The KSHV portal protein ORF43 is essential for the production of infectious viral particles

Daniela (Dana) Dünn-Kittenplon a,b,c, Inna Kalt a,c, Jean-Paul (Moshe) Lellouche b,c, Ronit Sarid a,c,*

a The Mina and Everard Goodman Faculty of Life Sciences, Bar Ilan University, Ramat-Gan 5290002, Israel
b Department of Chemistry, Bar Ilan University, Ramat-Gan 5290002, Israel
c Advanced Materials and Nanotechnology Institute, Bar Ilan University, Ramat-Gan 5290002, Israel

1. Introduction

Kaposi sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is one of eight known human herpesviruses. KSHV is a cancer-related gamma-2 herpesvirus which is etiologically implicated in all types of Kaposi’s sarcoma (KS). In addition, KSHV is the causative agent of other disorders, including primary effusion lymphoma (PEL), multicentric Castleman’s disease, and KSHV-inflammatory cytokine syndrome (Chang et al., 1994; Dittmer and Damania, 2013; Goncalves et al., 2017; Gramolelli and Schulz, 2015; Kalt et al., 2009; Mesri et al., 2010).

Like other herpesviruses, infectious KSHV virus particles are composed of a linear double-stranded DNA genome which is packed within a capsid that is coated with a proteinaceous tegument layer and an external phospholipid bilayer envelope spiked with viral glycoproteins (Heming et al., 2017). The KSHV capsid shares structural and morphological features with capsids of other herpesviruses, and its structural components are designated with reference to the herpes simplex virus type 1 (HSV-1) capsid (Trus et al., 2001). The capsid is approximately 125 nm in diameter with an icosahedral shell that consists of 955 copies of the major capsid protein (MCP) encoded by orf25, which forms 11 pentons and 150 hexons, comprising five and six MCP units, respectively (Wu et al., 2000). The pentons are located at 11 vertices of the icosahedral assembly while a 12-mer dodecameric cylindrical structure, formed by the portal complex, occupies a structurally unique 12th vertex (Chang et al., 2007; Trus et al., 2004). This ring-shaped structure forms a channel which functions as a gate for the cleavage, packaging and ejection of the viral genomic DNA. The MCP pentons and hexons are interconnected by 320 trimers, each composed of two ORF26 and one ORF62 molecules. The outer capsid shell is decorated with approximately 900 copies of the small capsid protein (SCP) encoded by orf65, which interacts with the MCP.

Assembly of the herpesvirus capsids is driven by interactions between the various capsid proteins. KSHV appears to share common features with the assembly mechanism of other herpesviruses (Baines, 2011; Brown and Newcomb, 2011; Deng et al., 2008; Heming et al., 2017). This process involves additional viral proteins that are commonly excluded from infectious viral particles, including the scaffold protein (SCAF) encoded by orf17.5, the protease protein encoded by orf17, and the tripartite terminase complex, which inserts and cleaves genomic viral DNA into the capsid through the portal, and is composed of proteins encoded by orf7, orf29 and orf67a. Formation of capsids appears to be initiated by interactions of the portal complex with MCP, which pairs with the SCAF that then brings together additional MCP and SCAF molecules to ensure accurate curvature and capsid size. The idea that the portal ring serves as a nucleating center for pro-capsid
assembly is supported by the observations of a single portal structure per capsid (Cardone et al., 2007; Chang et al., 2007; Deng et al., 2008; Motwani et al., 2017; Newcomb et al., 2005, 2003). Yet, in-vitro self-assembled capsids lacking the portal protein have been described (Newcomb et al., 2005; Perkins et al., 2008; Tatman et al., 1994; Thomsen et al., 1994), suggesting that the portal protein is not crucial for capsid assembly, and that capsids lacking the portal ring could potentially be assembled during natural infection. Capsid assembly results in the formation of sphere-shaped porous procapsids composed of the MCP, an inner shell which includes more than 1000 SCAF (ORF17.5) units and about 100 protease (ORF17) molecules, and an outer shell composed of the trimer of ORF26 and ORF62 proteins. Maturation of the viral capsids involves packaging of the viral genome along with cleavage of the inner SCAF and protease proteins, which results in deconstruction of the scaffolding complex that evacuates the pro-capsid (Cardone et al., 2012; Deng et al., 2008). However, only a small fraction of the assembled capsids contains DNA. In fact, three distinct types of capsids are produced, denoted A, B, and C. C-capsids are potentially infectious and are filled with viral genomic DNA, but lack scaffolding complexes. A-capsids are empty, perhaps as a result of inefficient DNA retention or unscheduled evacuation of SCAF components, and B-capsids lack DNA but contain variable amounts of residual scaffolding structures in different configurations (Deng et al., 2008; Nealon et al., 2001; Tandon et al., 2015; Tatman et al., 1994).

