A Conserved Mechanism of APOBEC3 Relocalization by Herpesviral Ribonucleotide Reductase Large Subunits.

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A Conserved Mechanism of APOBEC Counteraction by Herpesviruses Ribonucleotide Reductase Large Subunits

Running Title: APOBEC Counteraction by Herpesvirus RNRs (54 Char w/ space)

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Abstract

An integral part of the antiviral innate immune response is the APOBEC3 family of single-stranded DNA cytosine deaminases, which inhibits virus replication through deamination-dependent and -independent activities. Viruses have evolved mechanisms to counteract these enzymes such as HIV-1 Vif-mediated formation of a ubiquitin ligase to degrade virus-restrictive APOBEC3 enzymes. A new example is Epstein-Barr virus (EBV) ribonucleotide reductase (RNR)-mediated inhibition of cellular APOBEC3B (A3B). The large subunit of the viral RNR, BORF2, causes A3B relocalization from the nucleus to cytoplasmic bodies and thereby protects viral DNA during lytic replication. Here, we use co-immunoprecipitation and immunofluorescent microscopy approaches to ask whether this APOBEC neutralization mechanism is shared with the γ-herpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV) and the α-herpesvirus, herpes simplex virus-1 (HSV-1). The large RNR subunit of KSHV, ORF61, co-precipitated multiple APOBEC3s including A3B and APOBEC3A (A3A). KSHV ORF61 also caused relocalization of these two enzymes to perinuclear bodies (A3B) and to oblong cytoplasmic structures (A3A). The large RNR subunit of HSV-1, ICP6, also co-precipitated A3B and A3A and was alone sufficient to promote the relocalization of these enzymes from nuclear to cytoplasmic compartments. HSV-1 infection caused similar relocalization phenotypes, and this was fully dependent on ICP6. These relocalization phenotypes could be exacerbated by infection with viruses that overexpress ICP6 due to an *ICP4* deletion. These results combine to indicate that both γ- and α-herpesviruses counteract the antiviral activities of cellular APOBEC3 enzymes through a similar RNR-dependent mechanism.

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Importance

The APOBEC3 family of DNA cytosine deaminases constitutes a vital innate immune defense against a range of different viruses. A novel counter-restriction mechanism has recently been uncovered for the γ-herpesvirus EBV, in which a subunit of the viral protein known to produce DNA building blocks (ribonucleotide reductase) causes APOBEC3B to relocalize from the nucleus to the cytosol. Here, we extend these observations to a closely related γ-herpesvirus, KSHV, and to a more distantly related α-herpesvirus, HSV-1. Relocalization was also evident for these ribonucleotide reductases and APOBEC3A, which is 92% identical to APOBEC3B. These studies are important because they suggest a conserved mechanism of APOBEC3 evasion by large double-stranded DNA herpesviruses. Strategies to block this host-pathogen interaction may be effective for treating infections by these and other pathogenic herpesviruses.

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Introduction

There are nine described human herpesviruses (HHV-1 to -8, including HHV6A and 6B), each of which is capable of establishing a lifelong infection [1]. They are among the most prevalent viruses with some such as Epstein-Barr virus (EBV/HHV-4) exceeding 90% seroprevalence in the global population [2]. Primary infection by herpesviruses is often accompanied by mild clinical symptoms and is generally self-limiting before progressing into latency, where the virus persists asymptomatically and is capable of periodic cycles of reactivation [3]. A classic example is herpes simplex virus-1 (HSV-1/HHV-1) infection, which produces primary vesicular lesions in the oral mucosa and establishes latency in adjacent neural ganglia where reactivation of the virus leads to formation of repeated lesions in the same area over the course of the infected individual's life [4-6]. Other herpesviruses such as human cytomegalovirus (HCMV/HHV-5) and Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) establish latency in various myeloid and lymphoid compartments, often without symptoms associated with reactivation except in the cases of immunosuppression such as organ transplantation or untreated HIV-1 infection [7-13].

Overcoming or evading immune surveillance is critical to the survival of human herpesviruses, and these viruses utilize a diverse complement of immunoevasive proteins and RNAs to establish life-long infections [14]. An important arm of the innate immune response lies in the APOBEC family of single-stranded DNA cytosine deaminases [15-17]. Each of the seven APOBEC3 (A3) enzymes, A3A-D and A3F-H, have been implicated in the restriction and hypermutation of a variety of different viruses including lentiviruses (HIV-1, HIV-2, HTLV-1) [18-22], hepadnaviruses (HBV) [23, 24], endogenous retroviruses (LINE-1, Alu) [25, 26], small DNA tumor viruses (HPV, JC/BK-PyV) [27-31], and most recently EBV [32, 33]. It is difficult, if not impossible, to predict *a priori* which subset of APOBEC3 enzymes has the potential to engage a given virus and, furthermore, how that virus might counteract potentially restrictive A3 enzymes.

Each of the three herpesvirus subfamilies (α, β, and γ) encode both large and small RNR subunits with the exception of β-herpesviruses, which lack a small subunit (**Fig 1A**). These RNRs have the canonical function of synthesizing deoxyribonucleotides by reducing the 2'-hydroxyl from ribonucleotide substrates [34]. While they are essential for all cellular life, the requirement for endogenous viral RNRs differs tremendously across viral families. For example, RNRs are almost ubiquitous among large double-stranded DNA (dsDNA) viruses, such as herpesviruses, poxviruses, and tailed phages (caudovirales), presumably due to high dNTP requirements during DNA replication [35-37]. On the other hand, most small dsDNA viruses and single-stranded DNA viruses do not encode RNRs and instead rely on host-encoded RNRs for deoxyribonucleotide production [38, 39]. In addition to ribonucleotide reductase activity, some viral RNRs have been shown to engage in non-catalytic activities that result in

101   proviral phenotypes. For instance, the HSV-1 and HSV-2 large ribonucleotide reductase subunits, ICP6

102   and ICP10, respectively, have unique N-terminal extensions that block caspase-8 activity to inhibit

103   apoptosis and bind RIP3 to promote necroptosis [40-43] (**Fig 1B**). Interestingly, HCMV UL45 also has

104   anti-apoptotic and pro-necroptotic functions despite lacking a viral small subunit counterpart, suggesting

105   that the primary function of this RNR large subunit is no longer ribonucleotide reduction [43-45].

