1 **THE KSHV ORF20 PROTEIN INTERACTS WITH THE VIRAL PROCESSIVITY FACTOR ORF59 AND PROMOTE**
2 **VIRAL REACTIVATION.**

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7

8 **ABSTRACT**

9 Upon KSHV lytic reactivation, rapid and widespread amplification of viral DNA (vDNA) triggers
10 significant nuclear reorganization. As part of this striking shift in nuclear architecture, viral
11 replication compartments are formed as sites of lytic vDNA production along with remarkable
12 spatial remodeling and relocalization of cellular and viral proteins. These viral replication
13 compartments house several lytic gene products that coordinate viral gene expression, vDNA
14 replication, and nucleocapsid assembly. The viral proteins and mechanisms that regulate this
15 overhaul of the nuclear landscape during KSHV replication remain largely unknown. KSHV's
16 ORF20 is a widely conserved lytic gene among all herpesviruses suggesting it may have a
17 fundamental contribution to the progression of herpesviral infection. Here, we utilized a
18 promiscuous biotin ligase proximity labeling method to identify the proximal interactome of
19 ORF20, which includes several replication-associated viral proteins, one of which is ORF59, the
20 KSHV DNA processivity factor. Using co-immunoprecipitation and immunofluorescence assays,
21 we confirmed the interaction between ORF20 and ORF59 and tracked the localization of both
22 proteins to KSHV replication compartments. To further characterize the function of ORF20, we
23 generated an ORF20-deficient KSHV and compared its replicative fitness relative to wild type
24 virus. Virion production was significantly diminished in the ORF20-deficient virus as observed by
25 supernatant transfer assays. Additionally, we tied this defect in viable virion formation to a
26 reduction in viral late gene expression. Lastly, we observed an overall reduction in vDNA
27 replication in the ORF20-deficient virus implying a key role for ORF20 in the regulation of lytic
28 replication. Taken together, these results capture the essential role of KSHV ORF20 in
29 progressing viral lytic infection by regulating vDNA replication alongside other crucial lytic
30 proteins within KSHV replication compartments.

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32 INTRODUCTION

33 Human herpesvirus 8, also known as Kaposi's Sarcoma associated herpesvirus (KSHV),
 34 is an oncogenic virus that has the incredible ability of initiating and maintaining lifelong infections
 35 in its host [1]. In immunocompromised individuals, these lifelong infections can lead to the
 36 development of several malignancies including Kaposi's Sarcoma (KS), Primary Effusion
 37 Lymphoma (PEL), and Multicentric Castleman Disease (MCD) [2-4]. Similar to other
 38 herpesviruses, KSHV undergoes a bi-phasic lifecycle that features both latent and lytic phases
 39 [1]. During latency, KSHV can persist for decades within its infected host during which time only
 40 a few viral proteins are expressed. These latency-associated proteins in concert with host
 41 machinery coordinate viral genome maintenance and replication while evading detection by host
 42 immune surveillance. However, upon reactivation into the lytic phase, the vast majority of KSHV
 43 genes are expressed in an ordered cascade, rapidly amplifying the viral genome and triggering
 44 the assembly of new viral particles. As with other herpesviruses, KSHV lytic phase replication
 45 progresses *via* a mechanism distinct from latent viral DNA replication [5]. In particular, lytic
 46 replication initiates at a different origin of replication (ori-Lyt) on the viral genome and does not
 47 occur in synchrony with the host cell [6,7]. Instead, KSHV lytic replication requires at least eight
 48 viral proteins (ORF9, ORF6, ORF40, ORF44, ORF56, ORF50 and ORFK8) including its own
 49 virally-encoded DNA polymerase (ORF9) [1,8,9] and DNA polymerase processivity factor
 50 (ORF59) [10-12]. In particular, ORF59 has been shown to be recruited to the lytic origin of
 51 replication via its binding to ORF50 (RTA) [13] which leads to the recruitment of the viral DNA
 52 polymerase and initiation of replication [10,11,14]. Therefore, KSHV lytic DNA replication is
 53 reliant on the recruitment of ORF59 and the assembly of a replication complex comprised of
 54 multiple viral lytic factors.

55 To accommodate the large-scale amplification of the viral genome, lytic replication is
 56 accompanied by a drastic reorganization of the nucleus. This results in major nuclear and

nucleolar remodeling including relocalization of host chromatin and displacement of several host proteins such as Nucleolin and Nucleophosmin [15]. This represents one of the hallmarks of herpesvirus replication: the formation of a “bean-shaped” viral replication compartment (RC) [16-19].

Among the ~85 viral proteins encoded by KSHV, ORF20 belongs to the herpesviral core UL24 gene family, widely conserved throughout the Alpha-, Beta-, and Gammaherpesviridae [20,21]. Very little is known about the functions of KSHV ORF20 and its orthologs across the herpesvirus family. For ORF20 orthologs encoded by HSV-1 (UL24), HCMV (UL76), and MHV68 (ORF20), each have been reported to modulate cell cycle arrest and apoptosis [22-24]. Past studies have also shown that KSHV ORF20 and its orthologs UL24 and UL76 localize to the nucleoli of transfected cells [25,26],[27] with a demonstrably complex localization pattern [28]. Furthermore, UL24 was shown to contribute to nucleolar reorganization by affecting the expression pattern of key nucleolar proteins [29,30][31][25]. ORF20 mRNA expression kinetics seem to vary depending on the host cell demonstrating late gene kinetics upon *de novo* infection of primary human umbilical vein endothelial cells (HUVEC) and upon reactivation of a latently infected body cavity based lymphoma cell line (BCBL-1) [32]. However, more recently, upon lytic reactivation in endothelial cells, KSHV ORF20 was shown to be expressed as an immediate early viral gene [28]. It was also demonstrated that through its interaction with OASL, ORF20 enhances KSHV replication, possibly by regulating ribosome composition [28].