The portal protein, KSHV ORF43, shares 41–70% amino acid sequence similarity and predicted secondary and tertiary structure with known portal proteins that were already functionally characterized (Kornfeind and Visalli, 2018; Visalli and Howard, 2014). These include the prototype portal protein UL6, encoded by HSV-1, which was widely studied (Newcomb et al., 2001; Patel et al., 1996), pORF54 encoded by VZV (Visalli et al., 2014), pUL104 encoded by hCMV (Dittmer and Bogner, 2006; Holzenburg et al., 2009) and BBRF1 encoded by EBV (Pavlova et al., 2013), which were previously shown to be essential for the production of infectious progeny viruses. No detailed crystallographic data describing herpesvirus portal structures, including the KSHV portal protein, exist. Of note, recent cryoEM images of UL6 based on subnanometer resolution 3D reconstruction identified a unique portal-vertex-associated tegument complex, which is probably involved in genomic DNA retention (McElwee et al., 2018). Evidence for the existence of a single portal complex in KSHV capsids was previously obtained by using cryoelectron tomography (Deng et al., 2008). Molecular evidence supporting the function of KSHV ORF43 has not been published to date.

In the present study, we generated a KSHV ORF43-specific antisem which was used to detect expression and localization of ORF43 during infection. We show that ORF43 is nuclear when expressed in the absence of other KSHV proteins. We constructed a recombinant ORF43-null KSHV genome and demonstrate that this clone establishes latent infection but fails to produce infectious viral particles. This phenotype was restored, though at different efficiencies, by expression of several untagged and tagged ORF43 proteins.

2. Results

2.1. KSHV ORF43 is a late viral protein, which is localized in the nucleus

To characterize the expression kinetics and the cellular distribution of ORF43 during KSHV infection, we generated rabbit polyclonal antisem against an E.coli expressed protein consisting of the carboxyterminal KSHV ORF43 (amino acids 306–605). This anti-serum detected a ~60-kDa untagged and myc-tagged ORF43 proteins that were ectopically expressed in cells; these proteins were not recognized by the corresponding pre-immune serum (data not shown). Anti-myc antibody detected the same band of myc-tagged-ORF43. The observed molecular
mass of ~60-kDa was slightly lower than expected (68-kDa) based on the 605 amino acid comprising ORF43. We used the antiserum in western blot assay to determine the expression of ORF43 protein during lytic virus reactivation in HEK-293T and in iSLK cells which were infected with a BAC16 recombinant KSHV clone. Lytic infection was induced upon expression of the replication and transcription activator (RTA) protein combined with treatment with the histone deacetylase inhibitor, sodium butyrate. As shown in Fig. 1A and B, the expression level of ORF43 increased over time and shared a similar pattern with the small capsid protein ORF65, which is classified as late lytic protein. Similar pattern was observed in iSLK cells that were treated with Doxycycline and sodium butyrate to induce lytic reactivation. Furthermore, combined treatment with the viral DNA polymerase inhibitor phosphonoacetic acid (PAA), which inhibits expression of late viral genes, completely abolished the expression of ORF43. Similar results were obtained for the late lytic small capsid protein ORF65, whereas the expression of ORF45 and K-b-ZIP K8, representing immediate-early and early gene products, respectively, continued though at relatively lower levels. Similarly, RTA which is expressed in iSLK cells under the control of doxycycline, as well as an immediate-early lytic gene product, continued to be expressed. These findings support the classification of ORF43 as a late lytic protein (Fig. 1C).