106   Recently, we identified the ribonucleotide reductase (RNR) large subunit of the γ-herpesvirus

107   EBV as novel inhibitor of APOBEC3B (A3B) [32]. We found that EBV BORF2 functions by directly

108   binding A3B and relocalizing it from the nucleus to the cytoplasmic compartment, which prevents

109   A3B-mediated deamination of cytosines to uracils in viral genomic DNA during lytic replication. In the

110   absence of BORF2, we found that A3B was able to mutate EBV genomes and reduce viral titers and

111   infectivity. We also reported that the homologous protein from KSHV, ORF61, is similarly capable of

112   A3B co-immunoprecipitation and relocalization [32]. Here, we ask whether this novel virus protection

113   mechanism is specific or more general-acting by assessing interactions between γ-herpesvirus

114   BORF2/ORF61 and other human APOBEC3 enzymes and by determining whether the more distantly

115   related α-herpesvirus HSV-1 has a similar A3 neutralization mechanism. We found that, in addition to

116   binding and relocalizing A3B, both BORF2 and ORF61 were also capable of co-immunoprecipitation and

117   relocalization of A3A. More importantly, we found that the HSV-1 RNR large subunit ICP6 similarly

118   binds and relocalizes both A3B and A3A. Overexpression studies showed that ICP6 alone is sufficient for

119   A3B and A3A relocalization. Infection studies with mutants demonstrated that ICP6 is essential and that

120   no other viral protein is capable of this function. These data combined to indicate that pathogenic γ and α-

121   herpesviruses have evolved an effective APOBEC3 counteraction mechanism, which is governed by the

122   viral RNR large subunit.

123

124   **Results**

125   **EBV BORF2 and KSHV ORF61 bind and relocalize both A3B and A3A**

126   Our prior co-immunoprecipitation (co-IP) experiments indicated that EBV BORF2 interacts

127   strongly with A3B and weakly with A3A and A3F (see Fig. 1c in Cheng *et al*. [32]). EBV BORF2 was

128   both necessary and sufficient to relocalize A3B in a variety of different cell types including endogenous

129   A3B in the AGS gastric carcinoma cell line and the M81 B cell line [32]. However, our original studies

130   did not address whether EBV BORF2 could functionally interact with and relocalize any of these related

131   human A3 enzymes. We therefore performed immunofluorescent (IF) microscopy studies of U2OS cells

132   overexpressing A3-mCherry constructs with either empty vector or BORF2-FLAG. As reported, A3B is

133   nuclear, A3A has a cell-wide localization, A3H is cytoplasmic and nucleolar, and the other A3s are

134   cytoplasmic [46-50]. Also as expected, BORF2 caused a robust and complete relocalization of nuclear

A3B to perinuclear aggregates (**Fig 2**). Interestingly, BORF2 co-expression with A3A led to the presence of novel linear elongated structures concomitant with normal A3A localization. The localization patterns of the other five A3s were unchanged by BORF2 co-expression. Small BORF2 punctate structures were also noted in all conditions including the mCherry control, which is likely due to transfected BORF2 interacting with endogenous A3B (previously shown to be elevated in U2OS [32]). Similar A3B and A3A relocalization patterns were evident in Vero cells except that A3A relocalization became whole-cell without elongated structures (**Supplementary Fig 1**).

Like EBV BORF2, KSHV ORF61 was also shown to co-IP and relocalize A3B [32]. However, our original studies did not examine the specificity of this interaction by comparing with related human A3 enzymes. We therefore used co-IP experiments to evaluate KSHV ORF61 interactions with a full panel of human A3 enzymes. ORF61-FLAG was co-expressed with A3-HA family members in 293T cells, subjected to anti-FLAG affinity purification, and analyzed by immunoblotting (**Fig 3A**). The ORF61-FLAG pulldown resulted in A3B recovery as described [32]. However, the ORF61-FLAG IP also yielded a robust interaction with A3A and weaker interactions with A3D and A3F.

These KSHV ORF61-A3 interactions were then evaluated by IF microscopy experiments to look for changes in A3 localization in U2OS and Vero cells (**Fig 3B**). As expected [32], KSHV ORF61 caused A3B to relocalize to perinuclear aggregates. Moreover, as above for BORF2 and A3A, ORF61 co-expression caused a portion of the cellular A3A to localize to intense elongated linear structures in the cytosolic compartment (**Fig 3B**). No other A3 proteins showed altered subcellular localization in these experiments. These new results with EBV BORF2 and KSHV ORF61 combined to indicate that both A3B and A3A may be cellular targets of viral RNR-mediated neutralization.

HSV-1 ICP6 binds and relocalizes A3B and A3A

To test whether RNR-mediated APOBEC antagonism is a more broadly conserved mechanism, a series of co-IP experiments was done with the large RNR subunit of HSV-1, ICP6. FLAG-ICP6 was co-expressed with each of the seven different HA-tagged human A3s in 293T cells and subjected to anti-FLAG IP as above. The EBV BORF2-A3B interaction was used as a positive control and BORF2-A3G as a negative control to be able to compare the relative strengths of pulldowns between RNRs and A3s. HSV-1 ICP6 showed a strong interaction with A3A and weaker, but detectable, interactions with A3B, A3C, and A3D (**Fig 4A**).

Next, IF microscopy was used to assess functional interactions between HSV-1 ICP6 and each of the human A3 enzymes. Human U2OS osteosarcoma cells were co-transfected with mCherry-tagged A3s and either empty vector or FLAG-tagged HSV-1 ICP6 and analyzed by IF after 48 hours (**Fig 4B**). On its own EBV BORF2 shows a cytoplasmic distribution and, as shown previously [32], it was able to

169　completely relocalize A3B from the nucleus to cytoplasm. In comparison, HSV-1 FLAG-ICP6 showed a

170　broadly cytoplasmic localization that did not change significantly with co-expression of any A3.

171　However, co-expression of FLAG-ICP6 and A3B-mCherry or A3A-mCherry led to a near complete

172　relocalization of these DNA deaminases from the nucleus to the cytoplasm. HSV-1 ICP6 co-expression

173　with the other A3s did not lead to any remarkable change in localization. These results suggested that,

174　although HSV-1 ICP6 interacted by co-IP with several A3s to varying degrees, functionally relevant

175　interactions may only be occurring with A3B and A3A.

176

177　HSV-1 infection relocalizes A3B and A3A

178　　　　To address whether HSV-1 infection similarly promotes relocalization of A3B and A3A, U2OS

179　cells were transfected with A3-mCherry constructs 48 hours prior to either mock or HSV-1 infection. We

180　used K26GFP, a HSV-1 strain that has a GFP moiety fused to capsid protein VP26 to allow for

181　identification of infected cells [51]. Cells were analyzed by IF 8 hours post-infection (hpi) (**Fig 5**). Similar

182　to the ICP6 overexpression experiments described above, HSV-1 infection caused A3A to relocalize to

183　the cytoplasmic compartment and A3B to change from a predominantly nuclear localization to a more

184　cell-wide distribution. A3C also changed from a predominantly cytoplasmic localization to a more diffuse

185　whole cell distribution, whereas A3D, A3F, A3G, and A3H were unchanged by HSV-1 infection. Similar

186　relocalization patterns were found in HeLa cells following HSV-1 K26GFP infection (**Supplementary**

187　**Fig 2**). Moreover, time-course experiments showed that relocalization of A3A was detectable as early as

188　3 hpi, whereas A3B and A3C relocalization became apparent by 6 or 9 hpi (**Supplementary Fig 3**).