Here, we investigated the ORF20 microenvironment by proximity labeling using BioID and identified ORF59 as an ORF20 interactor. We then tracked the localization of ORF20 upon lytic reactivation and found it localizes to KSHV replication compartments where it colocalizes with ORF59. Furthermore, we found that cells infected with an ORF20-deficient virus have a severe defect in viral DNA replication, late gene expression, and subsequent virion formation. Taken together, our results indicate that ORF20 is an important regulator of KSHV lytic cycle. By

82 placing ORF20 at the replication compartment where it interacts with KSHV processivity factor
83 ORF59, our work support a role for ORF20 in KSHV complex lytic DNA replication.

84

85 RESULTS

86 **KSHV ORF20 proximal proteome identifies novel ORF20 interactors.** To better understand
87 the function of ORF20 during lytic KSHV infection, we used the proximity labeling method BioID
88 to characterize the ORF20 microenvironment. We first generated an ORF20-deficient virus
89 (ORF20_{STOP}) using the BAC16 Red recombinase system [33] by mutating ORF20 genomic
90 sequence 16 nucleotides downstream of its translation start site (**Fig 1A**). This mutation resulted
91 in the expression of a premature termination codon. This virus was used to establish a latently
92 infected cell line in iSLK cells that will herein be referred to as iSLK-ORF20_{STOP}. Using a newly
93 generated ORF20 antibody, we confirmed that ORF20 expression is abrogated in these cells
94 (**Fig 1B**). Because timing of ORF20 expression is crucial to set our experimental timeline, and
95 due to expression being previously inconsistent, we next tested when endogenous ORF20 was
96 made in iSLK.WT cells, a KSHV positive cell line similar to the one used to establish our
97 ORF20-deficient viral mutant. Based on observations of ORF20 expression in iSLK.WT cells
98 relative to the ORF20_{STOP} cells, the band corresponding to ORF20 is the upper band in the
99 doublet detected with this antibody (**Fig 1B**). Based on this pattern, we detected ORF20 protein
100 expression as soon as 24h post lytic reactivation, making ORF20 a early gene as was recently

101 reported [28]. We thus used this time point for all future complementation experiments (**Fig 1C**).

102 To investigate ORF20 proximal interactome, we used the proximity labeling method BioID
103 in which a promiscuous biotin ligase – BirA – is fused to the protein of interest. Biotinylated
104 proteins resulting from the action of this BirA ligase can then be selectively isolated and
105 identified by mass spectrometry [34]. We thus generated a plasmid in which the BirA_{R118G} biotin
106 ligase was fused at the C-terminus of ORF20 which was subsequently used to rescue ORF20
107 expression in the iSLK-ORF20_{STOP} cells. This setup was used to identify novel ORF20 direct
108 and indirect interactors. iSLK-ORF20_{STOP} cells were reactivated for 24h with doxycycline and
109 sodium butyrate to trigger KSHV lytic cycle and transfected with BirA-ORF20 (or mock
110 transfected) with excess biotin added to the media at the time of transfection. After 16h, cells
111 were lysed and streptavidin-conjugated beads were used to isolate biotinylated proteins. The
112 identity of the biotinylated proteins was determined by LC-MS/MS (**Fig 2A**). As shown in
113 Supplementary Table 1, after filtering, we identified 316 high-confidence unique proteins in
114 ORF20 microenvironment, of which 62 were previously identified with KSHV ORF20 [28]. Based
115 on GO-term analysis on ORF20 interactors, several functional categories emerged, including

several related to ribosomal regulation as previously reported (**Fig 2B**). Intriguingly, several categories placed ORF20 at the nucleosome and close to proteins regulating helicase activity. Moreover, we detected ORF59, KSHV lytic DNA processivity factor that we decided to explore further.

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KSHV ORF20 interacts with ORF59 and localizes in the replication compartment. To validate the interaction between ORF20 and ORF59 detected by BioID, we used co-immunoprecipitation (Co-IP): cell lysates were collected from iSLK.WT cells either in their latent or reactivated state when both ORF59 and ORF20 should be expressed. We immunoprecipitated ORF59 overnight and immunoblotted the eluates. We found that ORF59 and ORF20 interact during KSHV lytic phase (**Fig 3A**).

ORF59 has a distinctive subcellular localization during KSHV lytic cycle and localizes into replication compartments. These replication compartments are the site of the viral genome replication and are easily identifiable as dark areas in DAPI staining of host nuclei. Given that ORF20 was previously shown to localize to discrete nuclear foci, we hypothesized that ORF20 could colocalize with ORF59 at these RCs. We first investigated if we could detect a fraction of ORF20 in the RC in reactivated iSLK cells by immunofluorescence assay (IFA). In iSLK.WT cells transfected with Flag-ORF20, ORF20 expression was predominantly nuclear and distinctively present in nuclear puncta overlapping with DAPI-negative areas. Because in iSLK.WT cells, endogenous viral ORF20 expression may interfere with our Flag tagged construct, we confirmed this expression pattern in iSLK.ORF20_{STOP} cells, where we also found ORF20 expressed in DAPI negative areas of the nucleus (**Fig 3B**). Since this expression pattern is consistent with ORF59 localization patterns and our BioID and Co-IP indicate an interaction between ORF59 and ORF20, we next monitored the localization of both ORF59 and ORF20. To do so, we used the KSHV-positive TREX-BCBL-1 cells since they do not have any fluorescent

141 marker and thus allowed us to use an additional color channel. In these cells, ORF59 and
 142 ORF20 appear to co-localize in the nucleus, in areas reminiscent of replication compartments
 143 (Fig 3C).