To determine the subcellular localization of ORF43 protein during lytic reactivation, we used an immunofluorescence assay with ORF43 antiserum. Cells were co-stained with the small capsid protein ORF65 antibody to enable identification of cells undergoing lytic reactivation. As shown in Fig. 2, ORF43 co-localized with ORF65, and was predominantly expressed in the nucleus within typical viral assembly structures (Fig. 2). However, newly assembled virions could be detected by ORF65 antibody, whereas ORF43 antiserum failed to detect virions (Fig. 2). This might be due to the relatively low copies of ORF43 (12 protein units) within each viral particle as compared to the large copy number of ORF65 protein (~900 protein units), or due to lower sensitivity of the ORF43 antiserum. Alternatively, it is possible that the epitope/s which are recognized by ORF43 antibody, are hidden within the capsid structure, and therefore cannot be detected by our ORF43 antibody. This hypothesis is supported by cryoelectron tomography reconstructions which illustrated the KSHV portal vertex as an internal structure with respect to the capsid floor (Deng et al., 2007, 2008; Kornfeind and Visalli, 2018). Of note, immunofluorescence staining of ORF43 protein during de novo infection with recombinant BAC16-mCherry-ORF45 virions (Bergson et al., 2014), which incorporate mCherry-ORF45 in the tegument, detected ORF43 in a minor fraction of incoming virions, suggesting the occurrence of a rare virion conformation which enables access of the antibody to ORF43 (data not shown). In line with the cellular distribution of ORF43 during lytic reactivation, un-tagged and myc-tagged ORF43 proteins that were ectopically expressed in uninfected cells were predominantly nuclear, suggesting that nuclear targeting of ORF43 does not require viral protein expression (Fig. 3). However, the cellular distribution of ORF43 was largely affected by the addition of HA and mCherry tags, resulting in cytoplasmic localization of ORF43 protein, which also formed noticeable aggregates when fused to HA-tag (Fig. 3).

2.2. Construction of BAC16-ORF43-null recombinant viral clone

To construct a mutant virus that fails to express ORF43, we used the complete KSHV BAC16 clone, which enables genetic manipulation of the KSHV genome in E. coli using the two-step Red-mediated recombination approach (Brulois et al., 2012; Tischer et al., 2006). The N-terminal of ORF43 coding sequence overlaps the C-terminal coding sequence of ORF44, and it was not possible to generate an orf43-stop mutation right after its first methionine without affecting the overlapping reading frame. Therefore, we introduced two stop codons within a downstream sequence that does not overlap orf44 gene (TCC...
CTCTTT → TAGCTCTAG) (Fig. 4A). The resulting virus was expected to fail to express full-length ORF43 protein, but instead expresses a short 21-amino acid peptide. Restriction enzyme analysis with XbaI showed the insertion of a kanamycin-resistance cassette after the first recombination, and appropriate restriction pattern after the second recombination (Fig. 4B). The recombinant viral clone, designated BAC16-ORF43-null, was confirmed by diagnostic PCR and sequence analysis, thereby ensuring the mutations and appropriate junctions in the recombination domain (Fig. 4C).

