs; Cat. No. D12-110), and developed with Alexa-  
819 fluor594 conjugated tyramide at a 1:500 dilution for 10 minutes. To ensure that that HRP  
820 inactivation and antibody stripping was complete, matched slides that had gone through the  
821 previous staining steps did not receive the subsequent primary antibody, but were developed with  
822 all slides from that round. In each case we did not see staining with Alexa-fluor 488, Alexa-fluor  
823 350 or Alexa-fluor 594 tyramide, indicating that the microwave step completely  
824 removed/inactivated the *in situ* amplification tree/HRP complexes and removed all previous  
825 antibody/HRP complexes. Slides were coverslipped using Prolong Gold antifade mounting media  
826 (ThermoFisher; Cat. No. P36930), scanned using a Zeiss AxioScan Z1, and analyzed using Halo  
827 software (v2.3.2089.52; Indica Labs). Cell counts were quantified using the FISH Multiplex RNA  
828 v2.1 module and RhCMV RNA percent area quantification was performed using the Area  
829 Quantification FL v1.2 module. To calculate viral copy number, we used the HALO analysis  
830 program to analyze the average size (area) and fluorescent intensity of more than 10 individual  
831 virions within the spleen, which was used that to calculate a minimum estimate of RhCMV RNA  
832 copy numbers in the infected cells using the FISH Multiplex RNA v2.1 module. Statistical analysis  
833 was performed with GraphPad Prism v.7.04. Data are mean +/- S.E.M. as indicated.

834

835 **Intracellular cytokine staining (ICS)**

836 Our intracellular cytokine staining (ICS) protocol to examine immune cells isolated from RM  
837 tissues has been described previously (14, 75). Briefly, we isolated mononuclear cells from RM

838 tissues collected at necropsy and measured specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses by flow  
839 cytometric ICS. For this purpose, the isolated cells were incubated with mixtures of consecutive  
840 15mer peptides (overlapping by 11AA) of the *Mtb* Tb6Ag in the presence of the costimulatory  
841 molecules CD28 and CD49d (BD Biosciences) for 1 hour, followed by addition of brefeldin A  
842 (Sigma-Aldrich) for an additional 8 hours. As a background control we used cells co-stimulated  
843 without the peptide pool. Following incubation, cells were stored at 4°C until staining with  
844 combinations of fluorochrome-conjugated monoclonal antibodies including: anti-CD3 (SP34-2:  
845 Pacific Blue; BD Biosciences), anti-CD4 (L200: BV510; Biolegend), anti-CD8α (SK1: TruRed;  
846 eBioscience), anti-TNF-α (MAB11: PE; Life Tech), anti-IFN-γ (B27: APC; Biolegend) and anti-  
847 CD69 (FN50: PE/Dazzle 594; Biolegend), anti-Ki67 (B56: FITC; BD Biosciences). Data were  
848 collected on an LSR-II flow cytometer (BD Biosciences). FlowJo software (Tree Star) was used  
849 for data analysis. In all analyses, gating on the small lymphocyte population was followed by the  
850 separation of the CD3<sup>+</sup> T cell subset and progressive gating on CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets.  
851 Antigen-responding cells in both CD4<sup>+</sup> and CD8<sup>+</sup> T- cell populations were determined by their  
852 intracellular expression of CD69 and either or both of the cytokines interferon gamma (IFN-γ) and  
853 tumor necrosis factor alpha (TNF-α). Final response frequencies shown have been background  
854 subtracted and memory corrected as previously described (76).

855

#### 856 **RNA-seq Library Preparation, Sequencing and Data Analysis.**

857 Total RNA was isolated from *in vitro* cell cultures and *in vivo* tissues using the Trizol method.  
858 RNA next generational sequencing (NGS) was performed on polyA-fractionated RNA utilizing  
859 the TruSeq Stranded mRNA library prep kit (Illumina). Library was validated using Agilent DNA  
860 1000 kit on bioanalyzer according to manufacturer's protocol. RNA libraries were sequenced by

861 the OHSU Massively Parallel Sequencing Shared Resource Core using their Illumina HiSeq-2500  
862 using single-end 100 bp reads. Due to low fraction of viral reads relative to host, the libraries from  
863 parotid and submandibular glands were sequenced again to increase read depth. Sequence data  
864 were quality trimmed with Trimmomatic (77), and aligned to a custom reference genome  
865 comprised of the latest rhesus macaque genome build (MMul10; assembly ID GCA\_003339765.3)  
866 and the RhCMV68-1/Tb genome using the STAR aligner (78). For coverage analyses, GATK  
867 DepthOfCoverage (79) was used to produce a table of raw counts per base. Coverage across the  
868 RhCMV genome was visualized in R (3.6.1) for the three *in vitro* dataset (8, 24, and 72 hour post  
869 infection) using Gviz (1.3) (80). Base-pair level coverage data was smoothed using the ksmooth  
870 function of base R, a kernel-based regression smoothing technique, with the bandwidth parameter  
871 set to 500. Feature counting was performed using Subread featureCounts version 1.6.0 (81), using  
872 a GTF produced by concatenating Rhesus macaque Ensembl reference genes (build 98) with the  
873 ORFs annotated from RhCMV-68.1/Tb. For subread, the following options were used:  
874 fracOverlap of 0.1, minOverlap of 20, using both --largestOverlap, and --primary. To account for  
875 gene length differences that can bias the transcript counts, we normalized across the genes, for all  
876 samples by the gene length (computed as the total exon length from start to end positions). Finally,  
877 for an equivalent comparison across the *in vitro* and *in vivo* samples, used for the principal  
878 component analysis (PCA) as well as the combined heatmap (Fig.8A and B), we corrected for  
879 cross-sample variation by quantile normalization of the gene expression matrix. PCA was  
880 performed using the base R prcomp function on the normalized gene expression matrix as  
881 described in the data analysis section. The variance across the components was used to order and  
882 select for top components of interest.

883

884 **Author Contributions**

885 Conceptualization: HT, EM, BNB, LJP, DNS, KF, DM

886 Data Curation: HT, EM, SGH, AK, BNB, LJP, DNS, DM

887 Formal Analysis: HT, EM, MN, JS, PTE, SGH, BNB, DM

888 Funding Acquisition: LJP, KF

889 Investigation: HT, EM, CK, LSU, MRM, MJM, AB, MN, TW, EAS, LMS, DR, CMH, KAJ, ANS, ABV, YY, KS, RJS, SGH, BNB, DM

890

891 Methodology: EM, BNB, LJP, KF, DM

892 Project Administration: RJS, SGH, AK, DNS, DM

893 Resources: JS, MKA, JDE, SGH, AK, PAB, BNB, LJP

894 Software: EM, BNB

895 Supervision: SGH, DNS, DM

896 Validation: HT, EM, BNB, DM

897 Visualization: HT, EM, MJM, MN, SGH, BNB, DM

898 Writing – Original Draft Preparation: KF, DM

899 Writing – Review & Editing: HT, EM, MJM, RJS, JDE, SGH, AK, PAB, BNB, LJP, DNS, KF, DM

900

901

902 **Acknowledgements**

903 We would like to thank Dr. Thomas Shenk (Princeton University) for providing the RhCMV 68-  
904 1.2 BAC and the rhesus retinal pigment epithelium (RPE) cells. We would also like to thank the  
905 faculty and staff of the Departments of Veterinary Medicine and Collaborative Research at TNPRC  
906 for the excellent animal care and collection of research specimens. We are grateful to the OHSU  
907 molecular virology support core for generating RhCMV stocks and for assisting in the processing  
908 of tissue samples, to Dr. Robert Searles and the OHSU Massively Parallel Sequencing Shared  
909 Resource (MPSSR) for generating the NGS libraries and to Yibing Jia and the Molecular and  
910 Cellular Biology Core (MCB Core) at the ONPRC for analyzing the samples on their MiSeq next  
911 generation sequencer. Lastly, we would like to acknowledge the invaluable technical and scientific  
912 support provided by Katinka Vigh-Conrad, Larry Wilhelm, Shoko Hagen, Andrew Sylwester,  
913 Kyle Taylor, Junwei Gao, Jennie Womack, Nessy John and Hillary Cleveland-Rubeor that made  
914 this study possible. This work was supported by the National Institute of Allergy and Infectious  
915 Diseases (NIAID) (P01 AI129859U42, R01 AI095113, P01 AI094417, R37 AI054292, R01  
916 AI059457, OD023038), the National Institutes of Health Office of the Director (U42OD010426,  
917 P51OD011092, P51OD011104), the Eunice Kennedy Shriver National Institute of Child Health &  
918 Human Development (NICHD) (4DP2HD075699) and the Bill & Melinda Gates Foundation  
919 (OPP1033121, OPP1108533, OPP1152430).

920

921 **References**

922 1. Cannon MJ, Schmid DS, Hyde TB. Review of cytomegalovirus seroprevalence and  
923 demographic characteristics associated with infection. *Rev Med Virol*. 2010;20(4):202-13.

924 2. Boppana SB, Britt WJ. Synopsis of clinical aspects of human cytomegalovirus disease. In:  
925 Reddehase MJ, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*.  
926 2. 2nd ed. Norfolk, UK: Caister Academic Press/ Horizon Scientific Press; 2013. p. 1-25.

927 3. Acosta E, Bowlin T, Brooks J, Chiang L, Hussein I, Kimberlin D, et al. Advances in the  
928 Development of Therapeutics for Cytomegalovirus Infections. *J Infect Dis*.  
929 2020;221(Supplement\_1):S32-S44.

930 4. Staczek J. Animal cytomegaloviruses. *Microbiol Rev*. 1990;54(3):247-65.

931 5. Tirosh O, Cohen Y, Shitrit A, Shani O, Le-Trilling VT, Trilling M, et al. The Transcription  
932 and Translation Landscapes during Human Cytomegalovirus Infection Reveal Novel Host-  
933 Pathogen Interactions. *PLoS Pathog*. 2015;11(11):e1005288.

934 6. Nightingale K, Lin KM, Ravenhill BJ, Davies C, Nobre L, Fielding CA, et al. High-  
935 Definition Analysis of Host Protein Stability during Human Cytomegalovirus Infection  
936 Reveals Antiviral Factors and Viral Evasion Mechanisms. *Cell Host Microbe*.  
937 2018;24(3):447-60 e11.

938 7. Nobre LV, Nightingale K, Ravenhill BJ, Antrobus R, Soddy L, Nichols J, et al. Human  
939 cytomegalovirus interactome analysis identifies degradation hubs, domain associations and  
940 viral protein functions. *Elife*. 2019;8.

941 8. McGeoch DJ, Cook S, Dolan A, Jamieson FE, Telford EA. Molecular phylogeny and  
942 evolutionary timescale for the family of mammalian herpesviruses. *J Mol Biol*.  
943 1995;247(3):443-58.

944 9. Jurak I, Brune W. Induction of apoptosis limits cytomegalovirus cross-species infection.  
945 EMBO J. 2006;25(11):2634-42.

946 10. Malouli D, Nakayasu ES, Viswanathan K, Camp DG, 2nd, Chang WL, Barry PA, et al.  
947 Reevaluation of the coding potential and proteomic analysis of the BAC-derived rhesus  
948 cytomegalovirus strain 68-1. J Virol. 2012;86(17):8959-73.

949 11. Bialas KM, Tanaka T, Tran D, Varner V, Cisneros De La Rosa E, Chiuppesi F, et al.  
950 Maternal CD4+ T cells protect against severe congenital cytomegalovirus disease in a  
951 novel nonhuman primate model of placental cytomegalovirus transmission. Proc Natl Acad  
952 Sci U S A. 2015;112(44):13645-50.

953 12. Fruh K, Picker L. CD8+ T cell programming by cytomegalovirus vectors: applications in  
954 prophylactic and therapeutic vaccination. Curr Opin Immunol. 2017;47:52-6.

955 13. Hansen SG, Ford JC, Lewis MS, Ventura AB, Hughes CM, Coyne-Johnson L, et al.  
956 Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine.  
957 Nature. 2011;473(7348):523-7.

958 14. Hansen SG, Zak DE, Xu G, Ford JC, Marshall EE, Malouli D, et al. Prevention of  
959 tuberculosis in rhesus macaques by a cytomegalovirus-based vaccine. Nat Med.  
960 2018;24(2):130-43.

961 15. Hansen SG, Womack J, Scholz I, Renner A, Edgel KA, Xu G, et al. Cytomegalovirus  
962 vectors expressing Plasmodium knowlesi antigens induce immune responses that delay  
963 parasitemia upon sporozoite challenge. PLoS One. 2019;14(1):e0210252.

964 16. Hansen SG, Piatak M, Jr., Ventura AB, Hughes CM, Gilbride RM, Ford JC, et al. Immune  
965 clearance of highly pathogenic SIV infection. Nature. 2013;502(7469):100-4.

966 17. Hansen SG, Sacha JB, Hughes CM, Ford JC, Burwitz BJ, Scholz I, et al. Cytomegalovirus  
967        Vectors Violate CD8+ T Cell Epitope Recognition Paradigms. Science.  
968        2013;340(6135):1237874-.

969 18. Hansen SG, Wu HL, Burwitz BJ, Hughes CM, Hammond KB, Ventura AB, et al. Broadly  
970        targeted CD8(+) T cell responses restricted by major histocompatibility complex E.  
971        Science. 2016;351(6274):714-20.

972 19. Hansen SG, Marshall EE, Malouli D, Ventura AB, Hughes CM, Ainslie E, et al. A live-  
973        attenuated RhCMV/SIV vaccine shows long-term efficacy against heterologous SIV  
974        challenge. Sci Transl Med. 2019;11(501).

975 20. Marshall EE, Malouli D, Hansen SG, Gilbride RM, Hughes CM, Ventura AB, et al.  
976        Enhancing safety of cytomegalovirus-based vaccine vectors by engaging host intrinsic  
977        immunity. Sci Transl Med. 2019;11(501).