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146 **KSHV lacking ORF20 shows defect in virion formation and late gene expression.** Given
 147 that ORF20 function is poorly understood, we next wanted to better define its role during KHSV
 148 lytic cycle. Based on our data, ORF20 is expressed early in the lytic cycle and is expressed in
 149 RCs where it interacts with ORF59. Moreover, ORF20 is very well conserved in the herpesvirus
 150 family, suggesting that its function might be crucial for proper progression of viral infection so we
 151 hypothesized that ORF20 could be important for regulation of the viral replication. To address
 152 this question, we first monitored the ability of the iSLK.ORF20_{STOP} cells to produce progeny

virions by supernatant transfer assay and showed that compared to iSLK.WT cells, these almost completely failed to produce any viable virions (**Figure 4**).

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Provided that the ORF20_{STOP} cells were severely deficient in virion production, we next investigated whether ORF20 expression, or lack thereof, influences viral gene expression. We investigated the expression kinetics of two viral genes for which we have antibodies: the early viral protein ORF59 and the late major capsid protein K8.1. iSLK.WT or iSLK.ORF20_{STOP} cells were reactivated and either total RNA was extracted and used for qRT-PCR or cell lysates were collected and used for immunoblot. Compared with iSLK.WT cells, iSLK.ORF20_{STOP} cells displayed a drastic reduction of K8.1 expression both at the RNA (**Fig 5A**) and protein levels (**Fig 5B**) but only a marginal effect on ORF59. Because of this differential effect on an early (ORF59) vs. late gene (K8.1) expression and because ORF20 appears to be expressed in replication compartments, we hypothesized that ORF20 could have an impact on KSHV DNA replication. Therefore, we next quantified this process in both iSLK.WT cells and iSLK.ORF20_{STOP} cells. We observed a reduction in viral DNA replication in cells lacking ORF20 expression (**Fig 5C**) suggesting that the presence of ORF20 is required for proper progression of KSHV DNA replication and consequently, late gene expression and viable virion production.

173 **DISCUSSION**

174 In this study, we demonstrate that KSHV ORF20 localizes to replication compartments
175 where it interacts with KSHV DNA replication processivity factor ORF59. Our approach to better
176 define the contribution of ORF20 to KSHV lytic replication was to investigate ORF20
177 microenvironment by proximity labeling. Namely, we used BioID to uncover ORF20 proximal
178 interacting partners, complementing past work done to reveal the interactome of ORF20 [28].
179 We confirmed many of the previously identified ORF20 protein partners, including many
180 ribosomal factors as well as OASL as was previously reported [28]. A Go-term analysis of
181 ORF20 microenvironment also revealed several novel putative functions for ORF20, with
182 multiple relating to RNA binding. Since ORF20 was previously hypothesized to help bridge
183 specific mRNA to polysomes [28], it would be interesting to further investigate this aspect of
184 ORF20 biology. In addition, Nucleolin (NCL) was identified to be in close proximity to ORF20,
185 which is intriguing since ORF20 ortholog UL24 in HSV-1 was shown to be directly involved in
186 the dispersal of NCL during infection [29]. This suggests that this dispersal, which is also
187 observed during KSHV infection [35,36], might be a conserved function of ORF20.

188 We also show that ORF20 has a distinctive subcellular localization that we identified as
189 replication compartments. Of note, we also noticed that ORF20 subcellular localization was
190 heterogeneous and we observed that ORF20 could also be more diffused in the nucleus
191 dependent on experimental timing. This has been reported before [37] and suggests that ORF20
192 localization might be highly dynamic and only transiently needed at the replication compartment.

193 Replication compartments are enriched for many host and viral proteins involved lytic
194 DNA replication that shuttle in and out depending on their function during this process [38,39].
195 Formation of replication compartments is a complex but common process for viruses: these
196 membraneless compartments grow and coalesce over infection, using liquid-liquid phase
197 separation and pushing host chromatin to the nuclear periphery [40-42]. Replication

198 compartments are the site for viral genome replication, transcription, and packaging [19].
199 Finding ORF20 at this site thus brings up an important question: is ORF20 involved in KSHV
200 DNA replication? Past work on ORF20 orthologs have suggested that this viral protein is
201 important for viral replication [43], yet the precise mechanism is still unclear. However, ORF20
202 ortholog in MHV68 was not classified as an essential viral gene [44] suggesting that ORF20
203 contribution to DNA replication might vary slightly among the various herpesviruses.

204 One particularity of ORF20 is that it encodes a conserved PD-(D/E)XK endonuclease
205 domain [45] [46]. This signature nuclease domain is found in a number of proteins that can
206 cleave nucleic acids. The other known KSHV PD-(D/E)XK nuclease is the viral protein SOX,
207 which is responsible for host shutoff during the lytic phase [47] [48] [49]. It is thus interesting to
208 note that a second viral nuclease is encoded by KSHV and since we are placing ORF20 in the
209 replication compartment, this nuclease activity could be important for DNA replication. The
210 DNase activity of SOX was previously hypothesized to be responsible for initiating DNA
211 replication when a viral endonuclease is needed to create a nick in the origin of replication.
212 However, this could never be proven and instead, SOX DNase activity was shown to be
213 important for branch resolution during replication [50]. Similarly, in HSV-1, SOX ortholog UL12
214 was demonstrated to be involved in recombination [51] but has not yet been shown to be the
215 nickase necessary for rolling circle replication initiation. This therefore leaves open the question of
216 which protein – viral or cellular – carries out this single stranded cleavage at the origin of replication
217 during herpesviruses lytic replication. It would be interesting to test whether ORF20 PD-(D/E)XK
218 domain is functional, and whether this viral protein contributes to this process.

219 Like other herpesviruses, KSHV lytic DNA replication is distinct from latent viral DNA
220 replication both because it uses a specific lytic origin of replication and because KSHV relies of
221 viral and not cellular factors to regulate this process. Regulation of this process is complex and
222 extensive; more work is necessary to fully understand this crucial viral mechanism. As we

223 understand more and more about these previously uncharacterized viral proteins, such as
224 ORF20, we are getting closer to this goal. Given the frequently multifunctional roles of viral
225 proteins, it will be of interest to also explore possible additional activities of ORF20, including
226 how it may contribute to nuclear remodeling during KSHV replication and whether ORF20 has
227 an intrinsic nuclease activity.