189　These kinetic differences may reflect a differential affinity of the viral protein(s) to bind to these cellular

190　A3 enzymes and/or competitions with cellular interactors. Quantification of the mean fluorescence

191　intensity in nuclear and cytoplasmic compartments showed that the nuclear localization of A3A and A3B

192　was significantly decreased upon HSV-1 infection, but A3G localization remained unaltered

193　(**Supplementary Fig 4**).

194

195　HSV-1-mediated relocalization of A3B and A3A requires ICP6

196　　　　To investigate whether the HSV-1 large RNR subunit is necessary for A3A/B relocalization, we

197　next examined A3 localization in cells following infection with an HSV-1 KOS1.1 strain lacking ICP6

198　due to a deletion of the *UL39* gene (*UL39* encodes ICP6) [52]. Vero cells were transfected with

199　A3-mCherry constructs 48 hours prior to mock infection or infection with KOS1.1 or KOS1.1ΔICP6.

200　After 8 hours, cells were fixed, permeabilized, and subjected to IF analysis by staining for the HSV-1

201　immediate early protein ICP27 to mark infected cells, and monitoring A3 localization through mCherry

202　fluorescence. As above, HSV-1 infection caused the relocalization of A3A, A3B, and A3C (**Fig 6A**).

However, only the relocalization A3A and A3B was ICP6-dependent, whereas A3C redistributed regardless of the presence of ICP6. These results provide strong support for mechanistic conservation of the RNR large subunit-A3 interaction (A3A and A3B) and also indicated that A3C relocalization by HSV-1 is mechanistically distinct.

To further investigate the role of ICP6 in mediating A3A and A3B relocalization, U2OS cells were infected with an HSV-1 KOS mutant with a deletion in the *ICP4* gene [53]. ICP4, an immediate early protein, is the major transcriptional activator protein of HSV-1 [53]. *ICP4*-null mutants exhibit a strict block to expression of nearly all viral delayed-early and late genes, but are competent to express the viral immediate-early genes (*ICP0*, *ICP22*, *UL54*, and *US12*) as well the *UL39* gene, a delayed-early gene that is uniquely transactivated by ICP0 [54]. In fact, at intermediate and late times post-infection, *ICP4*-null mutants express abnormally high levels of both the immediate early proteins and ICP6 [53]. Similar to what was seen for wild-type HSV-1 infection, infection with the HSV-1 KOSΔICP4 mutant also led to A3A and A3B relocalization, but with noticeably more pronounced phenotypes (**Fig 6B**). For instance, this mutant virus caused A3B-mCherry to form perinuclear aggregates reminiscent of previously observed BORF2-A3B bodies [32] (**Fig 6B**). Interestingly, A3C localization remained predominantly nuclear upon HSV-1 KOSΔICP4 infection, suggesting that one of the other four immediate early proteins besides ICP4 induces its relocalization. Taken together, these data show that HSV-1 ICP6 is both necessary and sufficient for the relocalization of A3A and A3B, and that at least one other viral factor is responsible for A3C relocalization. Identification of this factor will be the subject of a future investigation.

Discussion

We previously described a novel mechanism of A3B counteraction by γ-herpesvirus RNR large subunits, EBV BORF2 and KSHV ORF61 [32]. These viral proteins interact directly with A3B, relocalize it from the nuclear to the cytoplasmic compartment, and protect lytically replicating viral genomes from A3B-mediated deamination and hypermutation. Here, we investigated the question of specificity by comparing interactions with the full repertoire of seven different human A3 enzymes, and we also addressed the potential for broader conservation by asking whether the α-herpesvirus HSV-1 has a similar APOBEC3 counterdefense mechanism. Although EBV BORF2 and KSHV ORF61 were able to interact with several different A3 proteins in co-IP experiments, these viral RNR large subunits only promoted the relocalization of A3B and A3A. HSV-1 ICP6 showed a similar range of co-IP interactions and also only promoted the relocalization of A3B and A3A. Wild-type but not ICP6 deletion mutant HSV-1 infections yielded similar A3B and A3A relocalization phenotypes. These studies combine to indicate that human γ- and α-herpesviruses possess a conserved A3B/A counterdefense .

237   The question of whether A3B, A3A, or both enzymes are most relevant to herpesvirus
238   pathogenesis is likely to vary among herpesvirus type and depend, at least in part, on the complex
239   interplay between viral tropism(s) and alternating modes of latent versus lytic replication. For EBV,
240   epithelial cells serve as the source of primary infection which are mandatory for establishing lytic
241   replication cycles for person-to-person spread and enabling secondary infection of B lymphocytes for
242   establishment of long-term latency [55]. B cells also support lytic reactivation for reinfection and
243   maintenance of EBV in the blood [56]. Here, A3B may be more important than A3A simply because its
244   expression is well-documented in these cell types [57, 58]. Likewise, KSHV infects epithelial and B cells,
245   but also engages in infection of clinically relevant endothelial cells which can lead to Kaposi's sarcoma
246   [59]. Additionally, monocytes are likely to be a secondary reservoir of latent infection in addition to the B
247   cell reservoir [60-62]. It would therefore not be surprising that KSHV requires the capacity to relocalize
248   both A3B and A3A (A3B neutralization for replication in B cells and A3A neutralization for replication
249   in monocytes/macrophages, where A3A can be expressed at extremely high levels) [57, 63, 64]. For
250   HSV-1, although neither A3B nor A3A expression has been reported in neural/CNS cells, lytic replication
251   in epithelial cells may require functional neutralization of A3B and/or A3A [65, 66]. Of course, dedicated
252   functional studies in the most disease relevant *in vivo* systems will be required to fully address the
253   question of whether A3B, A3A, or both enzymes are most relevant to herpesvirus pathogenesis.

254   Additional studies will also be required to determine if the β-herpesviruses are also able to
255   counteract the activities of APOBEC3 enzymes. Preliminary studies using the HCMV homologue, UL45,
256   showed no detection of interaction with A3B or A3A by co-IP (data not shown), although this alone does
257   not rule-out the absence of an A3 counteraction mechanism. The fact that all herpesviruses analyzed thus
258   far have an ability to relocalize A3B and A3A strongly suggests that these enzymes pose a significant
259   threat to the genetic integrity of these pathogens. Indeed, our prior studies showed that A3B causes
260   declines in both EBV viral titers and infectivity for viruses lacking BORF2. Given the strong mechanistic
261   conservation demonstrated here, we anticipate even larger virus replication phenotypes for RNR mutants
262   in relevant *in vivo* systems. However, such studies will require a structural understanding of the A3-RNR
263   interactions such that separation-of-function mutants can be engineered to dissociate phenotypes due to
264   A3 enzymes and those due to RNR activity as well as other important alternative functions.