978 21. Asher DM, Gibbs CJ, Jr., Lang DJ, Gajdusek DC, Chanock RM. Persistent shedding of  
979        cytomegalovirus in the urine of healthy Rhesus monkeys. Proc Soc Exp Biol Med.  
980        1974;145(3):794-801.

981 22. Chang WL, Barry PA. Cloning of the full-length rhesus cytomegalovirus genome as an  
982        infectious and self-excisable bacterial artificial chromosome for analysis of viral  
983        pathogenesis. J Virol. 2003;77(9):5073-83.

984 23. Oxford KL, Eberhardt MK, Yang KW, Strelow L, Kelly S, Zhou SS, et al. Protein coding  
985        content of the UL)b' region of wild-type rhesus cytomegalovirus. Virology.  
986        2008;373(1):181-8.

987 24. Assaf BT, Mansfield KG, Westmoreland SV, Kaur A, Oxford KL, Diamond DJ, et al.  
988 Patterns of acute rhesus cytomegalovirus (RhCMV) infection predict long-term RhCMV  
989 infection. *J Virol.* 2012;86(11):6354-7.

990 25. Lilja AE, Shenk T. Efficient replication of rhesus cytomegalovirus variants in multiple  
991 rhesus and human cell types. *Proc Natl Acad Sci U S A.* 2008;105(50):19950-5.

992 26. Oxford KL, Strelow L, Yue Y, Chang WL, Schmidt KA, Diamond DJ, et al. Open reading  
993 frames carried on UL/b' are implicated in shedding and horizontal transmission of rhesus  
994 cytomegalovirus in rhesus monkeys. *J Virol.* 2011;85(10):5105-14.

995 27. Nelson CS, Cruz DV, Tran D, Bialas KM, Stamper L, Wu H, et al. Preexisting antibodies  
996 can protect against congenital cytomegalovirus infection in monkeys. *JCI Insight.*  
997 2017;2(13).

998 28. Vera Cruz D, Nelson CS, Tran D, Barry PA, Kaur A, Koelle K, et al. Intrahost  
999 cytomegalovirus population genetics following antibody pretreatment in a monkey model  
1000 of congenital transmission. *PLoS Pathog.* 2020;16(2):e1007968.

1001 29. Alcendor DJ, Barry PA, Pratt-Lowe E, Luciw PA. Analysis of the rhesus cytomegalovirus  
1002 immediate-early gene promoter. *Virology.* 1993;194(2):815-21.

1003 30. Tarantal AF, Salamat MS, Britt WJ, Luciw PA, Hendrickx AG, Barry PA.  
1004 Neuropathogenesis induced by rhesus cytomegalovirus in fetal rhesus monkeys (*Macaca  
1005 mulatta*). *J Infect Dis.* 1998;177(2):446-50.

1006 31. Murrell I, Wilkie GS, Davison AJ, Statkute E, Fielding CA, Tomasec P, et al. Genetic  
1007 Stability of Bacterial Artificial Chromosome-Derived Human Cytomegalovirus during  
1008 Culture In Vitro. *J Virol.* 2016;90(8):3929-43.

1009 32. Dargan DJ, Douglas E, Cunningham C, Jamieson F, Stanton RJ, Baluchova K, et al.  
1010 Sequential mutations associated with adaptation of human cytomegalovirus to growth in  
1011 cell culture. *J Gen Virol.* 2010;91(Pt 6):1535-46.

1012 33. Assaf BT, Mansfield KG, Strelow L, Westmoreland SV, Barry PA, Kaur A. Limited  
1013 dissemination and shedding of the UL128 complex-intact, UL/b'-defective rhesus  
1014 cytomegalovirus strain 180.92. *J Virol.* 2014;88(16):9310-20.

1015 34. Caposio P, van den Worm S, Crawford L, Perez W, Kreklywich C, Gilbride RM, et al.  
1016 Characterization of a live-attenuated HCMV-based vaccine platform. *Sci Rep.*  
1017 2019;9(1):19236.

1018 35. Rivaillet P, Kaur A, Johnson RP, Wang F. Genomic sequence of rhesus cytomegalovirus  
1019 180.92: insights into the coding potential of rhesus cytomegalovirus. *J Virol.*  
1020 2006;80(8):4179-82.

1021 36. Barry PA, Deere JD, Yue Y, Chang WWL, Schmidt KA, Wussow F, et al.  
1022 Cytomegalovirus-vectored vaccines for HIV and other pathogens. *AIDS.* 2020;34(3):335-  
1023 49.

1024 37. McCormick AL, Skaletskaya A, Barry PA, Mocarski ES, Goldmacher VS. Differential  
1025 function and expression of the viral inhibitor of caspase 8-induced apoptosis (vICA) and  
1026 the viral mitochondria-localized inhibitor of apoptosis (vMIA) cell death suppressors  
1027 conserved in primate and rodent cytomegaloviruses. *Virology.* 2003;316(2):221-33.

1028 38. Gill RB, Jason Bowman J, Krogmann T, Wollenberg K, Asher DM, Cohen JI. Coding  
1029 potential of UL/b' from the initial source of rhesus cytomegalovirus Strain 68-1. *Virology.*  
1030 2013;447(1-2):208-12.

1031 39. Burwitz BJ, Malouli D, Bimber BN, Reed JS, Ventura AB, Hancock MH, et al. Cross-  
1032 Species Rhesus Cytomegalovirus Infection of Cynomolgus Macaques. *PLoS Pathog.*  
1033 2016;12(11):e1006014.

1034 40. Davison AJ, Holton M, Dolan A, Dargan DJ, Gatherer D, Hayward GS. Comparative  
1035 Genomics of Primate Cytomegaloviruses. In: Reddehase MJ, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*. 1. 2nd ed. Norfolk, UK: Caister Academic  
1036 Press/ Horizon Scientific Press; 2013. p. 1-22.

1037 41. Marsh AK, Willer DO, Ambagala AP, Dzamba M, Chan JK, Pilon R, et al. Genomic  
1038 sequencing and characterization of cynomolgus macaque cytomegalovirus. *J Virol.*  
1039 2011;85(24):12995-3009.

1040 42. Russell JN, Marsh AK, Willer DO, Ambagala AP, Dzamba M, Chan JK, et al. A novel  
1041 strain of cynomolgus macaque cytomegalovirus: implications for host-virus co-evolution.  
1042 *BMC Genomics.* 2016;17:277.

1043 43. Stanton RJ, Baluchova K, Dargan DJ, Cunningham C, Sheehy O, Seirafian S, et al.  
1044 Reconstruction of the complete human cytomegalovirus genome in a BAC reveals RL13  
1045 to be a potent inhibitor of replication. *J Clin Invest.* 2010;120(9):3191-208.

1046 44. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-  
1047 responsive promoters. *Proc Natl Acad Sci U S A.* 1992;89(12):5547-51.

1048 45. Zhong G, Wang H, Bailey CC, Gao G, Farzan M. Rational design of aptazyme riboswitches  
1049 for efficient control of gene expression in mammalian cells. *Elife.* 2016;5.

1050 46. Murrell I, Tomasec P, Wilkie GS, Dargan DJ, Davison AJ, Stanton RJ. Impact of sequence  
1051 variation in the UL128 locus on production of human cytomegalovirus in fibroblast and  
1052 epithelial cells. *J Virol.* 2013;87(19):10489-500.

1053

1054 47. Zhou M, Yu Q, Wechsler A, Ryckman BJ. Comparative analysis of gO isoforms reveals  
1055 that strains of human cytomegalovirus differ in the ratio of gH/gL/gO and gH/gL/UL128-  
1056 131 in the virion envelope. *J Virol.* 2013;87(17):9680-90.

1057 48. Zhang L, Zhou M, Stanton R, Kamil J, Ryckman BJ. Expression Levels of Glycoprotein  
1058 O (gO) Vary between Strains of Human Cytomegalovirus, Influencing the Assembly of  
1059 gH/gL Complexes and Virion Infectivity. *J Virol.* 2018;92(15).

1060 49. Wang D, Shenk T. Human cytomegalovirus UL131 open reading frame is required for  
1061 epithelial cell tropism. *J Virol.* 2005;79(16):10330-8.

1062 50. Li G, Nguyen CC, Ryckman BJ, Britt WJ, Kamil JP. A viral regulator of glycoprotein  
1063 complexes contributes to human cytomegalovirus cell tropism. *Proc Natl Acad Sci U S A.*  
1064 2015;112(14):4471-6.

1065 51. Sturgill ER, Malouli D, Hansen SG, Burwitz BJ, Seo S, Schneider CL, et al. Natural Killer  
1066 Cell Evasion Is Essential for Infection by Rhesus Cytomegalovirus. *PLoS Pathog.*  
1067 2016;12(8):e1005868.

1068 52. Malouli D, Hansen SG, Nakayasu ES, Marshall EE, Hughes CM, Ventura AB, et al.  
1069 Cytomegalovirus pp65 limits dissemination but is dispensable for persistence. *J Clin*  
1070 *Invest.* 2014;124(5):1928-44.

1071 53. Hansen SG, Vieville C, Whizin N, Coyne-Johnson L, Siess DC, Drummond DD, et al.  
1072 Effector memory T cell responses are associated with protection of rhesus monkeys from  
1073 mucosal simian immunodeficiency virus challenge. *Nat Med.* 2009;15(3):293-9.

1074 54. Cheng S, Caviness K, Buehler J, Smithey M, Nikolich-Zugich J, Goodrum F.  
1075 Transcriptome-wide characterization of human cytomegalovirus in natural infection and  
1076 experimental latency. *Proc Natl Acad Sci U S A.* 2017;114(49):E10586-E95.

1077 55. Hansen SG, Strelow LI, Franchi DC, Anders DG, Wong SW. Complete sequence and  
1078 genomic analysis of rhesus cytomegalovirus. *J Virol.* 2003;77(12):6620-36.

1079 56. Elde NC, Child SJ, Eickbush MT, Kitzman JO, Rogers KS, Shendure J, et al. Poxviruses  
1080 deploy genomic accordions to adapt rapidly against host antiviral defenses. *Cell.*  
1081 2012;150(4):831-41.

1082 57. Schultz EP, Lanchy JM, Day LZ, Yu Q, Peterson C, Preece J, et al. Specialization to cell-  
1083 free or cell-associated spread by BAC-cloned HCMV strains not determined by the UL128-  
1084 131 and RL13 loci. *bioRxiv.* 2019.

1085 58. Sijmons S, Thys K, Mbong Ngwese M, Van Damme E, Dvorak J, Van Loock M, et al.  
1086 High-throughput analysis of human cytomegalovirus genome diversity highlights the  
1087 widespread occurrence of gene-disrupting mutations and pervasive recombination. *J Virol.*  
1088 2015;89(15):7673-95.

1089 59. Cortese M, Calo S, D'Aurizio R, Lilja A, Pacchiani N, Merola M. Recombinant human  
1090 cytomegalovirus (HCMV) RL13 binds human immunoglobulin G Fc. *PLoS One.*  
1091 2012;7(11):e50166.

1092 60. Jackson JW, Hancock TJ, LaPrade E, Dogra P, Gann ER, Masi TJ, et al. The Human  
1093 Cytomegalovirus Chemokine vCXCL-1 Modulates Normal Dissemination Kinetics of  
1094 Murine Cytomegalovirus In Vivo. *mBio.* 2019;10(3).

1095 61. Barry PA, Lockridge KM, Salamat S, Tinling SP, Yue Y, Zhou SS, et al. Nonhuman  
1096 primate models of intrauterine cytomegalovirus infection. *ILAR J.* 2006;47(1):49-64.

1097 62. Itell HL, Kaur A, Deere JD, Barry PA, Permar SR. Rhesus monkeys for a nonhuman  
1098 primate model of cytomegalovirus infections. *Curr Opin Virol.* 2017;25:126-33.

1099 63. Chang WL, Kirchoff V, Pari GS, Barry PA. Replication of rhesus cytomegalovirus in life-  
1100 expanded rhesus fibroblasts expressing human telomerase. *J Virol Methods*.  
1101 2002;104(2):135-46.

1102 64. Yue Y, Kaur A, Lilja A, Diamond DJ, Walter MR, Barry PA. The susceptibility of primary  
1103 cultured rhesus macaque kidney epithelial cells to rhesus cytomegalovirus strains. *J Gen*  
1104 *Virol*. 2016;97(6):1426-38.

1105 65. Tischer BK, Smith GA, Osterrieder N. En passant mutagenesis: a two step markerless red  
1106 recombination system. *Methods Mol Biol*. 2010;634:421-30.

1107 66. Kolb P, Sijmons S, McArdle MR, Taher H, Womack J, Hughes C, et al. Identification and  
1108 Functional Characterization of a Novel Fc Gamma-Binding Glycoprotein in Rhesus  
1109 Cytomegalovirus. *J Virol*. 2019;93(4).

1110 67. Hirt B. Selective extraction of polyoma DNA from infected mouse cell cultures. *J Mol*  
1111 *Biol*. 1967;26(2):365-9.

1112 68. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al.  
1113 Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007;23(21):2947-8.

1114 69. Yao F, Svensjo T, Winkler T, Lu M, Eriksson C, Eriksson E. Tetracycline repressor, tetR,  
1115 rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates  
1116 inducible gene expression in mammalian cells. *Hum Gene Ther*. 1998;9(13):1939-50.

1117 70. Stanton RJ, McSharry BP, Armstrong M, Tomasec P, Wilkinson GW. Re-engineering  
1118 adenovirus vector systems to enable high-throughput analyses of gene function.  
1119 *Biotechniques*. 2008;45(6):659-62, 64-8.

1120 71. Sequar G, Britt WJ, Lakeman FD, Lockridge KM, Tarara RP, Canfield DR, et al.  
1121       Experimental coinfection of rhesus macaques with rhesus cytomegalovirus and simian  
1122       immunodeficiency virus: pathogenesis. *J Virol.* 2002;76(15):7661-71.