2.3. BAC16-ORF43-null-infected cells do not produce infectious viruses

To determine whether ORF43 is required for the production of infectious viral particles, we transfected the recombinant BAC16-ORF43-null DNA into HEK-293T cells. As a positive control, we transfected wild-type (WT) BAC16 DNA. Transfected cells were selected with hygromycin and, as expected, constitutively expressed GFP under the control of the cellular EF1-α promoter (Brulois et al., 2012). We independently established three WT and three mutant HEK-293T cell lines containing BAC16-ORF43-null and BAC16 DNA, respectively. To examine whether BAC16-ORF43-null-infected cells can undergo lytic induction and produce infectious viruses, we transduced these cells with a recombinant baculovirus that expresses RTA (BacK50), and treated with sodium butyrate to induce lytic virus reactivation. As shown in Fig. 5, at 72-hr post induction WT BAC16-infected cells demonstrated expression of ORF65 and ORF43 whereas BAC16-ORF43-null-infected cells expressed ORF65 but failed to express ORF43. Furthermore, 96-hr following lytic induction, we collected supernatants, removed cell debris by centrifugation and filtration, and examined the presence of infectious particles following inoculation of naïve SLK or iSLK cells by spinoculation. As expected, GFP-positive cells were obtained following exposure of SLK cells to supernatants from WT BAC16-infected cells. In contrast, not a single GFP-positive cell was detected in SLK cells that were inoculated with supernatants from BAC16-ORF43-null-infected cells. This suggests that BAC16-ORF43-null-infected cells fail to produce infectious progeny viruses.

To enable the packaging of the mutated BAC16-ORF43-null genome, we transfected different ORF43 expression vectors 24-hr prior to lytic induction, and assayed the presence of infectious progeny viruses in supernatants that were collected 96-hr post induction. WB analysis with ORF65 antisera confirmed lytic induction while ORF43 antisera confirmed expression of un-tagged and tagged ORF43 proteins (Fig. 6A). These experiments revealed that transfection of diverse ORF43 expression vectors, including un-tagged ORF43, N-terminal myc-tagged, N-terminal HA-tagged, and N- and C-terminal mCherry-tagged ORF43, enabled the production of infectious viral particles. Of note, we repeatedly observed relatively low levels of infectious viruses in supernatants from cells expressing mCherry-tagged ORF43, in particular C-terminal mCherry tag, while expression of untagged, myc or HA-tagged ORF43 enabled production of similar amounts of infectious particles (Fig. 6B). Using this approach, we established BAC16-ORF43-null-infected iSLK cells that expressed lytic proteins upon reactivation (Fig. 7A). To ensure that the infected iSLK cells carried a BAC16-ORF43-null genome rather than a WT BAC16 genome, we confirmed the desired mutations by sequence analysis of an amplified PCR product spanning this region. In addition, we designed two sets of PCR primers that selectively base-pair and amplify WT or mutated orf43. Using these sets, we obtained a PCR amplification product from WT BAC16-iSLK cells with the WT primer set, and not with the mutated orf43 primer set. In contrast, a PCR amplification product was obtained only with mutated orf43 primers when using DNA from iSLK cells that were infected with BAC16-ORF43-null virions, produced following ectopic expression of ORF43 with or with no tag (Fig. 7B). So far, we cannot rule out the occurrence of rare homologues recombination events between a segment of the ORF43 expression vector and the viral genome leading to the production of a small number of revertant genomes generating...
proper portal structures enabling packaging of the mutated BAC16-ORF43-null DNA. Nevertheless, our analysis suggests that vast majority of the virions obtained contain mutant BAC16-ORF43-null genome. Furthermore, by using these sets of primers we amplified packaged DNase-resistant WT orf43 which was concentrated from WT BAC16-infected cells, but failed to amplify DNase-resistant WT or mutated orf43 in concentrated supernatants from BAC16-ORF43-null infected cells (Fig. 7B). These findings, along with the failure of BAC16-ORF43-null virions to produce GFP-positive cells upon their inoculation, indicate that ORF43-null recombinant virus fails to package genomic viral DNA.

2.4. KSHV-ORF43-null cannot produce C-capsids

Three types of capsids are generated during herpesvirus assembly. Two types, A and B, lack genomic viral DNA and are either empty or contain different amounts of residual scaffold protein, respectively, whereas the third type, the C-capsid, is occupied with genomic viral DNA. The portal ring is required for the production of C-capsids (Deng et al., 2008). To examine capsid production and to characterize the capsid types that are produced by BAC16-ORF43-null-infected cells, we employed transmission electron microscopy (TEM) on iSLK cells that were induced to undergo lytic induction for 72-hr. As shown in Fig. 8, unlike WT BAC16 which produced A, B and C capsids, BAC16-ORF43-null mutant did not produce C-type capsids. Quantitative analysis of 566 capsids from 10 WT BAC16-infected cells revealed 5%, 67% and 28% A, B and C-type capsids, respectively, whereas similar analysis of 149 capsids from 10 BAC16-ORF43-null-infected cells revealed 2.5% and 97.5% A and B-type capsids, respectively. This indicates that capsids are assembled in cells that are infected with BAC16-ORF43-null viruses, but the viral genomic DNA is not inserted into the capsids.