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233 MATERIAL & METHODS

234 **Cells and transfections.** HEK293T cells (ATCC) were grown in DMEM (Invitrogen) supplemented with
235 10% FBS. The KHSV-infected renal carcinoma human cell line iSLK.BAC16 [52] (Kind gift from Dr.
236 Glaunsinger) bearing doxycycline-inducible RTA were grown in DMEM supplemented with 10% FBS. For
237 reactivation, BAC16-containing iSLK cells were treated with 1 µg/ml doxycycline and 1 mM sodium
238 butyrate for the indicated times. The KSHV-positive B cell line bearing a doxycycline-inducible version of
239 the major lytic transactivator RTA (TREX-BCBL-1) [53] was maintained in RPMI medium (Invitrogen)
240 supplemented with 10% fetal bovine serum (FBS; Invitrogen), 200 µM L-glutamine (Invitrogen), 100 U/ml
241 penicillin/streptomycin (Invitrogen), and 50 µg/ml hygromycin B (Omega Scientific). Lytic reactivation was
242 induced by treatment with 20 ng/ml 2-O-tetradecanoylphorbol-13-acetate (TPA; Sigma), 1 µg/ml
243 doxycycline (BD Biosciences), and 500 ng/ml ionomycin (Fisher Scientific) for 48h.

244 The KSHV ORF20 premature Stop codon (ORF20_{STOP}) mutant was engineered using the
245 scarless Red recombination system in BAC16 GS1783 *Escherichia coli* as previously described [52] ,
246 except using two gBlocks (IDT) to introduce the mutation. Each gBlock contained half of the kanamycin
247 resistance cassette, as well as the desired mutation, and was joined by short overlap-extension PCR
248 before being used as the linear insert in the established protocol. The BAC16 ORF20 mutant was
249 purified using the NucleoBond BAC 100 kit (Clontech). iSLK cell lines latently infected with the KSHV
250 ORF20_{STOP} virus were then established by coculture: HEK293T cells were transfected with 5µg of
251 ORF20_{STOP} BAC DNA. The following day, the cells were trypsinized and mixed 1:1 with the KSHV
252 negative iSLK-puro cells and then treated with 25nM 12-O-tetradecanoylphorbol-13-acetate (TPA) and
253 300 nM sodium butyrate for 4 days to induce lytic replication. iSLK cells were then selected using
254 selection media containing 300 µg/ml hygromycin B, 1 µg/ml puromycin, and 250 µg/ml G418. Media
255 were replaced every other day for ~2 weeks, gradually increasing the hygromycin B concentration until
256 there were no HEK293T cells remaining. These cells are herein referred to as iSLK-ORF20_{STOP}.

257 For DNA transfections, cells were plated and transfected after 24h when 70% confluent using
258 PolyJet (SignaGen).

Supernatant transfers were carried in iSLK cells. The indicated cells were reactivated for 48h, Supernatants from induced iSLK cells were syringe filtered through a 0.45- μ m-pore-size filter, and spininfected onto HEK293T cells at 1500rpm for 1.5 h at 37C. 24h later, cells were imaged on a fluorescent microscope.

Plasmids. The KSHV ORF20 ORF was obtained from the KSHV ORFeome [54] and cloned into a pcDNA4 Nter-3xFlag vector. For the BirA-ORF20 construct, the sequence of the BirA ligase (containing a mutation -R118G- allowing it to act as a promiscuous biotin ligase) was PCR amplified from pcDNA3.1 MCS-BirA(R118G)-HA and cloned at the C-terminus of ORF20 in the pcDNA4 Nter-3xFlag vector. All cloning steps were performed using in-fusion cloning (Clontech-Takara) and were verified by Sanger sequencing.

RT-qPCR. Total RNA was harvested using Trizol following the manufacture's protocol. cDNAs were synthesized from 1 μ g of total RNA using AMV reverse transcriptase (Promega), and used directly for quantitative PCR (qPCR) analysis with the SYBR green qPCR kit (Bio-Rad) on a QuantStudio3 real-time PCR machine. Signals obtained by qPCR were normalized to 18S. For DNA replication calculation, iSLK cells were incubated with 5 $\times$ proteinase K digestion buffer (50 mM Tris-HCl [pH 7.4], 500 mM NaCl, 5 mM EDTA, 2.5% SDS) and digested with proteinase K (80 μ g/ml) overnight at 55°C. The gDNA was isolated by using a Zymo Quick gDNA miniprep kit according to the manufacturer's instructions. DNA levels were quantified using relative standard curves with primers specific for KSHV ORF59 (5'-AATCCACAGGCATGATTGC -3' and 5'- CACACTTCCACCTCCCCTAA-3') or a region in the GAPDH promoter (5'-TACTAGCGGTTTTACGGGCG-3' and 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'). The relative genome numbers were normalized to GAPDH to account for loading differences and to uninduced samples to account for the starting genome copy number.

285 **Western Blotting.** Cell lysates were prepared in lysis buffer [NaCl 150mM, Tris 50mM, NP40 0.5%, DTT
286 1mM and protease inhibitor tablets] and quantified by Bradford assay. Equivalent amounts of each
287 sample were resolved by SDS-PAGE and western blotted with the following antibodies at 1:1000 in
288 TBST (Tris-buffered saline, 0.1% Tween 20): rabbit anti-ORF20 (Yenzym) rabbit anti-DHX9/RNA
289 Helicase A (Abcam), rabbit anti-GAPDH (Abcam). The rabbit anti-ORF59 and anti-K8.1 were used at
290 1:10,000 and 1:50,000 respectively and were a gift from the Glaunsinger lab. Primary antibody
291 incubations were followed by HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies
292 (Southern Biotechnology, 1:5000).

