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A Functional Global Gene Profiling of Kaposi's Sarcoma-Associated Herpesvirus

By

Marco A Paliza-Carre

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

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in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Fenyong Liu, Chair
Professor Lin He
Professor Sheng Luan

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Abstract

A Functional Global Gene Profiling of Kaposi's Sarcoma-Associated Herpesvirus

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Doctor of Philosophy in Comparative Biochemistry

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Professor Fenyong Liu, Chair

Kaposi's sarcoma-associated herpesvirus (KSHV) is associated with several human malignancies including the cancers Kaposi's sarcoma and primary effusion lymphoma. As with other herpesviruses, KSHV can enter either latency or undergo lytic replication within its host. Several latency-associated open reading frames (ORF) and a few lytic cycle-associated ORFs are relatively well-studied. However, the majority of the 140.5kb long unique region of the genome, which contains about 90 ORFs, is largely uncharacterized outside of in silico studies. We took advantage of the BAC16, a bacterial artificial chromosome clone of a KSHV genome, as well as the iSLK cell line, a permissive cell line with tight latency when infected and efficient reactivation upon chemical induction, in order to generate targeted KSHV mutants to better understand the functionality of KSHV ORFs.

In chapter 2, I explain how we generated 90 mutants using the BAC16, each characterized by the disruption of a single ORF. Mutant BACs were introduced into iSLK cells by transfection and classified as essential or nonessential for the maintenance of latency in this cell model. Then transfected cells were chemically induced into lytic replication and the amount of infectious virus generated and released into the supernatant was quantified. This allowed us to classify ORFs as essential or nonessential for reactivation and infectious virus generation. Notably, we identified several core herpesvirus ORFs as non-essential which had previously been classified as essential in other herpesviruses, including ORF 17, 42, 57, 64, 66, 67, 69, glycoprotein L, glycoprotein H, and glycoprotein B.

I then further characterized the nonessential mutants by using growth curve assays under conditions favoring latency, reactivation, and lytic replication in chapter 3. This allowed me to isolate the roles of KSHV ORFs to specific viral life cycle stages, and to take a high-level approach of understanding which ORFs disrupt or promote infectious virus generation under these conditions. We found that ORFs 16, 46, 58, 60, 61, and 67 are deficient in virus generation under lytic conditions and likely play an important role in the early stages of viral infection to prime the virus for successful reactivation. We also identified several KSHV-specific ORFs (K3, K4, and K5), which function to inhibit reactivation. Finally, we discovered that ORFs 49, 50, 56,

and K11 promote spontaneous reactivation during latency while ORFs 11AA, 72, K6, and K7 repress it, underscoring the biological importance of viral control of this phenomenon, which plays a large role in pathogenesis and is not yet fully understood. In chapter 4, I further dissected the effects of ORF disruption on the viral life cycle. Here we monitored lytic antigen expression of BAC16 or mutant infected cells by measuring the percentage of infected cells expressing immediate-early (ORF45), early (K8α), or late (K8.1A/B) lytic antigens under conditions favoring latency or reactivation. We found that ORF20 tempers lytic gene expression upon reactivation. Additionally, ORF 10.1, 20, 23, 27, 58, and 67 seem to facilitate the burst of lytic gene expression shortly after infection, while ORF 11, 28, K10, K10.5, and K9 promote the establishment of latency after this.

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Chapter 1: Literature Review

Kaposi's sarcoma-associated herpesvirus and associated diseases

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8, is a member of the Herpesviridae family, the Gammaherpesvirinae subfamily, and the Rhadinovirus genus. It is most closely related to herpesvirus saimiri and Equid herpesvirus-2, but the most closely related human pathogen is Epstein-Barr virus, also a gammaherpesvirus but in the Lymphocryptovirus genus[1]. KSHV infection can cause several malignancies, including the cancers Kaposi's sarcoma (KS) and primary effusion lymphoma[2,3]. It is also associated with the plasmablastic variant of multicentric Castleman's disease, a lymphoproliferative disorder, as well as the immunoregulatory disorder KSHV inflammatory cytokine syndrome[4,5].