3. Discussion

Productive viral infection involves expression of gene products that are not always essential. However, this is not the case with regard to a set of herpesviral structural proteins that participate in capsid assembly and maturation and in packaging of genomic viral DNA. We anticipated that the KSHV ORF43 protein, which shares homology with portal proteins of other herpesviruses, is essential for the production of infectious progeny viruses. In the present study, we used the full-length
KSHV clone BAC16 to generate a recombinant virus that contains two stop mutations within the coding sequence of ORF43. This recombinant virus, termed BAC16-ORF43-null, established latent infection upon transfection of its DNA, and upon lytic induction, produced capsids that were similar in size to their WT counterparts. Yet, this mutant virus failed to package viral DNA and to produce infectious progeny virions and no C-type particles with electron-dense cores were observed by TEM analysis. Accordingly, our findings suggest that assembly of the KSHV capsids in cells may initiate and proceed in the absence of ORF43, though this protein is essential for the production of infectious viral particles. These findings are consistent with other herpesviruses, revealing that viral capsids can be assembled but infectious particles cannot be produced in the absence of full-length portal protein (Lamberti and Weller, 1996; Patel et al., 1996; Pavlova et al., 2013).

Fig. 6. Ectopic expression of un-tagged and tagged ORF43 protein enables production of infectious virions. BAC16-ORF43-null infected HEK-293T cells were transfected with the indicated expression plasmids (ORF43, myc-ORF43, HA-ORF43, mCherry-ORF43 and ORF43-mCherry). 24-hr post transfection cells were transduced with a recombinant baculovirus which expresses RTA and treated with 1 mM sodium butyrate to induce lytic replication. WB analysis with antibodies to ORF65 and ORF43 confirmed lytic induction and ectopic expression of ORF43, respectively (asterisks indicate ORF43). Uninduced and induced BAC16-ORF43-null infected cells were used as controls (A). Supernatants were collected 96-hr post induction, concentrated, and used to infect SLK cells. Infected cells were detected as GFP-positive. Images were captured 72-hr post infection and GFP was quantified with ImageJ software. % GFP-positive area, representing average of 7 images, is shown. Control uninfected, WT BAC16-infected and BAC16-ORF43-null infected cells are also shown. Scale bars 100 μM. Results shown represent one experiment representative of three providing similar results (B).
Consistent with our results, previous in vitro studies demonstrated assembly of herpesviral capsids, including KSHV, in the absence of the portal protein (Motwani et al., 2017; Newcomb et al., 2005; Perkins et al., 2008; Tatman et al., 1994; Thomsen et al., 1994).

Ectopic expression of ORF43 enabled production of virions containing the mutated orf43 viral genome, which in-turn could infect cells and establish latent infection. Accordingly, we conclude that the BAC16-ORF43-null virus does not contain mutations beyond those that we deliberately introduced. Yet, it was surprising to find that all the ectopically expressed ORF43 proteins rescued the BAC16-ORF43-null mutated phenotype, despite their altered subcellular distribution though at different efficiencies. This was most unexpected for mCherry and HA-tagged ORF43 proteins that were shown to be predominantly expressed in the cytoplasm. Because we were concerned about the development of revertant wild-type (WT) genomes, we developed a PCR assay which discriminates between WT and mutated development of revertant wild-type (WT) genomes, we developed a PCR assay which discriminates between WT and mutated phenotype, despite their altered subcellular distribution.

Consistent with our results, previous in vitro studies demonstrated assembly of herpesviral capsids, including KSHV, in the absence of the portal protein (Motwani et al., 2017; Newcomb et al., 2005; Perkins et al., 2008; Tatman et al., 1994; Thomsen et al., 1994).