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294 **Immunoprecipitation.** Cells were lysed in low-salt lysis buffer [NaCl 150mM, NP-40 0.5%, Tris pH8
295 50mM, DTT 1mM, protease inhibitor cocktail] and protein concentrations were determined by Bradford
296 assay. Equivalent quantities of each sample and at least 400ug of total protein were incubated overnight
297 with the indicated antibody, and then with G-coupled magnetic beads (Life technologies) for 1h. Beads
298 were washed extensively with lysis buffer. Samples were resuspended in Western blot loading buffer
299 before resolution by SDS-PAGE.

300

301 **Immunofluorescence Assays.** iSLK-ORF20_{STOP} or TREX-BCBL1 cells were grown on coverslips, and
302 fixed in 4% formaldehyde for 20 min at room temperature. Cells were then permeabilized in 1% Triton-X-
303 100 and 0.1% sodium citrate in PBS for 10 min, saturated in BSA for 30 min and incubated with the
304 indicated antibodies at a 1:100 dilution. After 1h, coverslips were washed in PBS and incubated with
305 AlexaFluor594 or AlexaFluor488 secondary antibodies at 1:1500 (Invitrogen). Coverslips were washed
306 again in PBS and mounted in DAPI-containing Vectashield mounting medium (VectorLabs) to stain cell
307 nuclei before visualization by confocal microscopy on a Nikon A1 Resonant Scanning Confocal
308 microscope (A1R-SiMe). The microscopy data was gathered in the Light Microscopy Facility and Nikon
309 Center of Excellence at the Institute for Applied Life Sciences, UMass Amherst with support from the
310 Massachusetts Life Sciences Center.

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313 **BioID and Mass Spectrometry.** BioID was originally developed by Dr. Kyle Roux [Roux K. J., Kim D. I.,
314 Raida M., and Burke B. (2012) A promiscuous biotin ligase fusion protein identifies proximal and
315 interacting proteins in mammalian cells. *J. Cell Biol.* 196, 801–810 10.1083/jcb.201112098] and makes
316 use of a promiscuous biotin ligase: the BirA ligase. Samples were prepared as in [BioID: A Screen for
317 Protein-Protein Interactions]. Briefly, iSLK-ORF20_{STOP} cells were seeded in 10cm plates and reactivated
318 at the same time. 24h later, cells were transfected using PolyJet with 5ug of the ORF20-BirA construct
319 (or mock-BirA control vector) and 50μM Biotin was added to the media. The following day, cells were
320 harvested, lysed and affinity purification was performed using magnetic Streptavidin beads (Cell
321 Signaling) overnight at 4C. Beads were then extensively washed and trypsin digested overnight.
322 Samples were then cleaned up using C18 column and Mass Spectral data were obtained at the
323 University of Massachusetts Mass Spectrometry Center using a Orbitrap Fusion mass spectrometer.

324

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334 **FIGURE LEGENDS**

335 **Figure 1: Characterization of the expression of KSHV ORF20.** (A) Diagram showing the genomic
336 locus of ORF20, with surrounding genes ORF21 (which partially overlaps ORF20 on the opposite strand
337 of the KSHV genome) depicting the location of the introduced mutation. The mutation was confirmed by
338 Sanger sequencing. (B) ORF20 expression was assessed by western blot in both iSLK.WT and
339 ORF20_{STOP} cells whether in their latent phase (Lat) or lytic phase (Lyt). DHX9 served as a loading
340 control. (C) Western blot showing the expression kinetics of ORF20 relative in iSLK.ORF20_{STOP} cells
341 after reactivation with doxycycline and sodium butyrate for the indicated times. DHX9 served as a loading
342 control.

343 **Figure 2: Determination of ORF20 proximal interactome.** (A) Schematic representation of the BioID
344 assay where ORF20 is fused to the biotin ligase BirA which will transfer a biotin to proteins coming in
345 close proximity to ORF20. (B) iSLK.ORF20_{STOP} cells were transfected with ORF20-BirA and subjected to
346 BioID and mass spectrometry. The bar graph represents the top GO-terms associated with ORF20
347 interacting partners and the network, generated by Cytoscape represents ORF20 proximal interactome.
348 Proteins annotated in the categories highlighted in the bar graph are shown in clusters.

349 **Figure 3: ORF20 interacts and co-localizes with ORF59 in KSHV Replication Compartments.** (A)
350 Lysates of latently infected (Lat) or DOX-reactivated (Lyt) KSHV-positive iSLK.WT cells were subjected
351 to IP with an anti-ORF59 antibody, then Western blotted (WB) for ORF20 or ORF59. (B) Reactivated
352 KSHV-positive iSLK.WT or iSLK.ORF20_{STOP} cells were transfected with Flag-ORF20 and 24h later were
353 subjected to immunofluorescence assay using an anti-FLAG antibody (red), and DAPI staining to identify
354 nuclei (blue). White dotted circles denote DAPI-deficient nuclear domain representing KSHV replication
355 compartments. (C) DOX-treated KSHV-positive TREX-BCBL1 cells were transfected with Flag-ORF20
356 and 24h later subjected to immunofluorescence assay using an anti-Flag antibody (red), anti-ORF59
357 antibody (yellow) and DAPI staining to identify nuclei (blue).

358 **Figure 4: Lack of ORF20 reduces virion production.** (A) Diagram depicting the supernatant transfer
359 assay in iSLK cells. (B) Supernatant transfer assay was used as a proxy for virion production and

360 performed as described in panel A. Infection of HEK-293T cells was monitored by contrast (top) and GFP
361 imaging (bottom) on a fluorescence microscope. (C) Quantification of GFP-positive cells. Values
362 represent four independent views of the infected cells.