1123 72. Kaur A, Hale CL, Noren B, Kassis N, Simon MA, Johnson RP. Decreased frequency of  
1124       cytomegalovirus (CMV)-specific CD4+ T lymphocytes in simian immunodeficiency  
1125       virus-infected rhesus macaques: inverse relationship with CMV viremia. *J Virol.*  
1126       2002;76(8):3646-58.

1127 73. Cline AN, Bess JW, Piatak M, Jr., Lifson JD. Highly sensitive SIV plasma viral load assay:  
1128       practical considerations, realistic performance expectations, and application to reverse  
1129       engineering of vaccines for AIDS. *J Med Primatol.* 2005;34(5-6):303-12.

1130 74. Deleage C, Wietgrefe SW, Del Prete G, Morcock DR, Hao XP, Piatak M, Jr., et al. Defining  
1131       HIV and SIV Reservoirs in Lymphoid Tissues. *Pathog Immun.* 2016;1(1):68-106.

1132 75. Sylwester AW, Hansen SG, Picker LJ. Quantification of T cell Antigen-specific Memory  
1133       Responses in Rhesus Macaques, Using Cytokine Flow Cytometry (CFC, also Known as  
1134       ICS and ICCS): Analysis of Flow Data. *Bio Protoc.* 2014;4(8).

1135 76. Hansen SG, Sacha JB, Hughes CM, Ford JC, Burwitz BJ, Scholz I, et al. Cytomegalovirus  
1136       vectors violate CD8+ T cell epitope recognition paradigms. *Science.*  
1137       2013;340(6135):1237874.

1138 77. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence  
1139       data. *Bioinformatics.* 2014;30(15):2114-20.

1140 78. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast  
1141       universal RNA-seq aligner. *Bioinformatics.* 2013;29(1):15-21.

1142 79. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The  
1143 Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA  
1144 sequencing data. *Genome Res.* 2010;20(9):1297-303.

1145 80. Hahne F, Ivanek R. Visualizing Genomic Data Using Gviz and Bioconductor. *Methods*  
1146 *Mol Biol.* 2016;1418:335-51.

1147 81. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for  
1148 assigning sequence reads to genomic features. *Bioinformatics.* 2014;30(7):923-30.

1149 82. Huang ES, Kilpatrick B, Lakeman A, Alford CA. Genetic analysis of a cytomegalovirus-  
1150 like agent isolated from human brain. *J Virol.* 1978;26(3):718-23.

1151 83. Martin WJ, Ahmed KN, Zeng LC, Olsen JC, Seward JG, Seehrai JS. African green monkey  
1152 origin of the atypical cytopathic 'stealth virus' isolated from a patient with chronic fatigue  
1153 syndrome. *Clin Diagn Virol.* 1995;4(1):93-103.

1154 84. Blewett EL, White G, Saliki JT, Eberle R. Isolation and characterization of an endogenous  
1155 cytomegalovirus (BaCMV) from baboons. *Arch Virol.* 2001;146(9):1723-38.

1156 85. Blewett EL, Sherrod CJ, Texier JR, Conrad TM, Dittmer DP. Complete Genome  
1157 Sequences of *Mandrillus leucophaeus* and *Papio ursinus* Cytomegaloviruses. *Genome*  
1158 *Announc.* 2015;3(4).

1159 86. Davison AJ, Dolan A, Akter P, Addison C, Dargan DJ, Alcendor DJ, et al. The human  
1160 cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus  
1161 genome. *J Gen Virol.* 2003;84(Pt 1):17-28.

1162 87. Daniel MD, Melendez LV, King NW, Fraser CE, Barahona HH, Hunt RD, et al. Herpes  
1163 virus aotus: a latent herpesvirus from owl monkeys (*Aotus trivirgatus*) isolation and  
1164 characterization. *Proc Soc Exp Biol Med.* 1971;138(3):835-45.

1165 88. Rangan SR, Chaiban J. Isolation and characterization of a cytomegalovirus from the  
1166 salivary gland of a squirrel monkey (*Saimiri sciureus*). *Lab Anim Sci.* 1980;30(3):532-40.

1167 89. Vink C, Beuken E, Bruggeman CA. Complete DNA sequence of the rat cytomegalovirus  
1168 genome. *J Virol.* 2000;74(16):7656-65.

1169 90. Ettinger J, Geyer H, Nitsche A, Zimmermann A, Brune W, Sandford GR, et al. Complete  
1170 genome sequence of the english isolate of rat cytomegalovirus (Murid herpesvirus 8). *J*  
1171 *Virol.* 2012;86(24):13838.

1172 91. Geyer H, Ettinger J, Moller L, Schmolz E, Nitsche A, Brune W, et al. Rat cytomegalovirus  
1173 (RCMV) English isolate and a newly identified Berlin isolate share similarities with but  
1174 are separate as an anciently diverged clade from Mouse CMV and the Maastricht isolate of  
1175 RCMV. *J Gen Virol.* 2015;96(Pt 7):1873-0.

1176 92. Hartley JW, Rowe WP, Huebner RJ. Serial propagation of the guinea pig salivary gland  
1177 virus in tissue culture. *Proc Soc Exp Biol Med.* 1957;96(2):281-5.

1178 93. Smith MG. Propagation of salivary gland virus of the mouse in tissue cultures. *Proc Soc*  
1179 *Exp Biol Med.* 1954;86(3):435-40.

1180 94. Fooden J, Lanyon SM. Blood-protein allele frequencies and phylogenetic relationships in  
1181 *Macaca*: A review. *Am J Primatol.* 1989;17(3):209-41.

1182 95. Smith DG, McDonough JW, George DA. Mitochondrial DNA variation within and among  
1183 regional populations of longtail macaques (*Macaca fascicularis*) in relation to other species  
1184 of the fascicularis group of macaques. *Am J Primatol.* 2007;69(2):182-98.

1185 96. Li J, Han K, Xing J, Kim HS, Rogers J, Ryder OA, et al. Phylogeny of the macaques  
1186 (Cercopithecidae: *Macaca*) based on Alu elements. *Gene.* 2009;448(2):242-9.

1187 97. Murphy E, Shenk T. Human cytomegalovirus genome. *Curr Top Microbiol Immunol.*  
1188 2008;325:1-19.

1189 98. Caracausi M, Piovesan A, Antonaros F, Strippoli P, Vitale L, Pelleri MC. Systematic  
1190 identification of human housekeeping genes possibly useful as references in gene  
1191 expression studies. *Mol Med Rep.* 2017;16(3):2397-410.

1192

1193 **Figure Legends**

1194 **Figure 1: Construction of FL-RhCMV**

1195 The schematic depicts the repair steps performed to generate FL-RhCMV. Unaltered ORFs and  
1196 the unmodified viral genome are shown in orange while the terminal repeats are indicated in blue.  
1197 ORFs containing known mutations that were repaired in this study are highlighted in red. Genes  
1198 contained in the acquired inversion in the ULb' region are shown in green, while genes lost in 68-  
1199 1 but re-inserted into the genome during the repair are highlighted in purple. The transposon picked  
1200 up during the generation of 68-1.2 RhCMV is highlighted in yellow and the 180.92 RhCMV PRC  
1201 members used in the construction of 68-1.2 RhCMV are marked in grey. Repair 0: The frameshift  
1202 resulting in a premature stop codon in Rh61/60 of 68-1 RhCMV was repaired and the two missing  
1203 PRC members (Rh157.4 and Rh157.5) were inserted to generate 68-1.2 RhCMV as described  
1204 previously (25). Repair 1: Two DNA fragments combined spanning 6.9kb corresponding to the  
1205 genomic sequence of the ULb' homologous region in the circulating virus originally isolated from  
1206 sample 68-1 (38) were synthesized. Three undefined bases in the published nucleotide sequence  
1207 (KF011492) were taken from the consensus sequence of all sequenced low-passage RhCMV  
1208 isolates. A synthetic DNA fragment spanning the region upstream of Rh157.5 (UL128) to Rh161  
1209 (UL146G) in its original orientation was used to replace the corresponding gene region in 68-1.2  
1210 RhCMV. The resulting construct maintains the repaired Rh61/60 gene while also containing the  
1211 original isolate 68-1 genes Rh157.4 (UL128) and Rh157.5 (UL130) as well as the genes coding  
1212 for the UL146 homologs Rh158.2, Rh158.3 and Rh161.1. Repair 2: Two previously described  
1213 frameshift mutations in Rh13.1 (10) were repaired resulting in an intact Rh13.1 ORF. Repair 3: A  
1214 premature stop codon in the viral Fcγ-Receptor homolog Rh152/151 (10) was repaired restoring  
1215 the ORF to its original length. Repair 4: A nonsynonymous point mutations in Rh164 (UL141)

1216 initially predicted by us was confirmed by sequencing the original urine isolate. Hence, we restored  
1217 the natural DNA sequence. Repair 5: Full genome sequencing of the RhCMV 68-1.2 BAC revealed  
1218 that an *E. coli* derived transposon had inserted itself into the Rh167 ORF. The transposon was  
1219 removed by *en passant* mutagenesis and the intact Rh167 ORF was restored. Repair 6: The US14  
1220 homologue Rh197 contained a premature stop codon which was repaired.

1221

1222 **Figure 2: Sequence relationship of FL-RhCMV with NHP CMVs**

1223 A phylogenetic tree for FL-RhCMV and rodent and primate CMVs was constructed based on full  
1224 genome alignments using the Geneious Prime Tree Builder application. Sequences previously  
1225 published include RhCMV 180.92 (35) as well as the RhCMV isolates 19262, 19936 and 24514  
1226 and the Cynomolgus CMV isolates 31906, 31907, 31908 and 31909 (39). We also included the  
1227 published sequences for the CyCMV strains Ottawa (41) and Mauritius (42), the simian (African  
1228 green monkey) CMV isolates Colburn (82), GR2715 (40) and stealth virus 1 (83) as well as the  
1229 BaCMV strains OCOM4-37 (84) and OCOM4-52 (85) and the DrCMV strain OCOM6-2 (85). For  
1230 comparison we included the HCMV TR3 strain (34), the chimpanzee CMV strain Heberling (86)  
1231 and the only two complete genome sequences of new world NHP CMVs, Aotus betaherpesvirus  
1232 1 (AoHV-1) strain S34E (87) and Saimiriine betaherpesvirus 4 (SaHV-4) strain SqSHV (88). New  
1233 genome sequences included in this alignment are as follows: the two RhCMV isolates 34844 and  
1234 KF03, the CyCMV isolate 31709, the Japanese macaque CMV JaCMV 24655 and the two baboon  
1235 CMVs BaCMV 31282 and 34826. These CMVs were isolated from fibroblast co-cultures of urine  
1236 samples obtained from NHP housed either at the Oregon National Primate Research Center  
1237 (ONPRC) or the Tulane National Primate Research Center (TNPRC). Also included in the  
1238 alignment are the genomic sequences of the previously published RhCMV isolates UCD52 and

1239 UCD59 that originated at the UC Davis National Primate Research Center (29, 30). The rodent  
1240 CMVs include the rat CMV (RCMV) isolates Maastricht (89), England (90) and Berlin (91), the  
1241 guinea pig CMV (GPCMV) isolate 22122 (92) and the murine CMV (MCMV) strain Smith (93),  
1242 which was used as an outgroup.

1243

1244 **Figure 3: Viral ORFs contained in FL-RhCMV compared to other NHP CMVs**

1245 Full genome annotations of all listed old world NHP CMVs are shown. The leftmost column  
1246 indicates the HCMV nomenclature for CMV encoded genes. Each ORFs that has a defined  
1247 orthologue in HCMV and old world NHP CMVs is marked. If an orthologue cannot be clearly  
1248 identified, the homologous gene family is given. The second column identifies the old world NHP  
1249 CMV nomenclature. The same ORF nomenclature is used across all shown species, with the first  
1250 or the first two letters corresponding to the host species (e.g. Rh for rhesus macaque). The virus  
1251 strain analyzed is indicated. Green boxes indicate ORFs present in a particular strain, whereas red  
1252 boxes indicate ORFs that are absent. Frameshifts or point mutations leading to shortened or  
1253 elongated ORFs are highlighted in yellow or blue, respectively. Grey boxes indicate absent ORFs  
1254 due to missing genome sequence information whereas ORFs with partial sequences are highlighted  
1255 in purple. Orthologous ORFs that lack a conserved canonical start codon in some strains are  
1256 highlighted in orange.

1257

1258 **Figure 4: Conditional expression of the RL13 homolog Rh13.1 results in reduced spreading  
1259 and genomic rearrangements.**

1260 A) Deletion or reduced expression of Rh13.1 results in increased plaque size. Telomerized rhesus  
1261 fibroblasts (TRF) or TRF expressing the tet-repressor (tetR) were transfected with the indicated

1262 BACs. All recombinant BACs were engineered to express GFP from a P2A linker after UL36 (6).  
1263 18 days later plaque sizes were visualized by GFP expression, and measured using ImageJ.  
1264 Statistical significance was determined using an ordinary one-way ANOVA test with a p-value  
1265 significance of <0.05. B) Representative images of the GFP positive plaques produced by the  
1266 indicated constructs on either TRFs or TRFs expressing the tetR are shown. C) Genetic instability  
1267 of the genomic region surrounding the Rh13.1. Top: The position and relative frequencies of single  
1268 nucleotide changes were determined by NGS within the genomes of FL-RhCMV/Rh13.1apt after  
1269 two passages *in vitro* in the presence or absence of tetracycline. The lower panel depicts the  
1270 positions of deletions/insertions of multiple nucleotides. Frequencies for each deletion are given  
1271 as percentages of all reads analyzed. Since the short reads generated by the Illumina platform do  
1272 not cover the entire Rh13.1 locus it is not possible to determine which deletions co-occurred in  
1273 individual viral genomes resulting in combined frequencies of >100%.