265

266   **Materials and Methods**

267   **Generation of herpesvirus phylogenetic tree**. Amino acid sequences for herpesvirus ribonucleotide
268   reductase large subunits were obtained from NCBI Protein RefSeq with the following GenBank accession
269   numbers: HSV-1 ICP6 YP_009137114.1, HSV-2 ICP10 YP_009137191.1, VZV ORF19 NP_040142.1,
270   EBV BORF2 YP_401655.1, HCMV UL45 YP_081503.1, HHV6A U28 NP_042921.1, HHV6B U28

271   NP_050209.1, HHV7 U28 YP_073768.1, KSHV ORF61 YP_001129418.1. Alignment was generated

272   using MUSCLE: multiple sequence alignment with high accuracy and high throughput [67] and

273   phylogenetic tree was made using a neighbor-joining tree without distance corrections. Output was made

274   using FigTree using scaled branches [68].

275   **DNA constructs for expression in human cell lines.** The full set of pcDNA3.1(+) human APOBEC-HA

276   expression constructs has been described [69] [A3A (GenBank accession NM_145699), A3B

277   (NM_004900), A3C (NM_014508), A3D (NM_152426), A3F (NM_145298), A3G (NM021822), A3H

278   (haplotype II; FJ376615)]. The full set of APOBEC-mCherry expression constructs was PCR amplified

279   with Phusion High Fidelity DNA Polymerase (NEB M0530) from previously described A3-mCherry

280   constructs [46] and subcloned into pcDNA5/TO (Invitrogen V103320). The forward PCR primers are as

281   follows: A3A (5'-NNN NAA GCT TAC CAC CAT GGA AGC C-3'), A3B and A3C (5'-NNN NNA

282   AGC TTA CCA CCA TGA ATC CA-3'), A3D (5'-NNN NNA AGC TTA CCA CCA TGA ATC CA-3'),

283   A3F (5'-NNN NNA AGC TTA CCA CCA TGA AGC CT-3'), A3G (5'-NNN NAA GCT TAC CAC

284   CAT GAA GCC T-3'), and A3H (5'-NNN NAA GCT TAC CAC CAT GGC TCT G-3'). The reverse

285   PCR primer used was 5'-AGA GTC GCG GCC GCT TAC TTG TAC A-3'. PCR fragments were

286   digested with *Hind*III-HF (NEB R3104) and *Not*I-HF (NEB R3189) and ligated into pcDNA5/TO. The

287   full set of pLenti-iA3i-HA constructs were previously described except the puromycin resistance gene

288   was replaced with a hygromycin resistance gene [70]. Briefly, this is a lentiviral construct with an intron

289   spanning the A3 gene with a C-terminal 3xHA tag, arranged in the antisense direction, which is expressed

290   after reverse transcription and integration. This construct bypasses limitation of self-restriction by

291   A3-mediated deamination of its own plasmid.

292   EBV BORF2 (GenBank accession V01555.2) with a C-terminal 3x-FLAG (DYKDDDDK) tag

293   and EBV BaRF1 (Genbank accession V01555.2) with a C-terminal 3x-HA (YPYDVPDYA) tag was

294   previously described [32]. Other viral RNRs were subcloned with Phusion High Fidelity DNA

295   Polymerase from previously described pCMV-3F vectors [32].

296   KSHV ORF61 (GenBank accession U75698.1) was PCR amplified using primers 5'-NNN NGA

297   ATT CGC CAC CAT GTC TGT CCG GAC ATT TTG T-3' and 5'-NNN NGA ATT CGC CAC CAT

298   GTC TGT CCG GAC ATT TTG T-3', digested with *Eco*RI-HF (NEB R3101S) and *Not*I-HF, and ligated

299   into pcDNA4 with a C-terminal 3x- FLAG. The same construct was PCR amplified using primers

300   5'-NNN NGC GGC CGC GTC TGT CCG GAC ATT TTG T-3' and 5'-NNN NTC TAG ATT ACT GAC

301   AGA CCA GGC ACT C-3', digested with *Not*I-HF and *Xba*I, and ligated into a similar pcDNA4 vector

302   with N-terminal 3x- FLAG.

303   HSV-1 UL39 (GenBank accession JN555585.1) was PCR amplified using primers 5'-NNN NGA

304   TAT CCG CCA CCA TGG CCA GCC GCC CAG CC-3' and 5'-NNN NGC GGC CGC CCC AGC GCG

CAG CT-3', digested with *Eco*RV-HF (NEB R1395) and *Not*I-HF, and ligated into pcDNA4 (Invitrogen V102020) with a C-terminal 3x-FLAG [71]. The same construct was PCR amplified using primers 5'-NNN NGC GGC CGC GGC CAG CCG CCC AGC CGC A-3' and 5'-NNN NTC TAG ATT ACA GCG CGC AGC TCG TGC A-3', digested with *Not*I-HF and *Xba*I (NEB R0145S), and ligated into a similar pcDNA4 vector with N-terminal 3x-FLAG.

Human cell culture. Unless indicated, cell lines were derived from established lab collections. All cell cultures were supplemented with 10% heat-inactivated fetal bovine serum (Gibco 16140-063), 1x Pen-Strep (Thermo Fisher 15140122), and periodically tested for mycoplasma (Lonza MycoAlert PLUS LT07-710). No cell lines have ever been mycoplasma positive or previously treated. 293T and Vero cells were cultured in high glucose DMEM (Hyclone), U2OS cells were cultured in McCoy's 5A media (Hyclone), and HeLa cells were cultured in RPMI 1640 (Corning).

Co-immunoprecipitation experiments and immunoblots. Semi-confluent 293T cells were grown in 6-well plates and transfected with plasmids and 0.6 µL TransIT-LT1 (Mirus 2304) per 100 ng DNA in 100 µL serum-free Opti-MEM (Thermo Fisher 31985062). A titration series was performed to achieve roughly equivalent protein expression by immunoblot for the A3 panel and RNR homologue co-IP experiments. Growth medium was removed after 48 hrs and whole cells were harvested in 1 mL PBS-EDTA by pipetting. Cells were spun down, PBS-EDTA was removed, and cells were resuspended in 300 µL of ice-cold lysis buffer [150 mM NaCl, 50mM Tris-HCl, 10% glycerol, 1% IGEPAL (Sigma I8896), Roche cOmplete EDTA-free protease inhibitor cocktail tablet (Roche 5056489001), pH 7.4]. Cells were vortexed vigorously and left on ice for 30 minutes, then sonicated for 5 seconds in an ice water bath. 30 µL of whole cell lysate was aliquoted for immunoblot. Lysed cells were spun down at 13,000 rpm for 15 minutes to pellet debris and supernatant was added to clean tube with 25 µL resuspended anti-FLAG M2 Magnetic Beads (Sigma M8823) for overnight incubation at 4 °C with gentle rotation. Beads were then washed three times in 700 µL of ice-cold lysis buffer. Bound protein was eluted in 30 µL of elution butter [0.15 mg/mL 3xFLAG peptide (Sigma F4799) in 150 mM NaCl, 50 mM Tris-HCl, 10% glycerol, 0.05% Tergitol, pH 7.4]. Proteins were analyzed by immunoblot and antibodies used include mouse anti-FLAG 1:5000 (Sigma F1804), mouse anti-tubulin 1:10,000 (Sigma T5168), and rabbit anti-HA 1:3000 (Cell Signaling C29F4).