Ectopic expression of ORF43 enabled production of virions containing the mutated orf43 viral genome, which in-turn could infect cells and establish latent infection. Accordingly, we conclude that the BAC16-ORF43-null virus does not contain mutations beyond those that we deliberately introduced. Yet, it was surprising to find that all the ectopically expressed ORF43 proteins rescued the BAC16-ORF43-null mutated phenotype, despite their altered subcellular distribution though at different efficiencies. This was most unexpected for mCherry and HA-tagged ORF43 proteins that were shown to be predominantly expressed in the cytoplasm. Because we were concerned about the development of revertant wild-type (WT) genomes, we developed a PCR assay which discriminates between WT and mutated orf43 within the genomic viral DNA. Using this assay, we confirmed that iSLK cells that were infected with virions that were collected from BAC16-ORF43-null-infected cells upon lytic induction and ectopic expression of the different ORF43 proteins, contained mutated viral genomes. Yet, this assay cannot formally exclude the occurrence of rare recombination events producing wild type genomes that enable production of proper capsids which package mutated genomes. Accordingly, further studies are required to track the mechanism by which the different recombinant proteins rescued production of infectious viral particles.

Since capsid assembly takes place in the nucleus during late phases of lytic infection, ORF43 was expected to present late expression kinetics and to localize in this compartment during lytic replication. As predicted, using the antiserum we produced, we identified ORF43 as a late viral protein which is expressed in the nucleus during lytic reactivation. ORF43 expression kinetics and cell localization corresponded to that of the small capsid protein, ORF65. Similarly, ectopically expressed untagged and myc-tagged ORF43 proteins were predominantly localized in the nucleus, suggesting that nuclear localization of ORF43 does not require additional viral proteins. In contrast, HA and mCherry-tagged ORF43 were predominantly cytoplasmic, and large cytoplasmic aggregates were evident upon expression of HA-ORF43. Similar cytoplasmic-granular localization with Golgi enrichment has been previously described for ORF43 containing the His-myc tag at the carboxyl terminus (Sander et al., 2008). Of note, different bioinformatic tools failed to identify a nuclear localization signal in ORF43. Together, these results indicate that care should be taken with regard to ORF43 tagging since the tag, and even a small one, may affect protein solubility, cellular localization, and function.

Structural, as well as non-structural viral proteins can potentially serve as targets for therapeutic interventions. Indeed, specific thiourea compounds selectively target herpesviral portal proteins and inhibit virus propagation. Furthermore, selected mutations in the portal protein provide resistance to these compounds (Newcomb and Brown, 2002; van Zeijl et al., 2000; Visalli et al., 2003; Visalli and van Zeijl, 2003). The reagents generated in the present study are expected to enable further studies of ORF43. In addition, BAC16-ORF43-null virus may serve in the future as a platform to screen for specific ORF43 mutations that prevent proper capsid assembly or provide drug resistance to encapsidation-specific antiviral inhibitors.

4. Materials and methods

4.1. Plasmids

ORF43 expression plasmids containing different tags were generated (Table 1). DNA inserts were synthesized by PCR amplification using BAC16 DNA as template and appropriate primers (Sigma). DNA cloning employed digestion of inserts and vectors using restriction enzymes (New England Biolabs, Ipswich, MA) and ligation with T4 DNA ligase (Biogase Fast Ligation Kit, Bio-Lab). Ligation products were transformed into DH5α, grown on appropriate selection medium, and examined by colony PCR and restriction enzyme analysis of purified plasmid DNAs (AccuPrep Plasmid Mini-Extraction Kit, Bioneer). All cloned PCR products were verified by sequencing.

4.2. Cell culture and transfection

Human epithelial kidney HEK-293T cells, renal cell carcinoma SLK and iSLK cells (kindly provided by Don Ganem, Howard Hughes Medical Institute, UCSF, San Francisco, CA, and Rolf Renne, University
Fig. 8. TEM microphotographs of WT BAC16 and BAC16-ORF43-null-infected cells. iSLK cells infected with WT BAC16 or BAC16-ORF43-null were induced to undergo lytic infection by 1 µg/ml Doxycycline (Dox) and 1 mM sodium butyrate. Cells were harvested 72-hr post induction and examined by transmission electron microscopy.