1274

1275 **Figure 5: Growth of FL-RhCMV *in vitro*.**

1276 A) Multistep growth curve of FL-RhCMV in fibroblasts indicates growth comparable to 68-1 and  
1277 68-1.2 RhCMV. Primary rhesus fibroblasts were infected with RhCMV 68-1, 68-1.2 and FL-  
1278 RhCMV/Rh13.1/apt at an MOI of 0.01 on day 0. Cell culture supernatants were harvested on the  
1279 indicated days and virus titers were determined by TCID50. Results of two biological repeats  
1280 titrated in duplicate are shown and the standard error of the mean (SEM) is indicated by error bars.  
1281 B) FL-RhCMV shows increased infection of epithelial cells compared to 68-1 RhCMV. Primary  
1282 rhesus fibroblasts or rhesus retinal epithelial cells (RPE) were infected with MOIs of 0.3 or 10,  
1283 respectively, and all experiments were performed in triplicates. After 48 hours post infection, cells  
1284 were harvested, fixed, permeabilized, stained with a RhCMV specific antibody (52) and analyzed

1285 by flow cytometry. Statistical significance was shown using an unpaired t-test with a p-value  
1286 significance threshold of <0.05. C) Rh159, the RhCMV homologue of UL148, is upregulated in  
1287 FL-RhCMV. Relative mRNA copy numbers of Rh159 (UL148) and Rh137 (UL99) were  
1288 determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using  
1289 specific probes. The data shown represent the mean of triplicate repeats (+/- SEM). Unpaired  
1290 student t-tests with a p-value significance threshold of <0.05 were performed to show statistical  
1291 significance in both graphs comparing FL-RhCMV/Rh13.1/apt to either 68-1 RhCMV or 68-1.2  
1292 RhCMV at 48 hpi.

1293

1294 **Figure 6: *In vivo* replication of FL-RhCMV is similar to low passage isolates in RhCMV-  
1295 negative animals**

1296 A) Replication of RhCMV isolates UCD52, UCD59 and 180.92 in RhCMV seronegative RM.  
1297 Plasma RhCMV DNA load in three RhCMV-seronegative pregnant female RM inoculated i.v.  
1298 with  $2 \times 10^6$  TCID50 RhCMV 180.92,  $1 \times 10^6$  PFU RhCMV UCD52, and  $1 \times 10^6$  PFU RhCMV  
1299 UCD59. The plasma viral load (PVL) in each RM was determined at days 7, 14 and 21 using qPCR  
1300 for the exon 1 region of the immediate early gene as described previously (11, 72). B) Replication  
1301 of FL-RhCMV in RhCMV-seronegative RM.  $1.79 \times 10^6$  PFU of FL-RhCMV/Rh13.1/apt were  
1302 inoculated i.v. into three RhCMV-seronegative male RM and the PVL was determined at the  
1303 indicated days by qPCR of the RhCMV gB gene as described previously (71). The PVL for each  
1304 RM is shown. C) The mean PVL for all animals infected in A) and B) are shown in comparison  
1305 and compared at days 14 and 21 by non-parametric Mann-Whitney test. The data indicates that  
1306 FL-RhCMV can induce a PVL comparable to commonly used virulent RhCMV isolates.

1307

1308 **Figure 7: Spreading of FL-RhCMV *in vivo***

1309 A) Tissue genome copy numbers of FL-RhCMV. Three RhCMV-naïve RM (RM1-RM3) were  
1310 inoculated with  $10^7$  PFU FL-RhCMV $\Delta$ Rh13.1/TB6Ag while another three RhCMV-naïve RM  
1311 (RM4-RM6) were inoculated with  $10^7$  PFU of FL-RhCMV $\Delta$ Rh13.1/TB6Ag $\Delta$ Rh157.4-5 $\Delta$ Rh158-  
1312 161. All 6 RM were necropsied at day 14 post-infection and viral genome copy numbers per  $10^7$   
1313 cell equivalents were determined in the indicated tissues using ultra-sensitive nested qPCR specific  
1314 for TB6Ag. Statistical analysis was performed using a two-sided Wilcoxon tests (unadjusted p  
1315 values  $< 0.05$ ) excluding all tissues at the injection site and the nearest draining lymph nodes to  
1316 detect significant differences in dissemination. (B) *In situ* immunofluorescence phenotyping of  
1317 cells expressing RhCMV RNA was performed by multiplexing RNAscope *in situ* hybridization  
1318 with antibody detection of cellular markers specific for myeloid/macrophage cells (CD68/CD163),  
1319 endothelial cells (CD34), and mesenchymal cells (vimentin) in the spleen of macaques inoculated  
1320 with either FL-RhCMV $\Delta$ Rh13.1TB6Ag (FL-RhCMV) or FL RhCMV $\Delta$ Rh13.1/TB6Ag $\Delta$ Rh157.4-  
1321 5 $\Delta$ Rh158-161. The majority of cells inoculated with the FL-RhCMV were vimentin+ CD34-  
1322 CD68/CD163-, indicating they were of mesenchymal origin. (C) To quantify differences in  
1323 RhCMV infection and expression levels in macaques inoculated with either FL-RhCMV or FL  
1324 RhCMV $\Delta$ Rh13.1/TB6Ag $\Delta$ Rh157.4-5 $\Delta$ Rh158-161, we used three independent quantitative  
1325 approaches in the HALO image analysis platform from Indica Labs: i) the percent area of the tissue  
1326 occupied by infected cells, ii) the number of infected cells per  $10^5$  cells, and iii) an estimate of  
1327 RhCMV viral RNA copy number per infected cell. Statistical significance was calculated using an  
1328 unpaired t-test. D) Tissue distribution of Tb6Ag insert-specific CD4 $^+$  and CD8 $^+$  T cell responses  
1329 elicited by FL-RhCMV $\Delta$ Rh13.1TB6Ag versus FL RhCMV $\Delta$ Rh13.1/TB6Ag $\Delta$ Rh157.4-5 $\Delta$ Rh158-  
1330 161 vectors. Flow cytometric ICS (CD69, TNF- $\alpha$  and/or IFN- $\gamma$  readout) was used to determine the

1331 magnitude of the CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to peptide mixes corresponding to the six Mtb  
1332 antigens contained in the TB6Ag-fusion (Ag85A, ESAT-6, Rpf A, Rpf D, Rv2626, Rv3407).  
1333 Mononuclear cells were isolated from the indicated tissues from three RhCMV-naïve RMs  
1334 inoculated with 10<sup>7</sup> PFU FL-RhCMVΔRh13.1TB6Ag (blue bars) and three RMs inoculated with  
1335 10<sup>7</sup> PFU FL-RhCMVΔRh13.1/TB6AgΔRh157.4-5ΔRh158-161 (green bars) and all RM taken to  
1336 necropsy at either 14 or 15 days post infection. Response comparisons per tissue are shown as the  
1337 mean + SEM percentage of T cells specifically responding to the total of all peptide mixes  
1338 (background subtracted) within the memory CD4<sup>+</sup> or CD8<sup>+</sup> T cell compartment for each tissue  
1339 (n=3 per tissue, unless otherwise noted by \* n=1 or † n=2).  
1340

1341 **Figure 8: Viral gene expression profile of FL-RhCMV *in vitro* and *in vivo*.**

1342 A) Comparison of *in vitro* and *in vivo* gene expression profiles by principal component analysis.  
1343 *In vitro*: Rhesus fibroblasts were infected with an MOI=5 of FLRhCMVΔRh13.1/TB6Ag and the  
1344 cells were harvested at the indicated times. *In vivo*: RNA-was isolated from indicated tissues of  
1345 RM1-RM3 described in Fig. 7. Total RNA was isolated from all samples and RNAseq was  
1346 performed on libraries build from polyA-fractionated RNA using an Illumina HiSeq-2500 next  
1347 generation sequencer. PCA was done on the combined and quantile normalized expression matrix  
1348 (see Methods). We observed that PC1 and PC2, shown herein, combined capture over 70% of total  
1349 variance with distinct sets of co-regulated genes. B) *In vitro* and *in vivo* expression levels of each  
1350 ORF. Expression levels were normalized between the *in vitro* and *in vivo* samples using quantile  
1351 normalization (see Methods). C) For all samples analyzed in B) the ten viral ORFs showing the  
1352 highest mRNA coverage after normalization for ORF size are shown. All values are given as  
1353 percent of total viral reads mapping to all annotated ORFs normalized for size.

1354

1355 **Figure 9: Acquisition of genes and gene families in old world NHP CMVs during co-evolution**  
1356 **with their primate hosts.**

1357 Full genome annotations of old world NHP CMVs across different species allowed for  
1358 comparative genomics to identify single ORFs or entire gene families that were present in one or  
1359 several CMV species but absent in others. These differences clustered in six independent loci  
1360 across the genome. Examination of the phylogenetic relationship of the individual CMV species  
1361 as well as their host species reveals at which point in time these gene acquisitions and gene  
1362 duplications occurred. The phylogenetic tree depicted is based on the full genome alignment  
1363 shown in Fig. 1. The green circles indicate genetic events that took place during the evolution of  
1364 each species. The blue circle represents the acquisition of RL11K, a gene duplication found in  
1365 RhCMV and JaCMV but not in CyCMV. Since CyCMV appears to be more closely related to  
1366 RhCMV than JaCMV by full genome alignment (see Fig.1) this appears counterintuitive, however,  
1367 phylogenetic alignments of the corresponding macaque host species based on morphology (94),  
1368 mitochondrial DNA data (95) or Alu elements (96) reveals that Japanese macaques (*M. fuscata*)  
1369 and rhesus macaques (*M. mulatta*) speciated more recently compared to cynomolgus macaques  
1370 (*M. fascicularis*).

1371

1372 **Figure S1: Genome organization of NHP CMVs**

1373 The genome of HCMVs and the closely related CCMV comprise two unique coding regions (U<sub>L</sub>  
1374 and U<sub>S</sub>) that are separated by an internal repeat region and flanked by terminal repeats. The repeat  
1375 regions consist of the three repeated sequence units a, b and c that form overlapping inverted  
1376 repeats in the form *ab-U<sub>L</sub>-b'a'c-U<sub>S</sub>-c'a*. The HCMV genome can re-arrange to four different

1377 isomers varying in the relative orientation of the  $U_L$  and  $U_S$  regions to one another (97).  
1378 Intriguingly, while the  $U_L$  and  $U_S$  regions can still be identified in old world NHP CMVs, the  
1379 repeat organization is vastly different. The terminal direct repeats in these species are short while  
1380 the internal repeats are completely missing resulting in a single isomer that has been fixed during  
1381 evolution. All Asian NHP CMVs and the African green monkey (Simian) CMV (SCMV) occur in  
1382 the same isomeric form whereas the  $U_S$  region appears in the opposite orientation to the  $U_L$  region  
1383 in the closely related BaCMV and DrCMV. New world (NW) CMVs retained a genome  
1384 organization with terminal and internal repeats similar HCMV, but the repeats are organized as  
1385 non-overlapping inverted repeats flanking the  $U_L$  and  $U_S$  regions, allowing for isomerization.

1386

1387 **Figure S2: The RL11 gene family of NHP CMVs**

1388 A) Phylogenetic tree of RL11 family genes from representatives of each NHP CMV species. B)  
1389 ORF structure of the RL11 family genes in each NHP CMV species. Each gene is color-coded  
1390 using the same colors as in A) showing the presence/absence of each ORF in a given NHP CMV  
1391 species.

1392

1393 **Figure S3: The UL83 (pp65) gene family of NHP CMVs**

1394 A) Phylogenetic tree based on the protein sequences of NHP CMV genes homologous to HCMV  
1395 UL83 encoding the major tegument protein pp65 from representatives of each NHP CMV species.  
1396 B) ORF structure of the UL83 family genes in each NHP CMV species. Each gene is color-coded  
1397 using the same colors as in A) showing the presence/absence of each ORF in a given NHP CMV  
1398 species.

1399

1400 **Figure S4: The UL146/UL147 gene family of NHP CMVs**

1401 A) Phylogenetic tree based on the protein sequences of NHP CMV genes homologous to HCMV  
1402 chemokine-like genes UL146 and UL147 from representatives of each NHP CMV species. B)  
1403 ORF structure of the UL146/147 family genes in each NHP CMV species. Each gene is color-  
1404 coded using the same colors as in A) showing the presence/absence of each ORF in a given NHP  
1405 CMV species.

1406

1407 **Figure S5: The Rh166 gene family of NHP CMVs**

1408 A) Phylogenetic tree based on the protein sequences of Rh166 family genes from representatives  
1409 of each NHP CMV species. B) ORF structure of the Rh166 family genes in each NHP CMV  
1410 species. Each gene is color-coded using the same colors as in A) showing the presence/absence of  
1411 each ORF in a given NHP CMV species.

1412

1413 **Figure S6: The US12 gene family of NHP CMVs**

1414 A) Phylogenetic tree based on the protein sequences of NHP CMV genes homologous to the  
1415 HCMV US12 family encoding seven transmembrane proteins from representatives of each NHP  
1416 CMV species. B) ORF structure of the US12 family genes in each NHP CMV species. Each gene  
1417 is color-coded using the same colors as in A) showing the presence/absence of each ORF in a given  
1418 NHP CMV species.

1419

1420 **Figure S7: The US28 gene family of NHP CMVs**

1421 A) Phylogenetic tree based on the protein sequences of NHP CMV genes homologous to HCMV  
1422 US28 encoding G-protein coupled receptors from representatives of each NHP CMV species. B)

1423 ORF structure of the US28 family genes in each NHP CMV species. Each gene is color-coded  
1424 using the same colors as in A) showing the presence/absence of each ORF in a given NHP CMV  
1425 species.

1426

1427 **Figure S8: Exploratory analysis to assess equivalency across RNA-seq data.**

1428 To account for equivalency across the RNA-seq samples, A) background expression was assessed  
1429 using house-keeping genes ("ACTG1", "RPS18", "MRPL18", "TOMM5", "YTHDF1", "TPT1",  
1430 "RPS27") (98) which did not identify a specific trend across samples. Next, B) host (RM) library  
1431 size was assessed across samples which showed higher overall transcript levels that were tissue  
1432 specific to the salivary glands. However, this difference did not correlate with the viral gene  
1433 expression levels. Finally, C) we found that all three in vitro samples had a higher number of total  
1434 viral reads than tissue biopsies.