Generation and titration of HSV-1 viruses. The HSV-1 strains used were wild-type strain KOS1.1 [72], K26GFP [51], ICP6 deletion mutant ICP6Δ, and the ICP4 deletion mutant d120 [53]. HSV-1 infections were done at a multiplicity of infection of 5 PFU per cell, as previously described [73]. Titers of viral stocks were determined by plaque assay on either Vero cells (KOS1.1, K26GFP, and ICP6Δ) or ICP4-complementing E5-Vero cells [74].

Immunofluorescence microscopy. For immunofluorescence imaging of transfected cells, approximately 5×10^4 Vero, HeLa, or U2OS cells were plated on coverslips and after 24 hrs, transfected with 200 ng pcDNA4-RNR-3xFLAG, 200 ng pcDNA5/TO-A3-mCherry, or both. After 48 hrs, cells were fixed in 4% formaldehyde, permeabilized in 0.2% Triton X-100 in PBS for 10 minutes, washed three times for 5 minutes in PBS, and incubated in blocking buffer (0.0028 M KH_2PO_4, 0.0072 M K_2HPO_4, 5% goat serum (Gibco), 5% glycerol, 1% cold water fish gelatin (Sigma), 0.04% sodium azide, pH 7.2) for 1 hr. Cells were then incubated in blocking buffer with primary mouse anti-Flag 1:1000 overnight at 4 ℃ to detect FLAG-tagged RNRs. Cells were washed 3 times for 5 minutes with PBS, then incubated in secondary antibody goat anti-mouse AlexaFluor 488 1:1000 (Invitrogen A11001) diluted in blocking buffer for 2 hrs at room temperature in the dark. Cells were then counterstained with 1 µg/mL Hoechst 33342 for 10 minutes, rinsed twice for 5 minutes in PBS, and once in sterile water. Coverslips were mounted on pre-cleaned slides (Gold Seal Rite-On) using 20-30 µL of mounting media (dissolve 1g n-propyl gallate (Sigma) in 40 mL glycerol overnight, add 0.35 mL 0.1M KH_2PO_4, then pH to 8-8.5 with K_2HPO_4, Q.S. to 50mL with water). Slides were imaged on a Nikon Inverted Ti-E Deconvolution Microscope instrument and analyzed using NiS Elements.

For immunofluorescence imaging of HSV-1-infected cells, approximately 5×10^4 Vero, HeLa, or U2OS cells were plated on coverslips and after 24 hrs, transfected with 200 ng pcDNA5/TO-A3-mCherry. After 48 hours, cells were infected with HSV-1 K26GFP, HSV-1 KOS1.1, HSV-1 KOS1.1ΔICP6, or HSV-1 KOS1.1ΔICP4 at MOI 5. Cells were fixed in 4% formaldehyde 8 hours post-infection and then IF studies proceeded as above. Time course experiments were fixed at either 3, 6, 9, or 12 hours post-infection. HSV-1 K26GFP experiments did not require primary or secondary antibody staining steps. Cells infected with HSV-1 KOS1.1 and mutants were incubated in primary antibody mouse anti-HSV-1 ICP27 H1113 (Santa Cruz sc69807) 1:1000 overnight at 4 ℃ to detected HSV-1-infected cells. Secondary antibody staining, counterstaining with Hoechst, mounting, and imaging proceeded as above.

For quantification of A3 nuclear to cytoplasmic ratio, IF images were analysed using Fiji software to obtain mean fluorescence intensities (MFI) of nuclear compartments determined by Hoechst stain outline and cytoplasmic compartments determined by cell outline. MFI values were divided and plotted on Prism. Statistical analysis was performed using unpaired Student's t-test.

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373

Author Contributions

375 Conceptualization, AZC, SAR, and RSH; Investigation, AZC, SNM, and CA; Resources, AZC, CJB, and

376 SAR; Software, MCJ; Supervision, LF, CJB, SAR, and RSH; Validation, AZC, SNM, CA, JY-M, MB,

377 GG, and VDO; Visualization, AZC; Writing – Original Draft, AZC and RSH; Writing – Review &

378 Editing, AZC, SNM, CA, JY-M, MCJ, MB, GG, VDO, LF, CJB, SAR, and RSH.

379

389

Competing Interests

391 I have read the journal's policy and the authors of this manuscript have the following competing interest:

392 RSH is a co-founder, shareholder, and consultant of ApoGen Biotechnologies Inc. The other authors have

393 declared that no competing interests exist.

394

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576

Figure Legends

578

579   **Fig 1. Herpesvirus ribonucleotide reductases conservation.**

580   (**A**) Amino acid sequences from ribonucleotide reductase large subunits were aligned using Multiple

581   Sequence Comparison by Log-Expectation (MUSCLE) and phylogeny was constructed using

582   neighbor-joining tree without distance corrections and scaled for equal branch lengths (scale bar = 1).

583   Shaded boxes indicate herpesvirus subfamilies, which group closely to established phylogenetic trees.

584   Protein names for human herpesvirus ribonucleotide reductase large and small subunits shown on the

585   right.

586   (**B**) Schematic of representative RNR large subunit polypeptides from α-, β-, and γ-herpesviruses with

587   conserved core sequences (colored) and unique N- and C-terminal extensions (gray). Diagram is

588   approximately to scale with a ~190 amino acid portion of HSV-1 ICP6 omitted to fit the figure. Scale bar

589   is 100 amino acids.

590

591   **Fig 2. EBV BORF2 relocalizes A3B and A3A.**

592   Representative images of U2OS cells transfected with either A3-mCherry or BORF2-FLAG constructs.

593   Cells were fixed 48 hours post-transfection, permeabilized, and stained with anti-FLAG antibody and

594   Hoechst. A3 localization was compared in the presence and absence of EBV BORF2-FLAG

595   co-transfection.

596

597   **Fig 3. KSHV ORF61 relocalizes A3B and A3A.**

598   (**A**) Co-immunoprecipitation of transfected KSHV ORF61-FLAG with the indicated A3-HA constructs in

599   293T cells. Cells were lysed 48 hours post-transfection for anti-FLAG pulldown and resulting proteins

600   were analyzed by immunoblot. EBV FLAG-BORF2 transfected with A3B and A3G were used as positive

601   and negative co-IP controls, respectively.

602   (**B**) Representative images of U2OS cells transfected with either A3-mCherry or FLAG-RNR constructs.