### Table 1
List of expression plasmids and primers used to obtain inserts.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction Enzymes</th>
<th>Vector</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCherry- orf43</td>
<td>EcoRI-BamHI</td>
<td>mCherry-C1</td>
<td>5’-TTTGAATCTTGTAGGATGAAACCGGGGCT-3’</td>
</tr>
<tr>
<td>orf43-mCherry</td>
<td>NheI-Xhol</td>
<td>mCherry-N1</td>
<td>5’-TTTGAGCTAGGATGAAACCGGGGCT-3’</td>
</tr>
<tr>
<td>myc- orf43-Zeo</td>
<td>BamHI-EcoRI</td>
<td>pcDNA3.1/Zeo</td>
<td>5’TTCGGATCCCTGACGACTTCAGGACAAG-3’</td>
</tr>
<tr>
<td>pETMBPH-orf43C</td>
<td>BamHI-EcoRI</td>
<td>pETMBPH</td>
<td>5’TTCGGATCCCTGACGACTTCAGGACAAG-3’</td>
</tr>
<tr>
<td>HA- orf43</td>
<td>NheI-EcoRI</td>
<td>pcDNA3.1/Zeo</td>
<td>5’-TTCGGATCCCTGACGACTTCAGGACAAG-3’</td>
</tr>
<tr>
<td>orf43</td>
<td>BamHI-EcoRI</td>
<td>pcDNA3.1/Zeo</td>
<td>5’TTCGGATCCCTGACGACTTCAGGACAAG-3’</td>
</tr>
</tbody>
</table>
of Florida, Gainesville, FL) (Myoung and Ganem, 2011; Sturzl et al., 2013) were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Biological Industries, Israel) containing 50 IU/ml penicillin and 50 µg/ ml streptomycin (Biological industries, Israel), and supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biological Industries, Israel). iSLK cells were grown in the presence of 250 µg/ml G418 and 1 µg/ml Puromycin (A.G. Scientific Inc.) to maintain the Tet-on transactivator and the RTA expression cassette, respectively. The growth medium of BAC16-infected HEK-293T and iSLK cells was supplemented with 200 and 600 µg/ml hygromycin (MegaPharm, San Diego, CA), respectively, to maintain KSHV episomes. 0.5 µM Phosphonoacetic acid (PAA) (Sigma) was added to inhibit viral DNA replication. Transient transfections of plasmid DNA employed the calcium phosphate precipitation method. 4.3. Production of rabbit antibodies to ORF43

The ORF43 carboxyl terminal fragment (amino acids 306–605) was cloned into the pETMPBH expression plasmid. This plasmid was transformed into BL21-NT+ E. coli cells which are enriched for extra copies of tRNA genes that are rare in E. coli along with a chloramphenicol resistance gene. Plasmid transformed bacteria were grown on medium containing 34 µg/ml chloramphenicol and 15 µg/ml Kanamycin. To obtain sufficient amounts of purified protein, a single amphenicol resistance gene. Plasmid transformed bacteria were grown transformed into BL21-NT+