1435

1436 **Figure S9: Sequence coverage of FL-RhCMVΔRh13.1/TB6Ag in cultured fibroblasts**

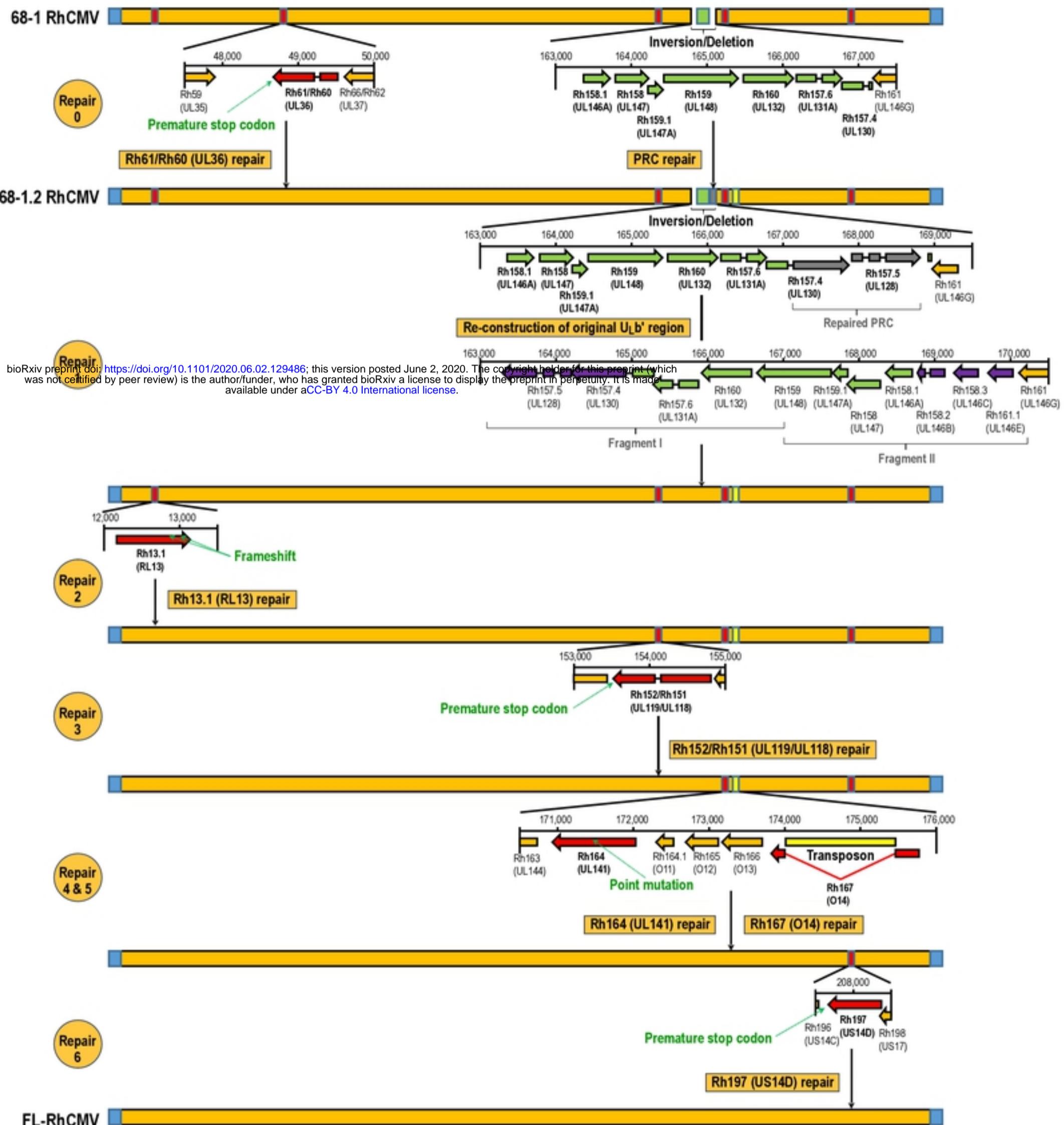
1437 Sequence coverage of FL-RhCMVΔRh13.1/TB6Ag in cultured fibroblasts, infected *in vitro* and  
1438 sampled at three timepoints post-infection (8, 24, 72 hours). Coverage per base is plotted with a  
1439 log10 scale. Colors: blue = 8hpi, magenta = 24hpi, green = 72hpi.

1440

1441 **Table S1: List of all RhCMV ORFs affected by changes made to the previously published**  
1442 **genome annotation using comparative genomics.**

1443

1444 **Table S2: Absolute number of RNAseq reads aligning to annotated RhCMV ORFs for all *in***  
1445 ***vitro* and *in vivo* samples.**



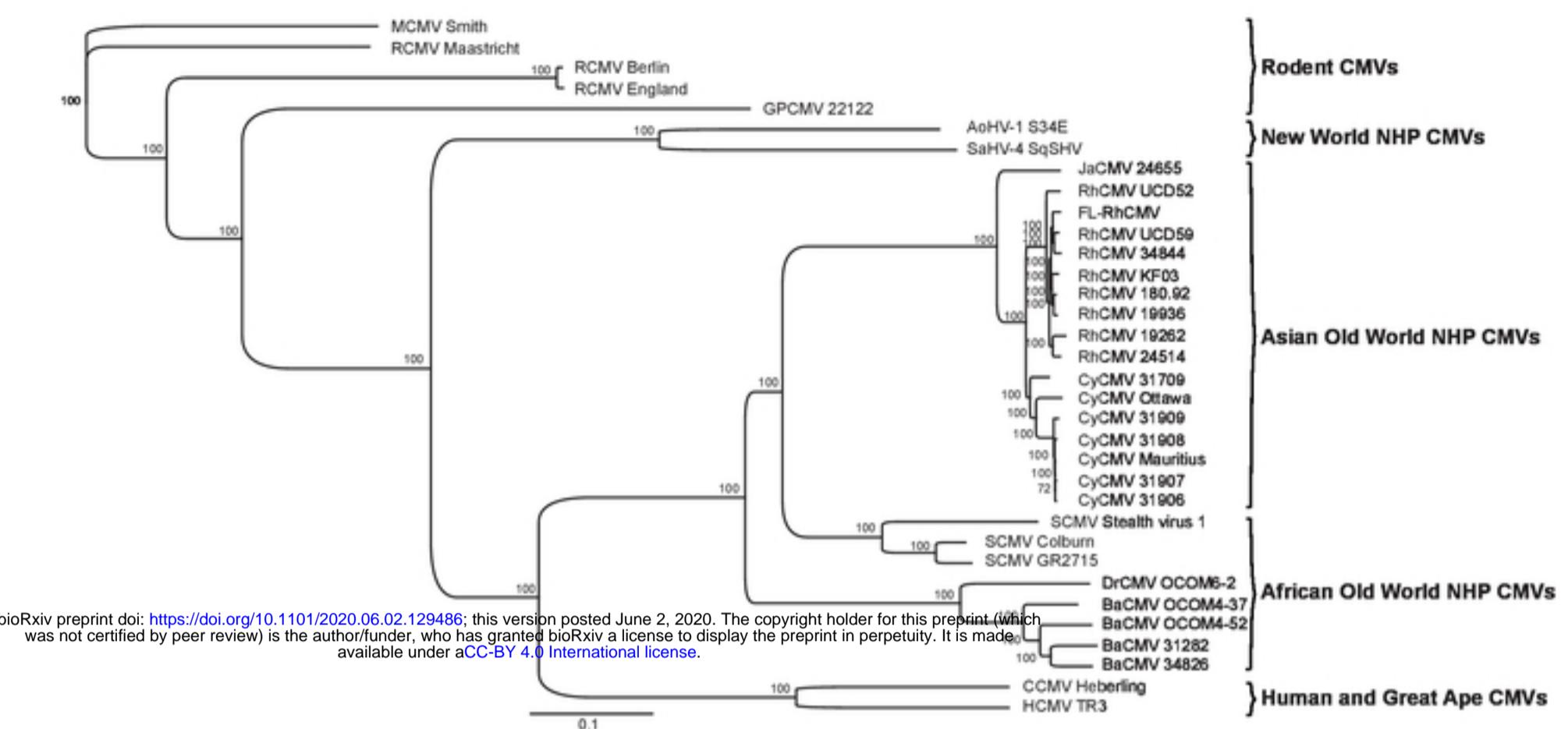
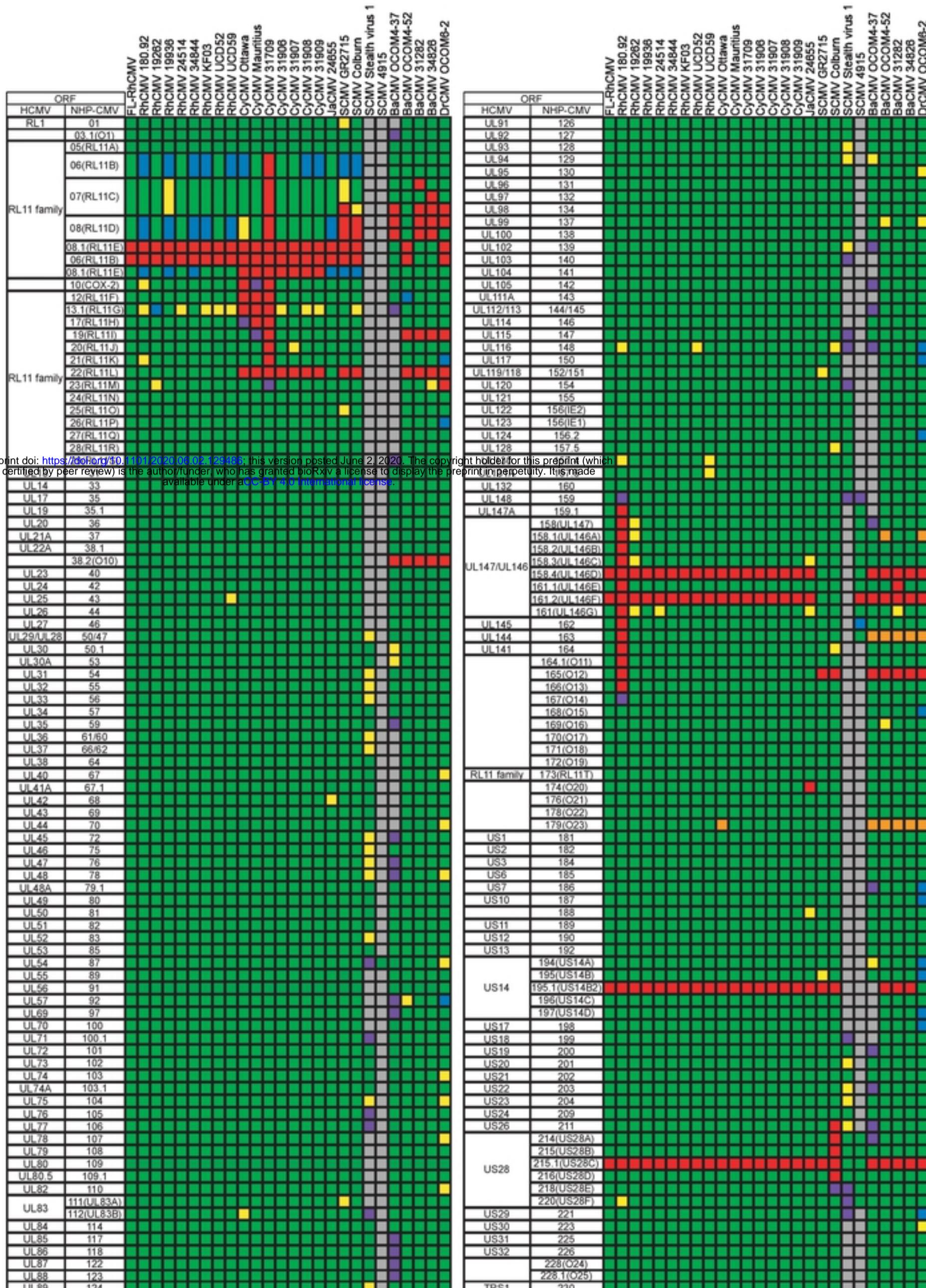


Fig.2



█ ORF conserved      █ ORF absent  
█ ORF shortened compared to consensus      █ ORF elongated compared to consensus  
█ Only partial sequence data available      █ No sequence data available  
█ ORF conserved, but consensus start codon is missing