603   Cells were fixed 48 hours post-transfection, permeabilized, and stained with anti-FLAG antibody and

604   Hoechst. Co-transfection with A3B-mCherry and EBV BORF2-FLAG was used as positive controls for

605   relocalization from nuclear to cytoplasmic aggregates. A3 localization was compared in the presence and

606   absence of KSHV ORF61-FLAG co-transfection.

607

608   **Fig 4. HSV-1 ICP6 binds and relocalizes A3B and A3A.**

609   (**A**) Co-immunoprecipitation of transfected HSV-1 FLAG-ICP6 with the indicated A3-HA constructs in

610   293T cells. Cells were lysed 48 hours post-transfection for anti-FLAG pulldown and resulting proteins

611 were analyzed by immunoblot. EBV FLAG-BORF2 transfected with A3B and A3G were used as positive

612 and negative co-IP controls, respectively.

613 (**B**) Representative images of U2OS cells transfected with either A3-mCherry or FLAG-RNR constructs.

614 Cells were fixed 48 hours post-transfection, permeabilized, and stained with anti-FLAG antibody and

615 Hoechst. Co-transfection with A3B-mCherry and EBV FLAG-BORF2 was used as positive controls for

616 relocalization from nuclear to cytoplasmic aggregates. A3 localization was compared in the presence and

617 absence of HSV-1 FLAG- ICP6 co-transfection.

618

619 **Fig 5. HSV-1 infection relocalizes A3B and A3A.**

620 Representative images of U2OS cells transfected with A3-mCherry constructs, followed by mock or

621 HSV-1 K26GFP infection 48 hours post-transfection. Cells were fixed 8 hpi and stained with Hoechst,

622 then imaged directly. The viral capsid protein VP26 is tagged with GFP which marks infected cells.

623

624 **Fig 6. A3B and A3A relocalization is dependent on HSV-1 ICP6.**

625 (**A**) Representative images of Vero cells transfected with A3-mCherry constructs, followed by mock,

626 wild-type HSV-1 KOS1.1, or HSV-1 KOS1.1ΔICP6 infection 48 hours post-transfection. Cells were

627 fixed 8 hours after HSV-1 infection, permeabilized, and stained with anti-ICP27 antibody to mark

628 infected cells and Hoechst.

629 (**B**) Representative images from an experiment similar to that described in panel A, except using U2OS

630 cells and the mutant virus HSV-1 KOS1.1ΔICP4.

631

632

633

Supporting Information

635 **Supplementary Fig 1. EBV BORF2 relocalizes A3B and A3A.**

636 Representative immunofluorescence microscopy images of Vero cells transfected with either A3-mCherry

637 or EBV BORF2-FLAG constructs. Cells were fixed 48 hours post-transfection, permeabilized, and

638 stained with anti-FLAG antibody and Hoechst to stain the nuclear compartment.

639

640 **Supplementary Fig 2. HSV-1 infection relocalizes A3B and A3A.**

641 Representative images of Vero cells transfected with A3-mCherry constructs, followed by mock or

642 HSV-1 K26GFP infection 48 hours post-transfection. Cells were fixed 8 hpi and then imaged directly.

643 The viral capsid protein VP26 is tagged with GFP to mark infected cells.

644

645 **Supplementary Fig 3. Time course of HSV-1-mediated relocalization of A3B and A3A.**

646 Representative images of U2OS cells transfected with A3-mCherry constructs, followed by mock or

647 HSV-1 KOS1.1 infection 48 hours post-transfection. Cells were fixed at either 3, 6, 9, or 12 hpi and

648 stained with anti-ICP27 antibody to mark infected cells and Hoechst to stain the nuclear compartment.

649

650 **Supplementary Fig 4. A3B and A3A localization is altered by HSV-1 infection.**

651 Quantification of A3 localization patterns with or without HSV-1 infection by dividing the mean

652 fluorescence intensity of the nuclear and cytoplasmic compartments. Data points are pooled evenly from

653 immunofluorescence microscopy images shown in Figs. 5 and 6. Statistical analysis was performed using