cloned into the pETMBPH expression plasmid. This plasmid was

4.4. Antibodies and western blot analysis

Cells were washed twice in cold phosphate-buffered saline (PBS), and suspended in radio-immunoprecipitation assay (RIPA) lysis buffer, or solubilization buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 1.5 mM MgCl2), containing the protease inhibitors PMSF and a combination protocol (Bergson et al., 2016; Tischer et al., 2006). To introduce inducible Red recombination enzymes and I-SceI endonuclease, Rsal) was added. The construct was incubated at 15 min at 4 °C, to remove cell debris. Sodium-dodecyl sulfate (SDS) loading buffer was added, and the samples were boiled for 5 min. Protein lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes using Trans-blot Turbo RTA Midi Nitrocellulose Transfer Kit (Bio-Rad, Berkeley, CA). The protein content of the different samples was verified by Ponceau S staining. The membranes were blocked with 5% dry milk in Tris-buffered saline (TBS) and then incubated with rabbit anti-ORF43 or mouse antibody to GFP (Covance Research Products, Denver, CO), ORF45 (Zhu et al., 2005), ORF K8 (Wang et al., 2008) (a kind gift from Prof. Yan Yuan), ORF65 (Ye et al., 2011) (a kind gift from Shou Jiang Gao), or RTA (Ueda et al., 2002) (kindly provided by Keiji Ueda). Immunoreactive bands were detected by anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). The bands were visualized with Clarity™ Western ECL substrate (Bio-Rad).
4.7. Transfection of KSHV BAC16 and BAC16-ORF43-null DNA and virus reconstitution

BAC16 and BAC16-ORF43-null reconstitution was carried out as previously described (Bergson et al., 2016; Gelgor et al., 2015). Briefly, BAC DNA was transfected into HEK-293T cells using the Lipofectamine 2000 transfection reagent (Life Technologies, Invitrogen), and transfected cells were selected with 200 µg/ml hygromycin B (A. G. Scientific Inc.). Plasmids expressing un-tagged or tagged ORF43 protein were transfected into HEK-293T containing the BAC16-ORF43-null genome. Lytic reactivation was induced 24-hr post transfection with re-combinant baculovirus that constitutively expresses RTA (Back50) (kindly provided by Prof. David Lukac) (Lukac et al., 2001) and 1 mM sodium butyrate. Supernatants containing virions were collected 96-hr later, and cleared of cells and debris by centrifugation (700 × g for 10 min at 4°C) and filtration (0.45-μm cellulose acetate filters; Corning). iSLK cells were infected in the presence of 8 µg/ml polybrene by spinnoculation (centrifugation at 1500 X g at 25°C for 60 min), which was followed by 1-hr incubation at 37°C, before changing the supernatant to fresh medium containing 5% FCS. Infected cells were selected in medium containing 600 µg/ml hygromycin B. KSHV-infected iSLK cells were treated with 1 µg/ml doxycycline and 1 mM sodium butyrate (Sigma), in the absence of hygromycin, puromycin, and G418, to induce RTA transgene expression and lytic cycle reactivation.

4.8. Distinction between WT and BAC16-ORF43-null genomes by PCR

To ensure that cells were infected with a mutated BAC16-ORF43-null genome, two sets of PCR primers were employed to selectively amplify WT or mutated orf43. DNA was amplified with 2xPCRBio HS Taq Mix Red PCR (Biocytosis) which allows direct amplification of unprocessed cell samples by using 5'-TTGCAGGATCTCAAAGAGGG-3' or 5'-TCCTGCAGTCCCCGTTATTAC-3' forward primer together with 5'-TTGCAGGATCTCCTAGAGCT-3' or 5'-TTGCAGGATCTCAAAGAGGG-3' reverse primers, which amplify WT or mutated null genome, two sets of PCR primers were employed to selectively amplify WT or mutated null genome.

60°C for 3 days. Thin sections were collected on copper grids and stained with Lead citrate. Cells were visualized with FEI Tecnai G2 120 kV transmission electron microscope.

Acknowledgements

We thank Dr. Julia Guez-Haddad for her valuable advice and help in bacterial expression and purification of ORF43 protein, Dr. Avi Jacobs and Dr. Irit Shoval for their assistance with confocal microscopy, Dr. Ayelet Atkins from the BIU TEM Unit, and Dr. Ilana Lowinger for her advice on PCR settings. We also thank Anastasia Gelgor for her constructive advice and help. We gratefully acknowledge Prof. Jae Jung, Don Ganem, Shou Jiang Gao, Yan Yuan, Keiji Ueda and David Lukac for gifts of reagents. This work was supported by a grant from the Israel Science Foundation (Grant no. 1365/15).

References

Mesi, E.A., Cesarman, E., Boshoff, C., 2010. Kaposi’s sarcoma and its associated her-