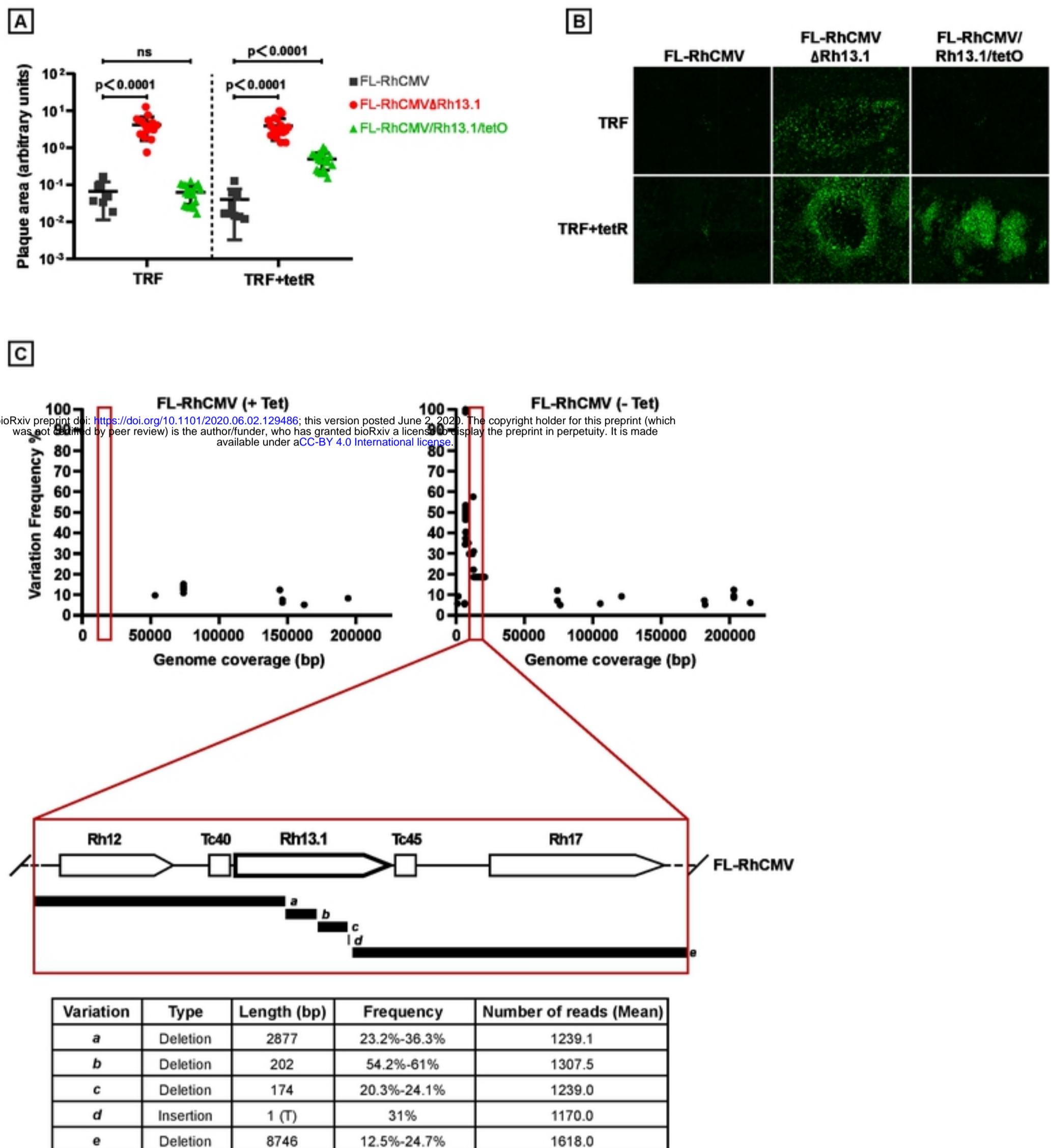
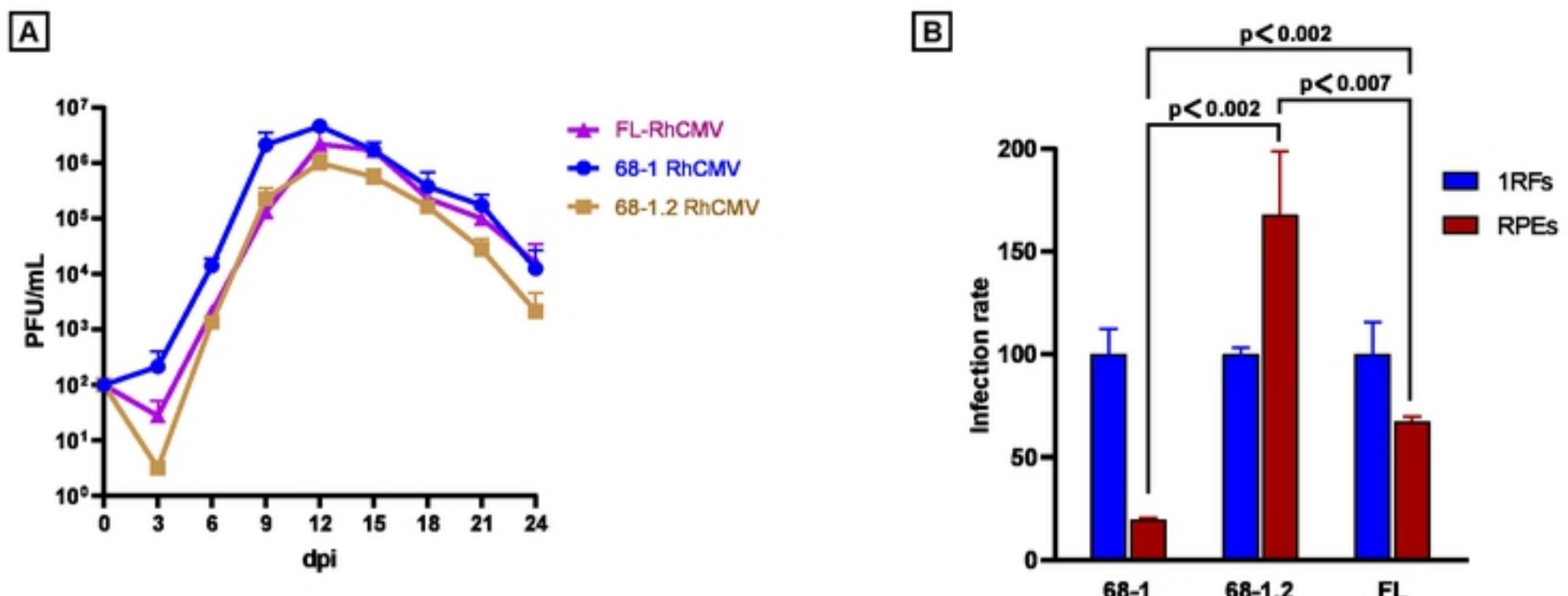
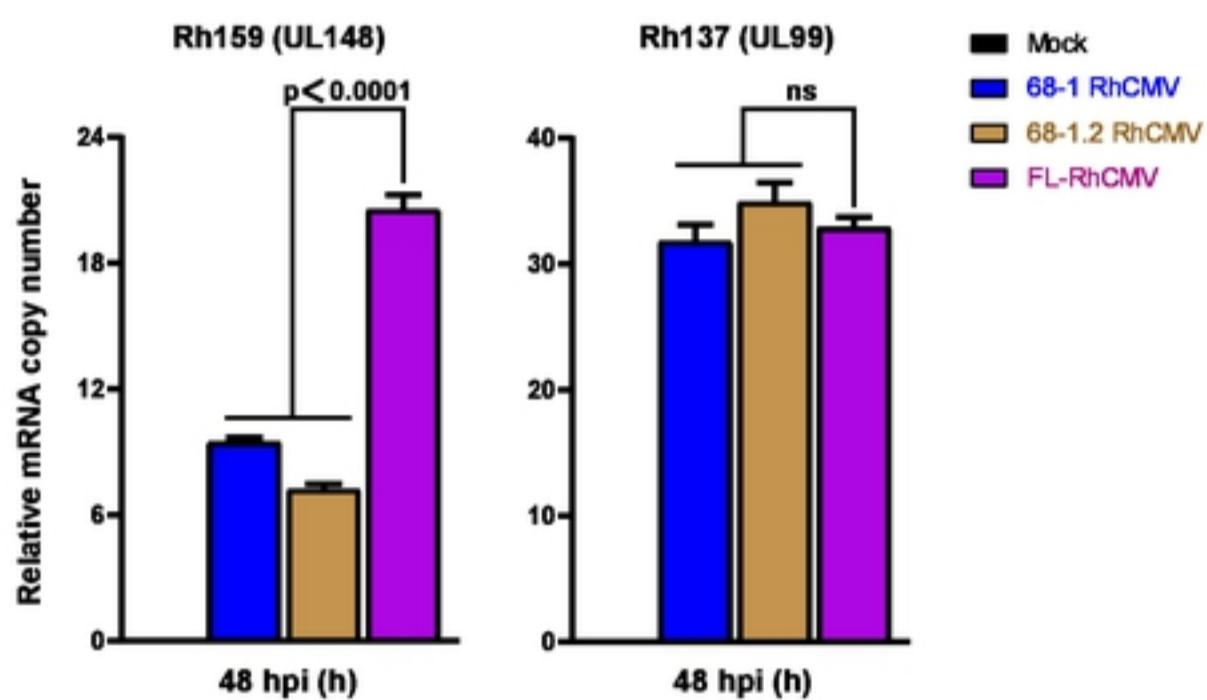
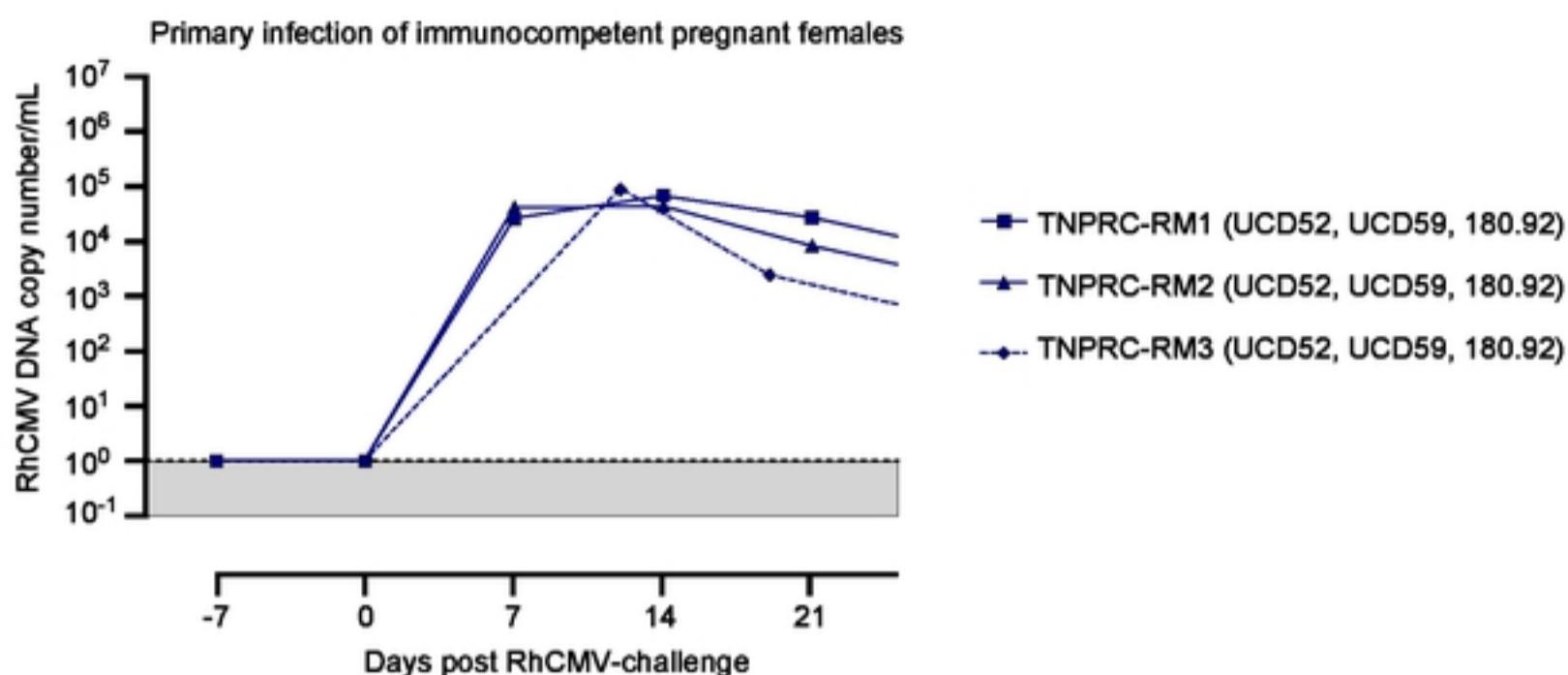
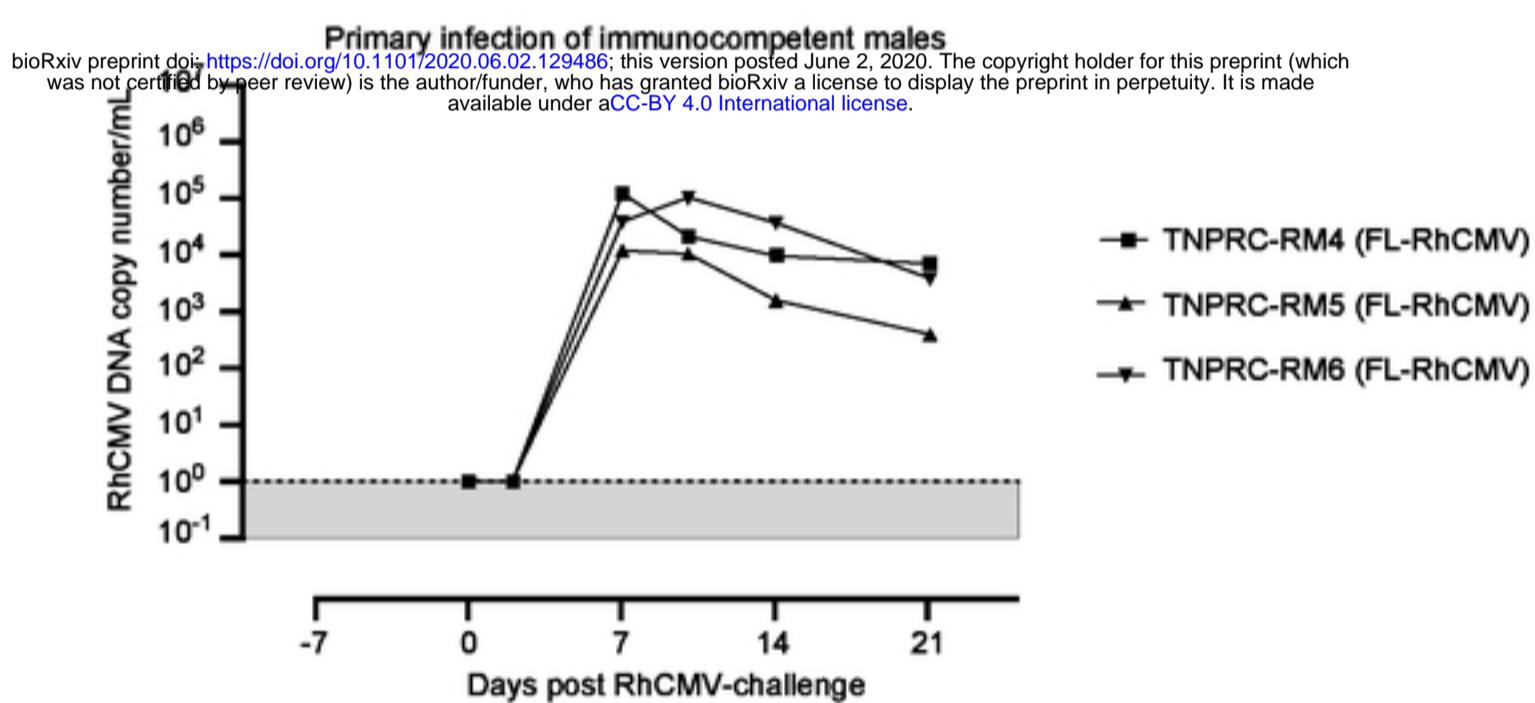
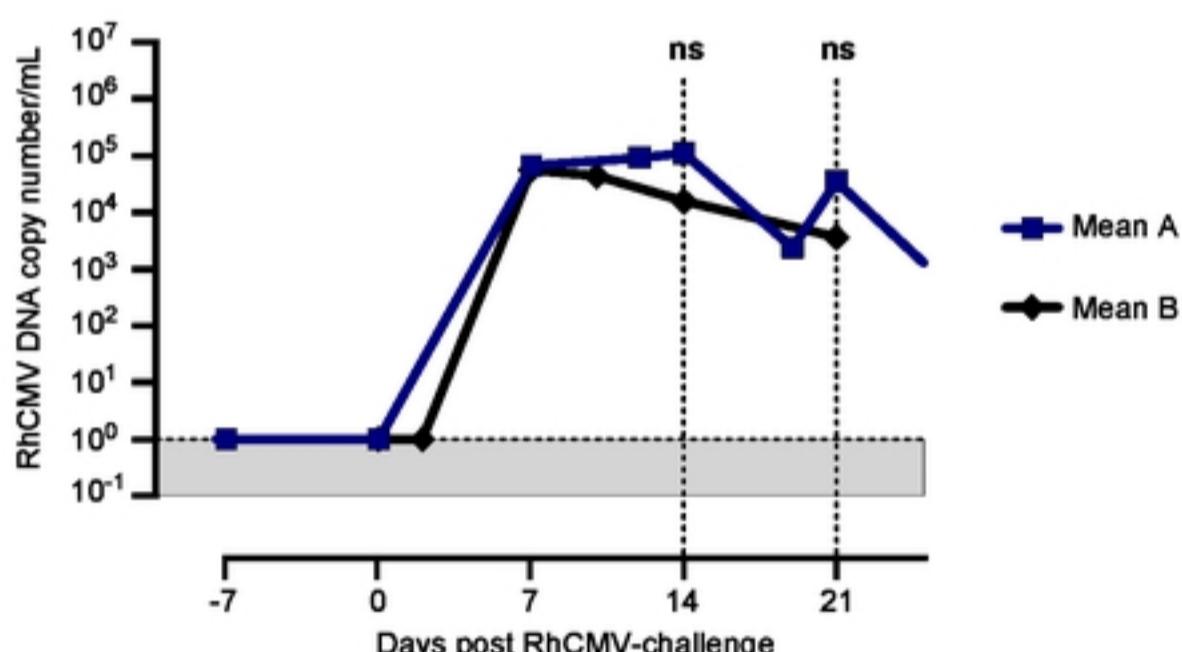