654 an unpair Student's t-test between indicated groups, NS = not significant).
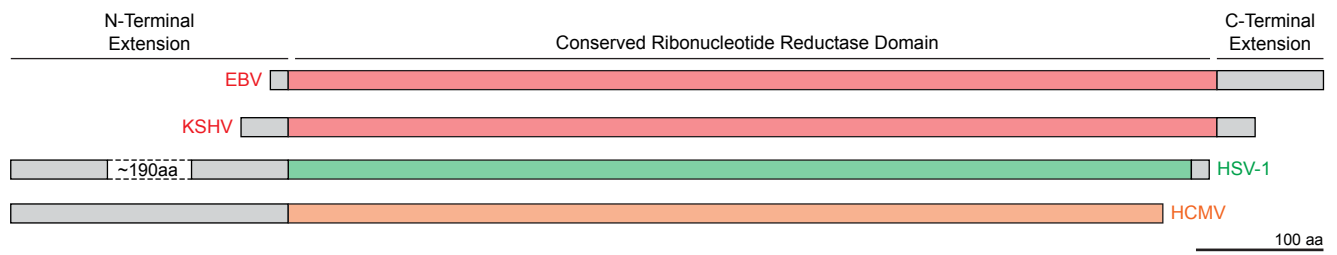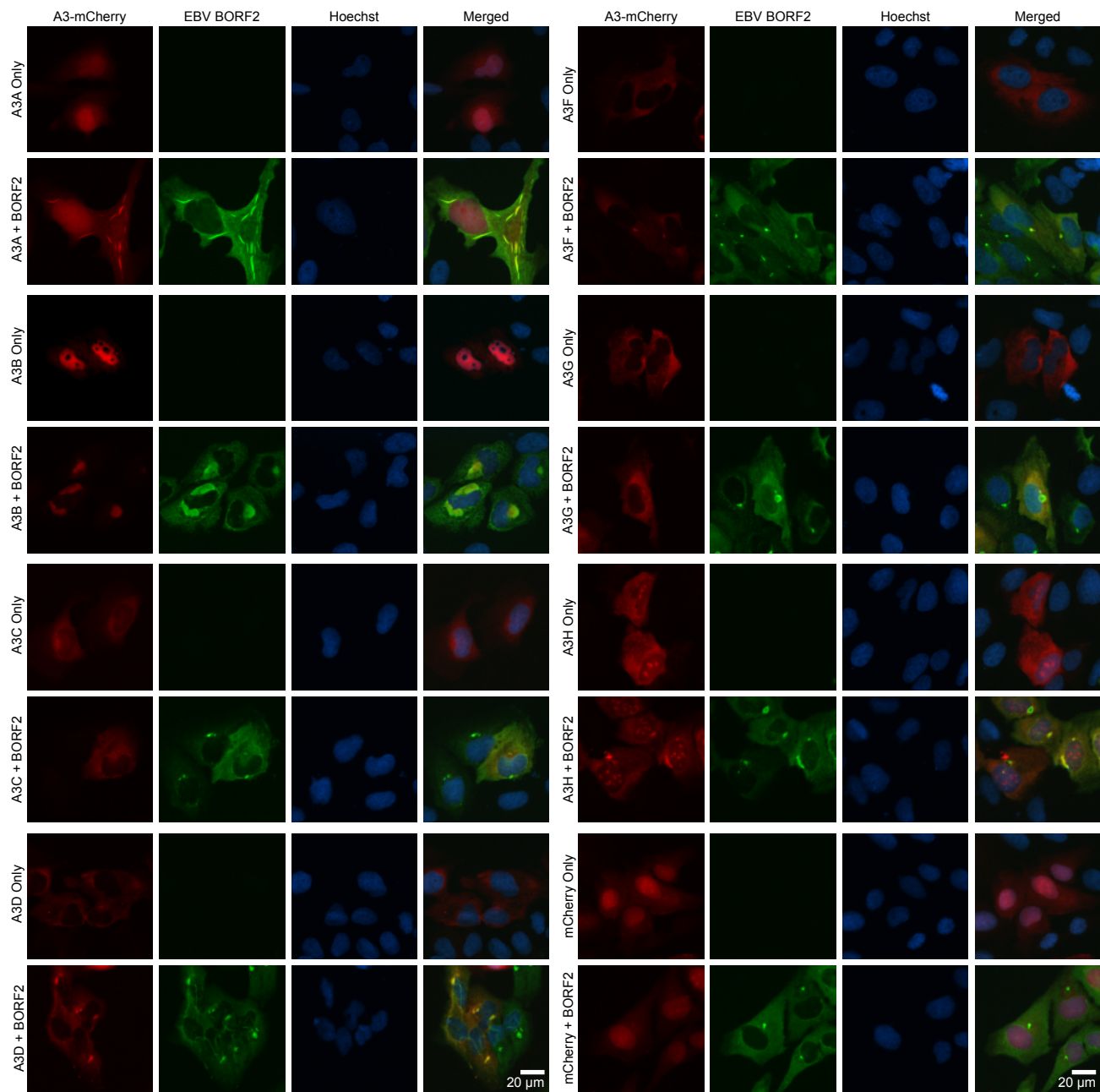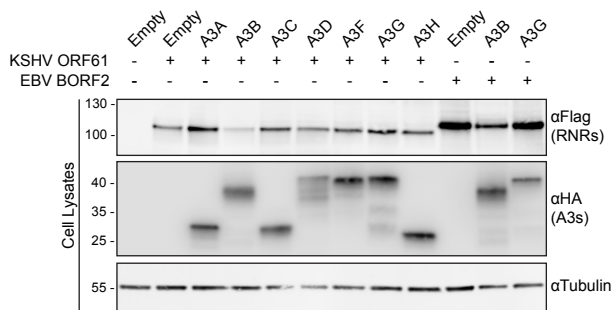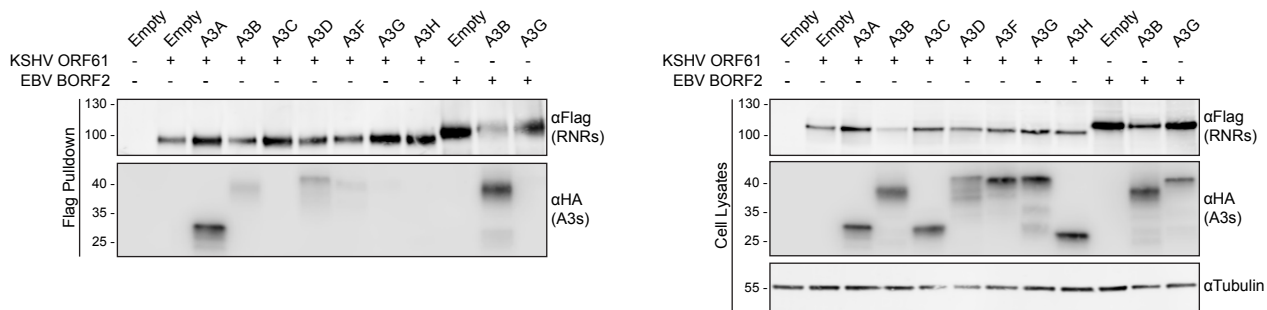
655

Fig 1

A

γ-herpesviruses		
RNR Subunit	EBV	KSHV
Large	BORF2	ORF61
Small	BaRF1	ORF60

α-herpesviruses			
RNR Subunit	HSV-1	HSV-2	VZV
Large	ICP6	ICP10	ORF19
Small	UL40	UL40	ORF18

β-herpesviruses			
RNR Subunit	HCMV	HHV6A/B	HHV7
Large	UL45	U28	U28
Small	None	None	None

B

Fig 2

Fig 3

A

B

Fig 4

A

B

Fig 5

20 μm

Fig 6

A

	Mock Infection		HSV-1 Infection		HSV-1 ΔICP6 Infection	
	A3-mCherry	Merged	A3-mCherry	Merged	A3-mCherry	Merged

A3A, A3B, A3C, A3D

20 μm

B

	Mock Infection		HSV-1 Infection		HSV-1 ΔICP4 Infection	
	A3-mCherry	Merged	A3-mCherry	Merged	A3-mCherry	Merged

A3A, A3B, A3C, A3D

20 μm

Supplementary Fig 1

20 μm

20 μm

Supplementary Fig 2

Supplementary Fig 3

	Mock Infection			HSV-1 K26GFP Infection		
	A3s-mCherry	VP26-GFP	Merged	A3s-mCherry	VP26-GFP	Merged
A3A						
A3B						
A3C						
A3D						
A3F						
A3G						
A3H						
mCherry			20 µm			20 µm