363 **Figure 5: ORF20 is involved in regulating DNA replication and subsequent late gene expression.**

364 (A & B) Total RNA was extracted from unreactivated and reactivated iSLK.WT or ORF20_{STOP} cells. (A)
365 RNA was then subjected to RT-qPCR to quantify expression of the indicated viral genes. Bars represent
366 the relative mRNA fold change over unreactivated cells. (B) Western blot showing the expression of
367 ORF59 and K8.1 in iSLK.WT or ORF20_{STOP} cells after reactivation with doxycycline and sodium butyrate.
368 DHX9 served as a loading control. (C) DNA replication was measured by qPCR of the viral genome
369 before and after reactivation of the lytic cycle.

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376 REFERENCES

- 377 1. Purushothaman P, Uppal T, Verma SC. Molecular biology of KSHV lytic reactivation. *Viruses*.
378 Multidisciplinary Digital Publishing Institute; 2015;7: 116–153. doi:10.3390/v7010116
- 379 2. Dupin N, Fisher C, Kellam P, Ariad S, Tulliez M, Franck N, et al. Distribution of human herpesvirus-8 latently
380 infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. *Proc*
381 *Natl Acad Sci USA*. 1999;96: 4546–4551. doi:10.1073/pnas.96.8.4546
- 382 3. Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, et al. KSHV-induced
383 oncogenesis. Cambridge: Cambridge University Press; 2007.
- 384 4. Greene W, Kuhne K, Ye F, Chen J, Zhou F, Lei X, et al. Molecular biology of KSHV in relation to AIDS-
385 associated oncogenesis. *Cancer Treat Res*. NIH Public Access; 2007;133: 69–127.
- 386 5. Fields BN, Knipe DM, Howley PM. *Fields virology*. 1996.
- 387 6. Lin CL, Li H, Wang Y, Zhu FX, Kudchodkar S, Yuan Y. Kaposi's sarcoma-associated herpesvirus lytic origin
388 (ori-Lyt)-dependent DNA replication: identification of the ori-Lyt and association of K8 bZip protein with the
389 origin. *J Virol*. American Society for Microbiology (ASM); 2003;77: 5578–5588. doi:10.1128/JVI.77.10.5578-
390 5588.2003
- 391 7. Nicholas J, Zong JC, Alcendor DJ, Ciufo DM, Poole LJ, Sarisky RT, et al. Novel organizational features,
392 captured cellular genes, and strain variability within the genome of KSHV/HHV8. *J Natl Cancer Inst*
393 *Monographs*. 1998;: 79–88.
- 394 8. AuCoin DP, Colletti KS, Cei SA, Papoušková I, Tarrant M, Pari GS. Amplification of the Kaposi's sarcoma-
395 associated herpesvirus/human herpesvirus 8 lytic origin of DNA replication is dependent upon a cis-acting
396 AT-rich region and an ORF50 response element and the trans-acting factors ORF50 (K-Rta) and K8 (K-
397 bZIP). *Virology*. 2004;318: 542–555. doi:10.1016/j.virol.2003.10.016
- 398 9. Cousins E, Nicholas J. Molecular biology of human herpesvirus 8: novel functions and virus-host
399 interactions implicated in viral pathogenesis and replication. *Recent Results Cancer Res*. Berlin, Heidelberg:
400 Springer Berlin Heidelberg; 2014;193: 227–268. doi:10.1007/978-3-642-38965-8_13
- 401 10. Chan SR, Chandran B. Characterization of human herpesvirus 8 ORF59 protein (PF-8) and mapping of the
402 processivity and viral DNA polymerase-interacting domains. *J Virol*. American Society for Microbiology
403 *Journals*; 2000;74: 10920–10929. doi:10.1128/jvi.74.23.10920-10929.2000
- 404 11. Lin K, Dai CY, Ricciardi RP. Cloning and Functional Analysis of Kaposi's Sarcoma-Associated Herpesvirus
405 DNA Polymerase and Its Processivity Factor. *J Virol*. 1998;72: 6228.
- 406 12. Chan SR, Bloomer C, Chandran B. Identification and characterization of human herpesvirus-8 lytic cycle-
407 associated ORF 59 protein and the encoding cDNA by monoclonal antibody. *Virology*. 1998;240: 118–126.
408 doi:10.1006/viro.1997.8911
- 409 13. Rossetto CC, Susilarini NK, Pari GS. Interaction of Kaposi's sarcoma-associated herpesvirus ORF59 with
410 oriLyt is dependent on binding with K-Rta. *J Virol*. 2011;85: 3833–3841. doi:10.1128/JVI.02361-10
- 411 14. McDowell ME, Purushothaman P, Rossetto CC, Pari GS, Verma SC. Phosphorylation of Kaposi's sarcoma-
412 associated herpesvirus processivity factor ORF59 by a viral kinase modulates its ability to associate with
413 RTA and oriLyt. *J Virol*. 2013;87: 8038–8052. doi:10.1128/JVI.03460-12
- 414 15. Kobiler O, Weitzman MD. Herpes simplex virus replication compartments: From naked release to
415 recombining together. Spindler KR, editor. *PLoS Pathog*. Public Library of Science; 2019;15: e1007714.
416 doi:10.1371/journal.ppat.1007714
- 417 16. Wu FY, Ahn JH, Alcendor DJ, Jang WJ, Xiao J, Hayward SD, et al. Origin-independent assembly of
418 Kaposi's sarcoma-associated herpesvirus DNA replication compartments in transient cotransfection assays