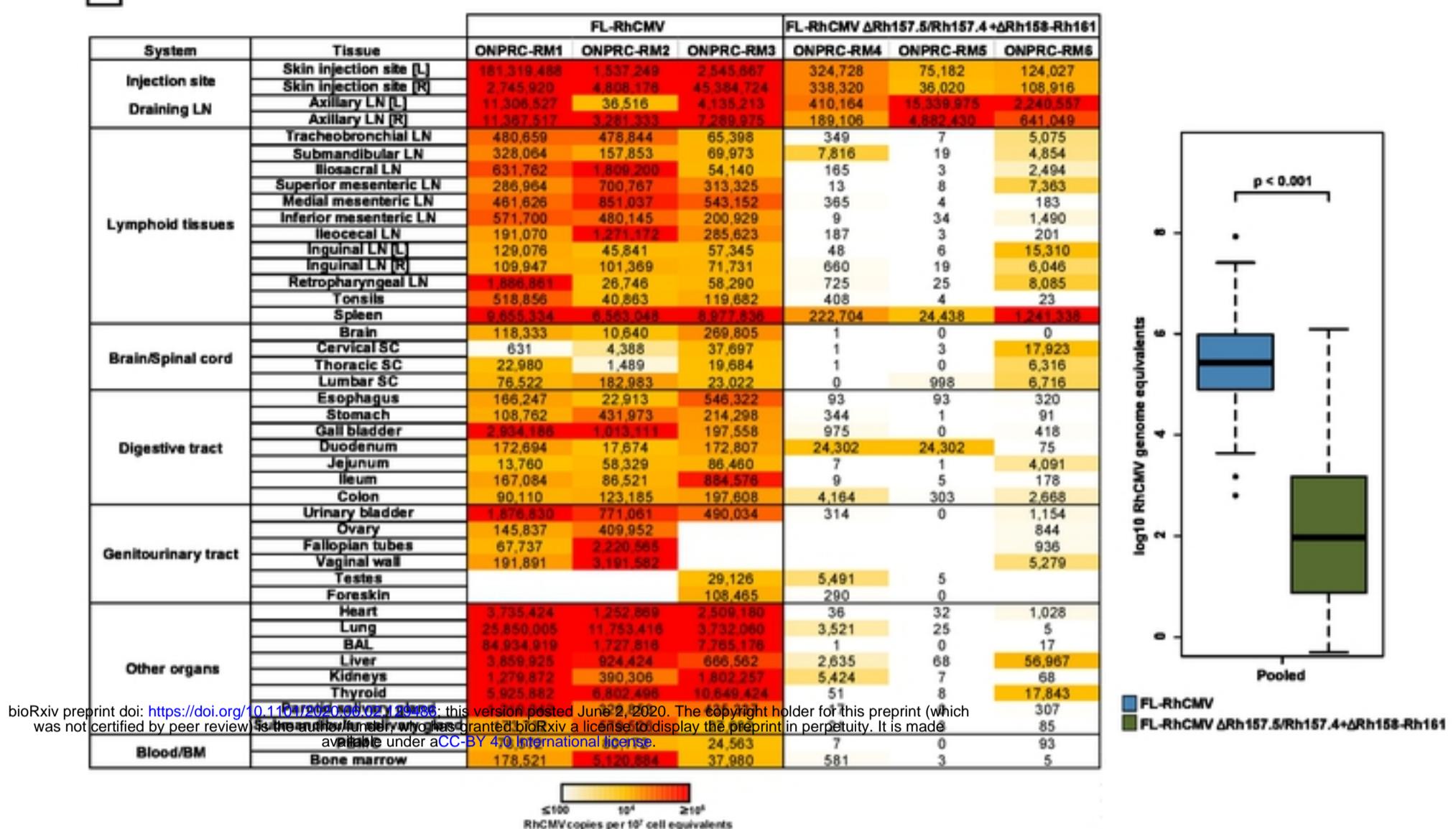
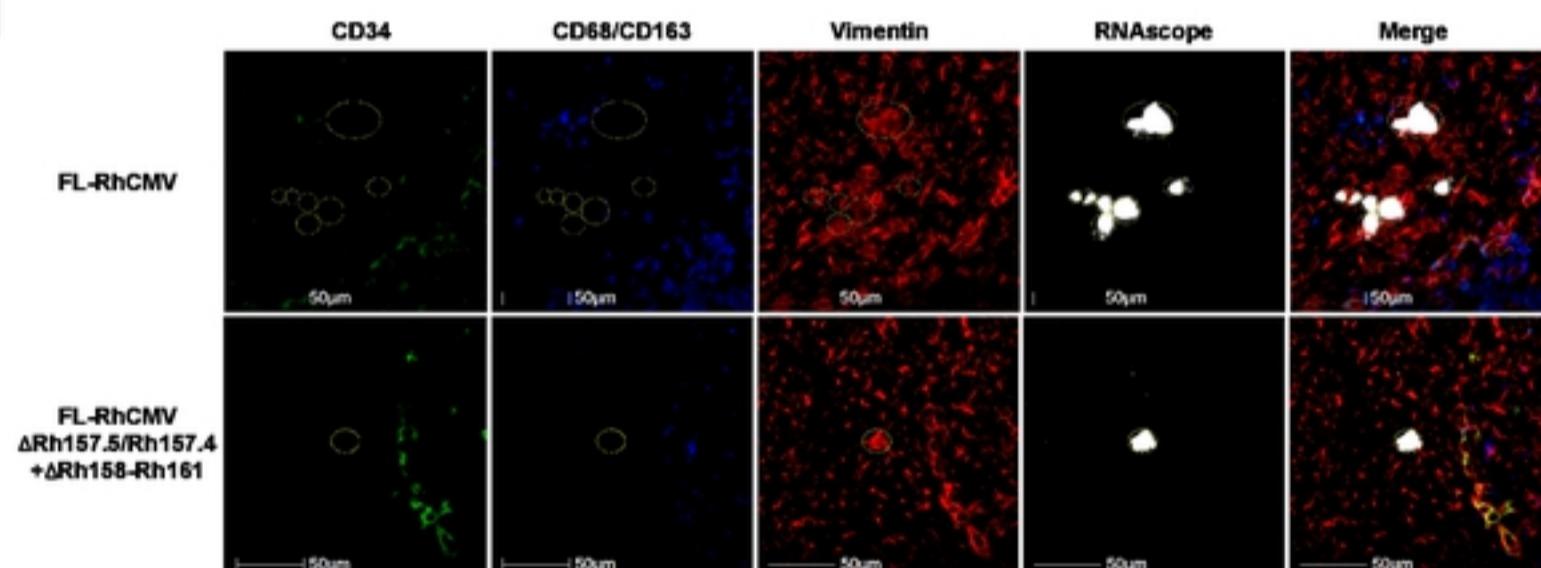
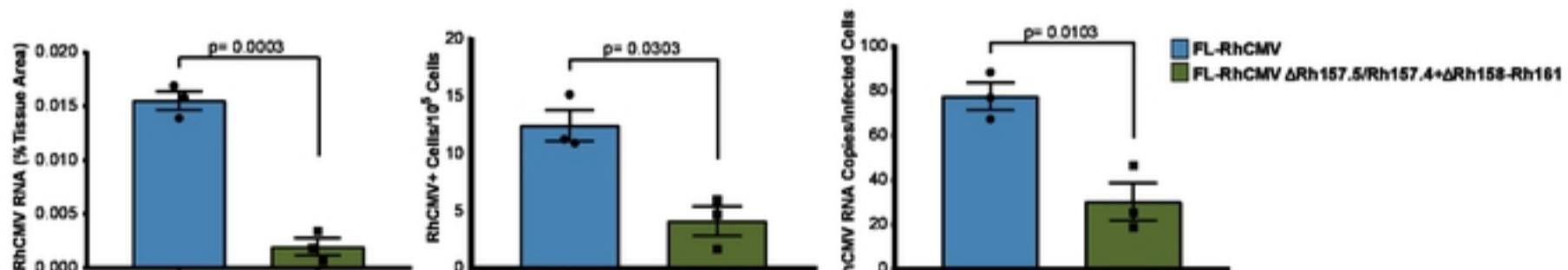
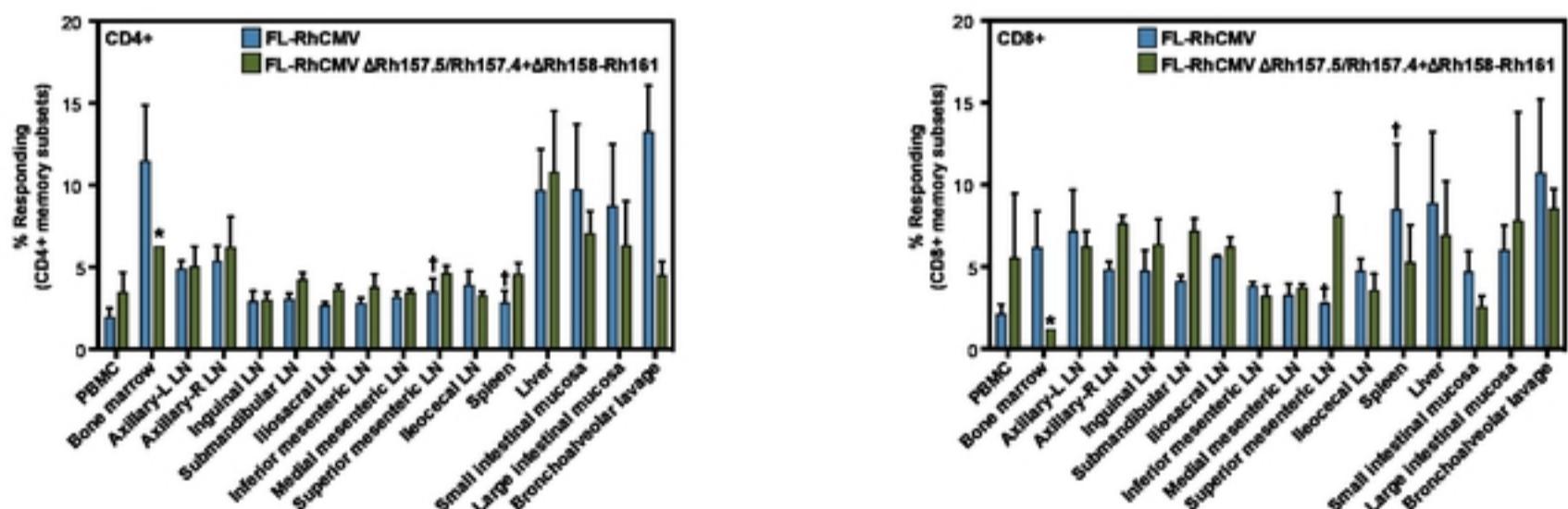
Fig.4