Supplementary Fig 4

Scale bar: 20 µm
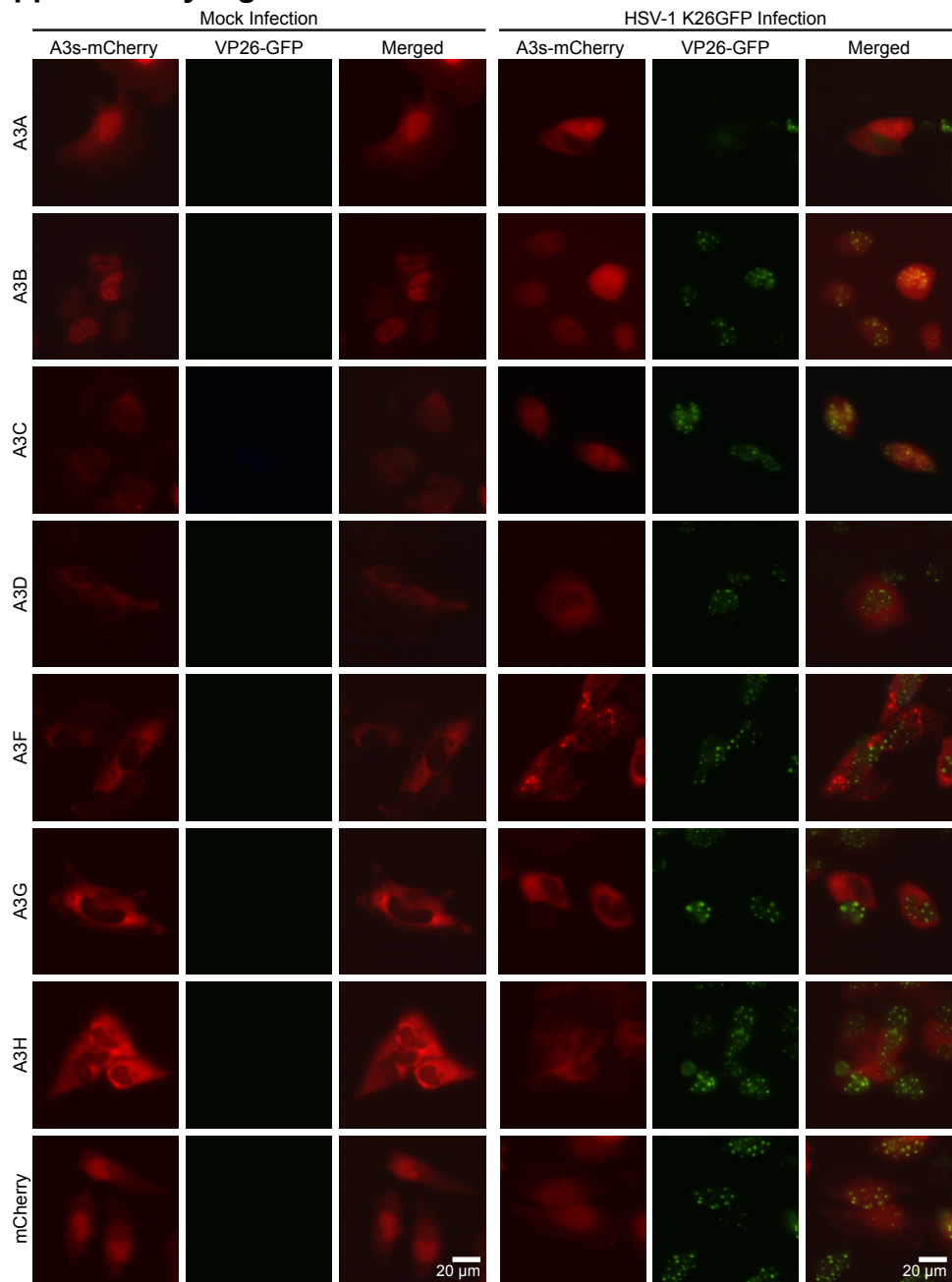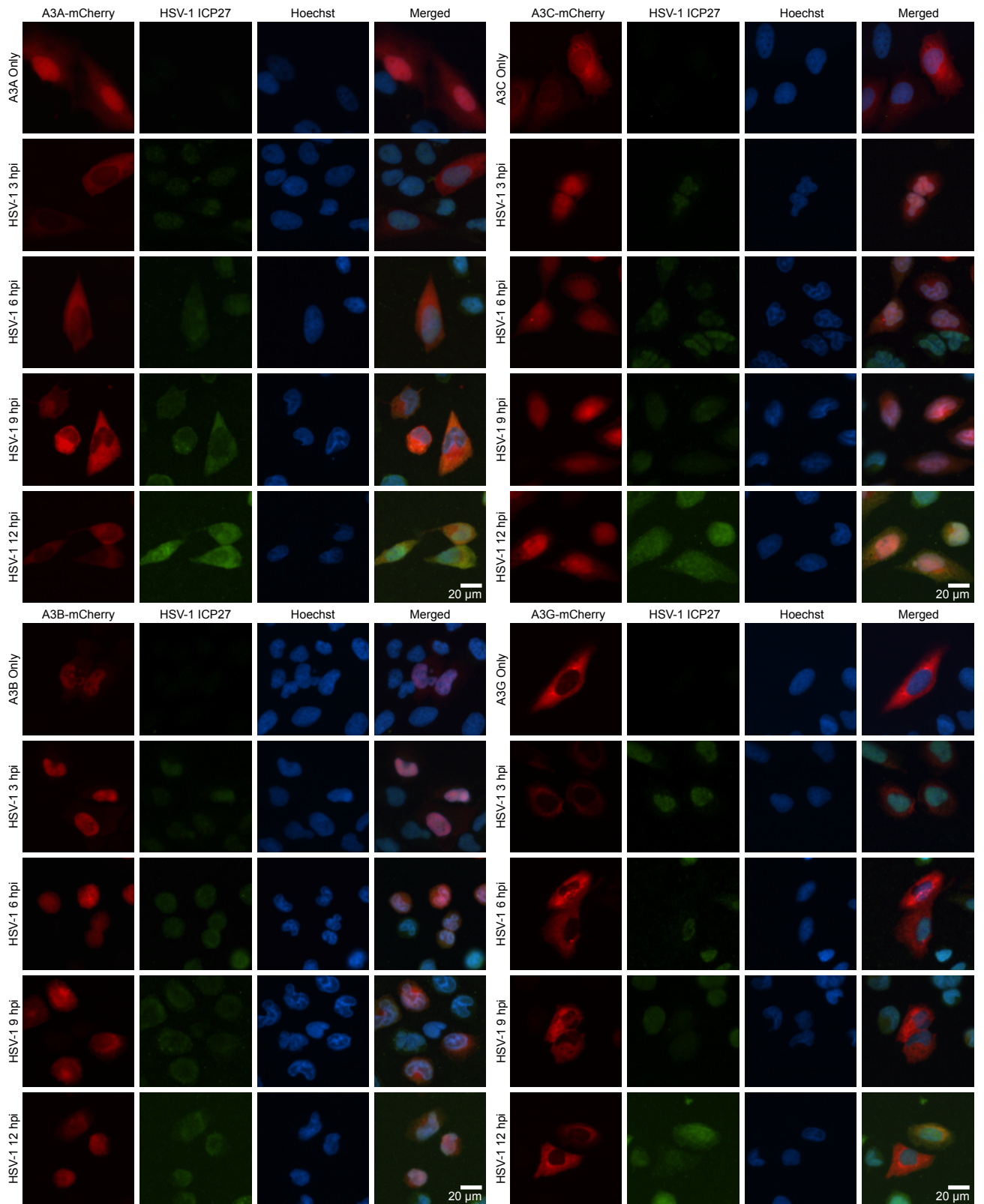
Supplementary Fig 4

Supplementary Fig 5

A

B