- and association with the ORF-K8 protein and cellular PML. *J Virol. American Society for Microbiology Journals*; 2001;75: 1487–1506. doi:10.1128/JVI.75.3.1487-1506.2001
17. Zhong L, Hayward GS. Assembly of complete, functionally active herpes simplex virus DNA replication compartments and recruitment of associated viral and cellular proteins in transient cotransfection assays. *J Virol. American Society for Microbiology (ASM)*; 1997;71: 3146–3160.
 18. Hollingworth R, Horniblow RD, Forrest C, Stewart GS, Grand RJ. Localization of Double-Strand Break Repair Proteins to Viral Replication Compartments following Lytic Reactivation of Kaposi's Sarcoma-Associated Herpesvirus. Jung JU, editor. *J Virol.* 2017;91: 1186. doi:10.1128/JVI.00930-17
 19. Schmid M, Speiseder T, Dobner T, Gonzalez RA. DNA virus replication compartments. *J Virol.* 4 ed. 2014;88: 1404–1420. doi:10.1128/JVI.02046-13
 20. Jacobson JG, Martin SL, Coen DM. A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. *J Virol. American Society for Microbiology (ASM)*; 1989;63: 1839–1843.
 21. Jacobson JG, Chen SH, Cook WJ, Kramer MF, Coen DM. Importance of the herpes simplex virus UL24 gene for productive ganglionic infection in mice. *Virology.* 1998;242: 161–169. doi:10.1006/viro.1997.9012
 22. Nascimento R, Dias JD, Parkhouse RME. The conserved UL24 family of human alpha, beta and gamma herpesviruses induces cell cycle arrest and inactivation of the cyclinB/cdc2 complex. *Arch Virol. Springer Vienna*; 2009;154: 1143–1149. doi:10.1007/s00705-009-0420-y
 23. Siew V-K, Duh C-Y, Wang S-K. Human cytomegalovirus UL76 induces chromosome aberrations. *J Biomed Sci. BioMed Central*; 2009;16: 107–10. doi:10.1186/1423-0127-16-107
 24. Nascimento R, Parkhouse RME. Murine gammaherpesvirus 68 ORF20 induces cell-cycle arrest in G2 by inhibiting the Cdc2-cyclin B complex. *J Gen Virol. Microbiology Society*; 2007;88: 1446–1453. doi:10.1099/vir.0.82589-0
 25. Pearson A, Coen DM. Identification, localization, and regulation of expression of the UL24 protein of herpes simplex virus type 1. *J Virol.* 2002;76: 10821–10828. doi:10.1128/jvi.76.21.10821-10828.2002
 26. Ben Abdeljelil N, Rochette P-A, Pearson A. The UL24 protein of herpes simplex virus 1 affects the sub-cellular distribution of viral glycoproteins involved in fusion. *Virology.* 2013;444: 263–273. doi:10.1016/j.virol.2013.06.021
 27. Lin S-R, Jiang MJ, Wang H-H, Hu C-H, Hsu M-S, Hsi E, et al. Human cytomegalovirus UL76 elicits novel aggresome formation via interaction with S5a of the ubiquitin proteasome system. *J Virol.* 5 ed. 2013;87: 11562–11578. doi:10.1128/JVI.01568-13
 28. Bussey KA, Lau U, Schumann S, Gallo A, Osbelt L, Stempel M, et al. The interferon-stimulated gene product oligoadenylate synthetase-like protein enhances replication of Kaposi's sarcoma-associated herpesvirus (KSHV) and interacts with the KSHV ORF20 protein. Robertson ES, editor. *PLoS Pathog. Public Library of Science*; 2018;14: e1006937. doi:10.1371/journal.ppat.1006937
 29. Lymberopoulos MH, Pearson A. Involvement of UL24 in herpes-simplex-virus-1-induced dispersal of nucleolin. *Virology.* 2007;363: 397–409. doi:10.1016/j.virol.2007.01.028
 30. Lymberopoulos MH, Bourget A, Ben Abdeljelil N, Pearson A. Involvement of the UL24 protein in herpes simplex virus 1-induced dispersal of B23 and in nuclear egress. *Virology.* 2011;412: 341–348. doi:10.1016/j.virol.2011.01.016
 31. Pearson A, Bourget A, Ben Abdeljelil N, Lymberopoulos MH. Role of the viral protein UL24 in nucleolar modifications induced by herpes simplex virus 1. *BMC Proc. BioMed Central*; 2011;5: P102–1. doi:10.1186/1753-6561-5-S1-P102

462 32. Yoo SM, Zhou F-C, Ye F-C, Pan H-Y, Gao S-J. Early and sustained expression of latent and host
463 modulating genes in coordinated transcriptional program of KSHV productive primary infection of human
464 primary endothelial cells. *Virology*. 2005;343: 47–64. doi:10.1016/j.virol.2005.08.018

465 33. Brulois KF, Chang H, Lee AS-Y, Ensser A, Wong L-Y, Toth Z, et al. Construction and manipulation of a new
466 Kaposi's sarcoma-associated herpesvirus bacterial artificial chromosome clone. *J Virol*. 2012;86: 9708–
467 9720. doi:10.1128/JVI.01019-12

468 34. Roux KJ, Kim DI, Burke B, May DG. BioID: A Screen for Protein-Protein Interactions. *Curr Protoc Protein*
469 *Sci*. John Wiley & Sons, Ltd; 2018;91: 19.23.1–19.23.15. doi:10.1002/cpps.51

470 35. Muller M, Hutin S, Marigold O, Li KH, Burlingame A, Glaunsinger BA. A ribonucleoprotein complex protects
471 the interleukin-6 mRNA from degradation by distinct herpesviral endonucleases. Conrad NK, editor. *PLoS*
472 *Pathog*. Public Library of Science; 2015;11: e1004899. doi:10.1371/journal.ppat.1004899

473 36. Atari N, Rajan KS, Chikne V, Cohen-Chalamish S, Orbaum O, Jacob A, et al. Lytic Reactivation of the
474 Kaposi's sarcoma-associated herpesvirus (KSHV) is Accompanied by Major Nucleolar Alterations. *bioRxiv*.
475 2020;: 2020.05.15.097808.