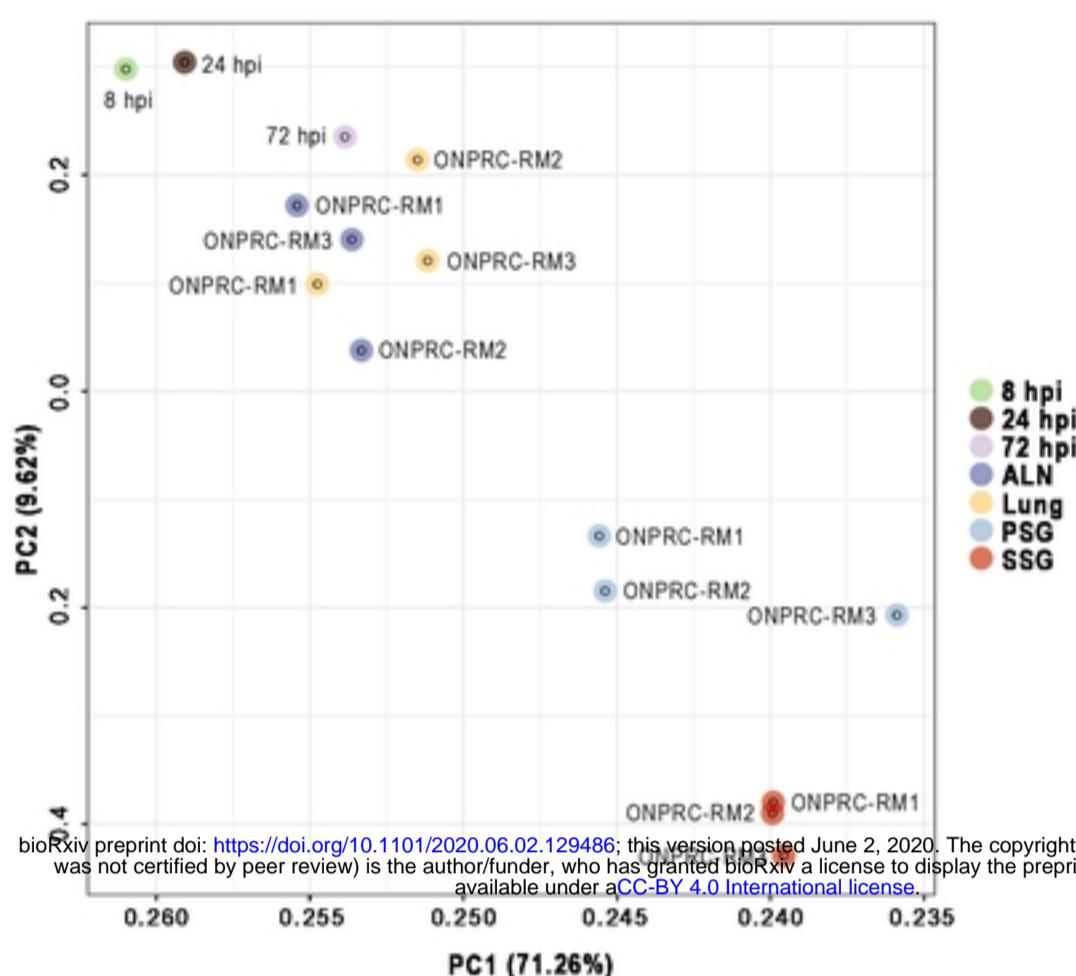
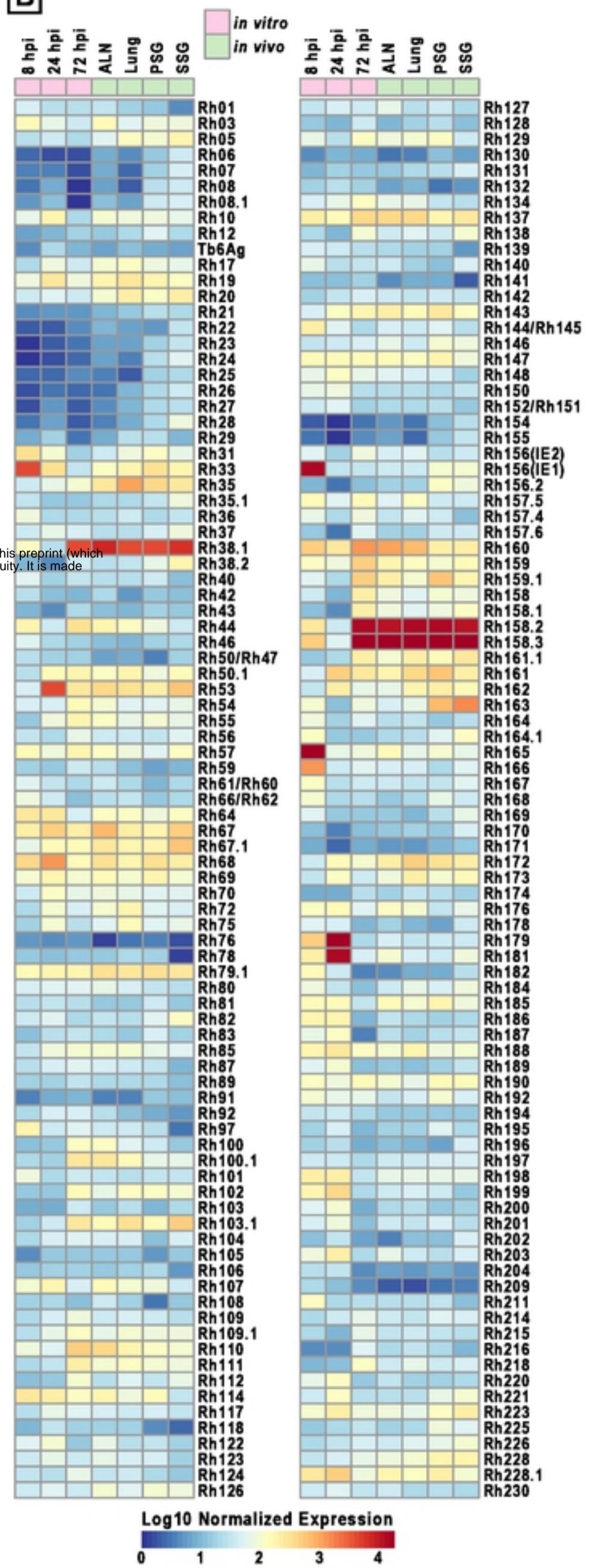


bioRxiv preprint doi: <https://doi.org/10.1101/2020.06.02.129486>; this version posted June 2, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



**A****B****C**

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

**A****B****C**

Rhesus Fibroblasts 8 hpi		Rhesus Fibroblasts 24 hpi		Rhesus Fibroblasts 72 hpi	
Rh165 (O12)	6.28%	Rh179 (O23)	3.14%	Rh158.3 (UL146C)	40.68%
Rh156 (UL123)	4.57%	Rh181 (US1)	2.38%	Rh158.2 (UL146B)	30.64%
Rh33 (UL14)	4.28%	Rh53 (UL30A)	2.11%	Rh38.1 (UL22A)	9.42%
Rh166 (O13)	3.58%	Rh68 (UL42)	2.10%	Rh160 (UL132)	2.60%
Rh179 (O23)	3.41%	Rh161 (UL146G)	1.99%	Rh159 (UL148)	0.88%
Rh160 (UL132)	2.87%	Rh228.1 (O25)	1.92%	Rh110 (UL82)	0.84%
Rh158.3 (UL146C)	2.84%	Rh67 (UL40)	1.90%	Rh159.1 (UL147A)	0.77%
Rh68 (UL42)	2.38%	Rh199 (US18)	1.89%	Rh137 (UL99)	0.75%
Rh31 (UL13)	2.25%	Rh33 (UL14)	1.86%	Rh44 (UL26)	0.49%
Rh228.1 (O25)	2.05%	Rh160 (UL132)	1.75%	Rh100.1 (UL71)	0.46%

Axillary Lymph Node (ALN)		Lung		Parotid Salivary Gland (PSG)		Submandibular Salivary Gland (SSG)	
Rh158.3 (UL146C)	13.93%	Rh158.3 (UL146C)	21.35%	Rh158.3 (UL146C)	16.80%	Rh158.3 (UL146C)	13.96%
Rh158.2 (UL146B)	11.92%	Rh158.2 (UL146B)	17.97%	Rh158.2 (UL146B)	11.45%	Rh158.2 (UL146B)	11.42%
Rh38.1 (UL22A)	5.62%	Rh38.1 (UL22A)	6.62%	Rh38.1 (UL22A)	6.13%	Rh38.1 (UL22A)	7.39%
Rh160 (UL132)	2.48%	Rh35 (UL17)	2.42%	Rh163 (UL144)	2.54%	Rh163 (UL144)	3.45%
Rh67 (UL40)	2.42%	Rh160 (UL132)	1.78%	Rh33 (UL14)	1.85%	Rh33 (UL14)	2.77%
Rh137 (UL99)	2.08%	Rh172 (O19)	1.60%	Rh35 (UL17)	1.67%	Rh67 (UL40)	2.09%
Rh53 (UL30A)	2.02%	Rh161 (UL146G)	1.57%	Rh172 (O19)	1.63%	Rh53 (UL30A)	1.79%
Rh79.1 (UL48A)	1.92%	Rh137 (UL99)	1.35%	Rh161 (UL146G)	1.46%	Rh160 (UL132)	1.67%
Rh110 (UL82)	1.85%	Rh53 (UL30A)	1.22%	Rh79.1 (UL48A)	1.36%	Rh67.1 (UL41A)	1.63%
Rh67.1 (UL41A)	1.44%	Rh19 (RL11)	1.15%	Rh143 (UL111A)	1.30%	Rh33 (UL14)	1.62%

**Fig.8**

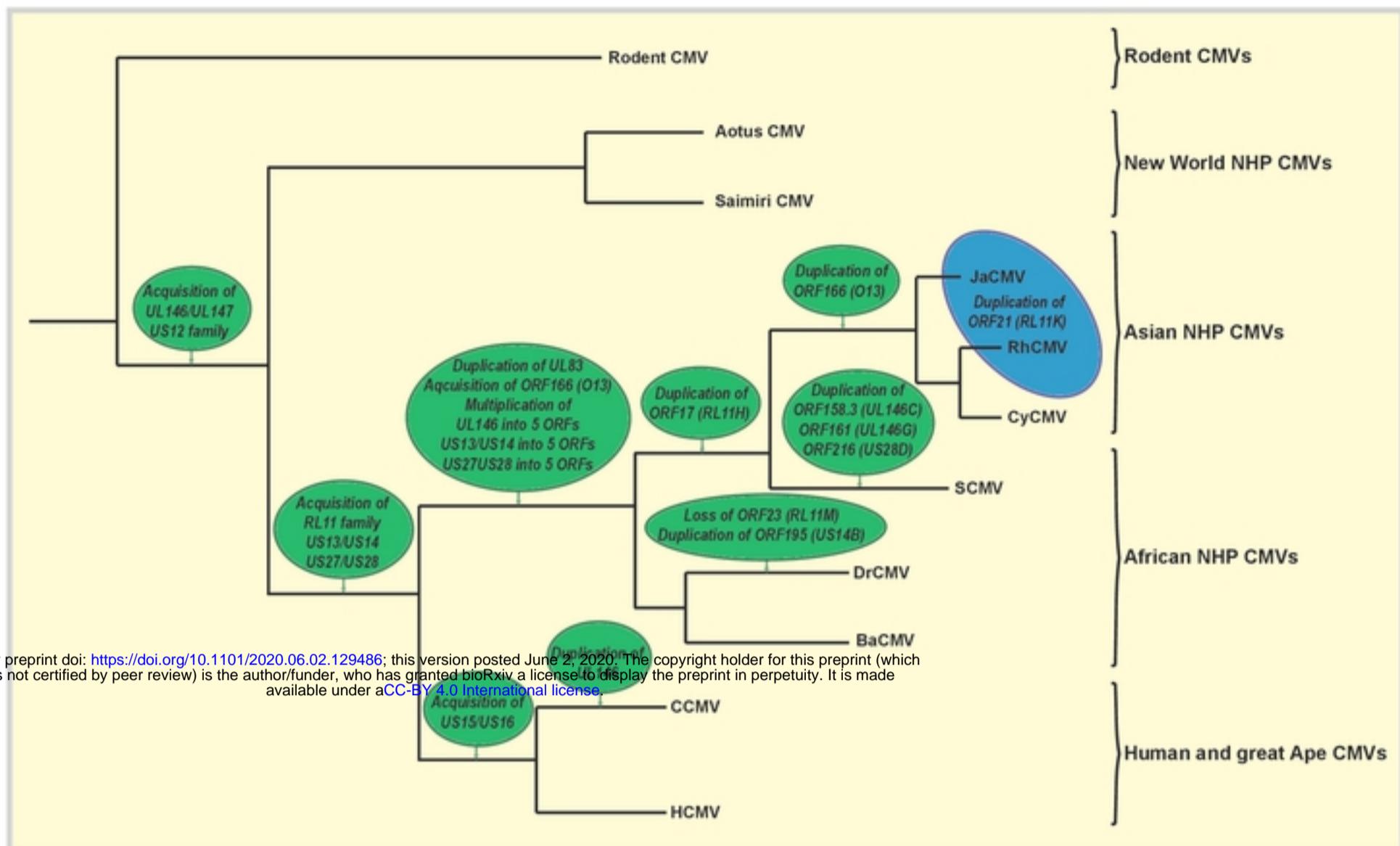


Fig.9