476 37. Diekmann U. Characterisation of the KSHV protein ORF20 and its role in innate immunity.
477 doi:10.24355/dbbs.084-201903041425-0

478 38. Dembowski JA, DeLuca NA. Selective recruitment of nuclear factors to productively replicating herpes
479 simplex virus genomes. Everett RD, editor. *PLoS Pathog*. Public Library of Science; 2015;11: e1004939.
480 doi:10.1371/journal.ppat.1004939

481 39. Li D, Fu W, Swaminathan S. Continuous DNA replication is required for late gene transcription and
482 maintenance of replication compartments in gammaherpesviruses. Robertson ES, editor. *PLoS Pathog*.
483 Public Library of Science; 2018;14: e1007070. doi:10.1371/journal.ppat.1007070

484 40. Taylor TJ, McNamee EE, Day C, Knipe DM. Herpes simplex virus replication compartments can form by
485 coalescence of smaller compartments. *Virology*. 2003;309: 232–247. doi:10.1016/s0042-6822(03)00107-7

486 41. Kobiler O, Brodersen P, Taylor MP, Ludmir EB, Enquist LW. Herpesvirus replication compartments originate
487 with single incoming viral genomes. Nemerow G, editor. *MBio*. 3rd ed. American Society for Microbiology;
488 2011;2: 857. doi:10.1128/mBio.00278-11

489 42. Chang L, Godinez WJ, Kim I-H, Tektonidis M, de Lanerolle P, Eils R, et al. Herpesviral replication
490 compartments move and coalesce at nuclear speckles to enhance export of viral late mRNA. *Proc Natl Acad*
491 *Sci USA*. National Academy of Sciences; 2011;108: E136–44. doi:10.1073/pnas.1103411108

492 43. Leiva-Torres GA, Rochette P-A, Pearson A. Differential importance of highly conserved residues in UL24 for
493 herpes simplex virus 1 replication in vivo and reactivation. *J Gen Virol*. Microbiology Society; 2010;91:
494 1109–1116. doi:10.1099/vir.0.017921-0

495 44. Song MJ, Hwang S, Wong WH, Wu T-T, Lee S, Liao H-I, et al. Identification of viral genes essential for
496 replication of murine gamma-herpesvirus 68 using signature-tagged mutagenesis. *Proc Natl Acad Sci USA*.
497 2005;102: 3805–3810. doi:10.1073/pnas.0404521102

498 45. Knizewski L, Kinch L, Grishin NV, Rychlewski L, Ginalski K. Human herpesvirus 1 UL24 gene encodes a
499 potential PD-(D/E)XK endonuclease. *J Virol*. 2006;80: 2575–2577. doi:10.1128/JVI.80.5.2575-2577.2006

500 46. Bertrand L, Leiva-Torres GA, Hyjazie H, Pearson A. Conserved residues in the UL24 protein of herpes
501 simplex virus 1 are important for dispersal of the nucleolar protein nucleolin. *J Virol*. 2010;84: 109–118.
502 doi:10.1128/JVI.01428-09

503 47. Mendez AS, Vogt C, Bohne J, Glaunsinger BA. Site specific target binding controls RNA cleavage efficiency
504 by the Kaposi's sarcoma-associated herpesvirus endonuclease SOX. *Nucleic Acids Res*. 2018;46: 11968–
505 11979. doi:10.1093/nar/gky932

- 506 48. Glaunsinger B, Chavez L, Ganem D. The exonuclease and host shutoff functions of the SOX protein of
507 Kaposi's sarcoma-associated herpesvirus are genetically separable. *J Virol. American Society for*
508 *Microbiology*; 2005;79: 7396–7401. doi:10.1128/JVI.79.12.7396-7401.2005
- 509 49. Bagnris C, Briggs LC, Savva R, Ebrahimi B, Barrett TE. Crystal structure of a KSHV-SOX-DNA complex:
510 insights into the molecular mechanisms underlying DNase activity and host shutoff. *Nucleic Acids Res.*
511 2011;39: 5744–5756. doi:10.1093/nar/gkr111
- 512 50. Uppal T, Meyer D, Agarwal A, Verma SC. The DNase Activity of Kaposi's Sarcoma-Associated Herpesvirus
513 SOX Protein Serves an Important Role in Viral Genome Processing during Lytic Replication. Jung JU,
514 editor. *J Virol. American Society for Microbiology Journals*; 2019;93: 1865. doi:10.1128/JVI.01983-18
- 515 51. Schumacher AJ, Mohni KN, Kan Y, Hendrickson EA, Stark JM, Weller SK. The HSV-1 exonuclease, UL12,
516 stimulates recombination by a single strand annealing mechanism. Everett RD, editor. *PLoS Pathog. Public*
517 *Library of Science*; 2012;8: e1002862. doi:10.1371/journal.ppat.1002862
- 518 52. Myoung J, Ganem D. Generation of a doxycycline-inducible KSHV producer cell line of endothelial origin:
519 maintenance of tight latency with efficient reactivation upon induction. *J Virol Methods.* 2011;174: 12–21.
520 doi:10.1016/j.jviromet.2011.03.012
- 521 53. Nakamura H, Lu M, Gwack Y, Souvlis J, Zeichner SL, Jung JU. Global changes in Kaposi's sarcoma-
522 associated virus gene expression patterns following expression of a tetracycline-inducible Rta transactivator.
523 *J Virol. American Society for Microbiology (ASM)*; 2003;77: 4205–4220. doi:10.1128/JVI.77.7.4205-
524 4220.2003
- 525 54. Davis ZH, Verschueren E, Jang GM, Kleffman K, Johnson JR, Park J, et al. Global mapping of herpesvirus-
526 host protein complexes reveals a transcription strategy for late genes. *Mol Cell.* 2015;57: 349–360.
527 doi:10.1016/j.molcel.2014.11.026

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