KS was initially described by Moritz Kaposi in 1872[6]. However, it wasn't until the AIDS epidemic of the 1980s, when a more aggressive form of KS named AIDS-associated KS emerged, that scientists searched for the causative agent of this disease. Kaposi's sarcoma-associated herpesvirus, the causative agent of KS, was first described in 1994 by Chang et al. who used representational difference analysis to find herpesvirus-like DNA sequences in KS tissues from AIDS patients[2].

KS is a cancer characterized by highly vascularized typically cutaneous nodules. These tumors are highly vascular and contain characteristic spindle cells. HIV coinfection and immunocompromised status are both high risk factors for disease. KS can be divided into 4 different types based off epidemiology and clinical presentation – classic KS, endemic KS, AIDS-associated KS, and iatrogenic KS. Classic KS was first described by Moritz Kaposi as a relatively benign disease found in elderly Mediterranean men in which lesions typically occur in the lower extremities[6]. Endemic KS is a more aggressive form of the disease found in sub-Saharan Africa. AIDS-associated KS emerged in the early 1980s during the AIDS epidemic and was defined by HIV coinfection and disseminated cutaneous disease. Iatrogenic KS can be found among immunocompromised patients – typically organ recipients receiving immunosuppressant drugs. For immunocompromised patients, a return to immunocompetent status is usually sufficient for disease regression – this typically involves antiretroviral treatment for AIDS patients[7].

Primary effusion lymphoma is a diffuse large B-cell lymphoma caused by KSHV, typically found in HIV-positive individuals. The tumor growth presents in a liquid phase in a body cavity and typically shows coinfection with Epstein-Barr virus[8]. Combination chemotherapy with a cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP)-like regimen is a typical treatment[9] and HAART treatment before diagnosis has been associated with better clinical outcomes[10]. Novel therapies using immunomodulatory molecules are currently in clinical trials.

The plasmablastic variant of multicentric Castleman disease is a lymphoproliferative disorder caused by KSHV infection and typically associated with HIV infection[4]. This variant involves the

expansion of polyclonal KSHV-infected, but Epstein-Barr virus negative, plasmablasts in the lymph nodes. Rituximab is the most common treatment[11].

KSHV inflammatory cytokine syndrome was more recently described and typically occurs in KSHV and HIV-infected patients. The pathophysiology includes elevated viral IL-6, human IL-6, IL-10, and KSHV viral load but lacks the lymphadenopathy seen in multicentric Castleman disease[5]. Treatments similar to those targeting multicentric Castleman disease may be effective[12].

Genome and virion structure

The genetic material for KSHV is in the form of DNA, and the genome consists of a coding region of about 140.5kb containing at least 70 primary ORFs as well other features including upstream and small ORFs and noncoding RNAs[13,14]. However, the study performing this ORF annotation was performed using ribosome profiling on KSHV-infected iSLK cells, which are neither endothelial nor b-cells, the two cell types which play the biggest role in KSHV-associated disease. It is possible that KSHV-infection in different cell types may cause expression of different ORFs. Notably, the first study to annotate KSHV ORFs was performed bioinformatically from the genome's DNA sequence and yielded 81 ORFs, some of which were not found in subsequent studies[13]. Twelve pre-miRNAs are also encoded by KSHV which can form 25 mature miRNAs[15–18] The coding region is flanked by a terminal repeat region containing about 35-45 repeats of 803bp each[19].

A KSHV virion consists of a nucleocapsid containing a linear double-stranded DNA genome surrounded by a tegument layer, which is surrounded by a lipid bilayer containing glycoproteins, similar to other herpesviruses. The capsid is icosahedral with a T=16 triangulation number and contains capsomeres consisting of pentamers or hexamers of the major capsid protein, ORF25. These capsomeres are connected by triplexes of two ORF26 and one ORF62 protein. The small capsid protein, ORF65 binds on the tips of the capsomeres[20]. The tegument consists of both viral and cellular proteins that are released into the cell upon infection and can vary from performing immunomodulatory functions to modulating expression of viral genes. Early studies used mass spectrometry to find 24 viral proteins, including capsid proteins and envelope glycoproteins, and a few cellular proteins associated with the virion[21,22]. A more recent study in 2020 detected an additional 17 viral proteins in the virion, using recent advances in proteomic technologies[23]. These recent advancements in the identification of novel virion-associated proteins demonstrates that research is still needed in this area, as the function of many of these ORFs as they are released from the virus into the host cell has not been studied, and due to temporal and spatial restriction could have different biological significance.

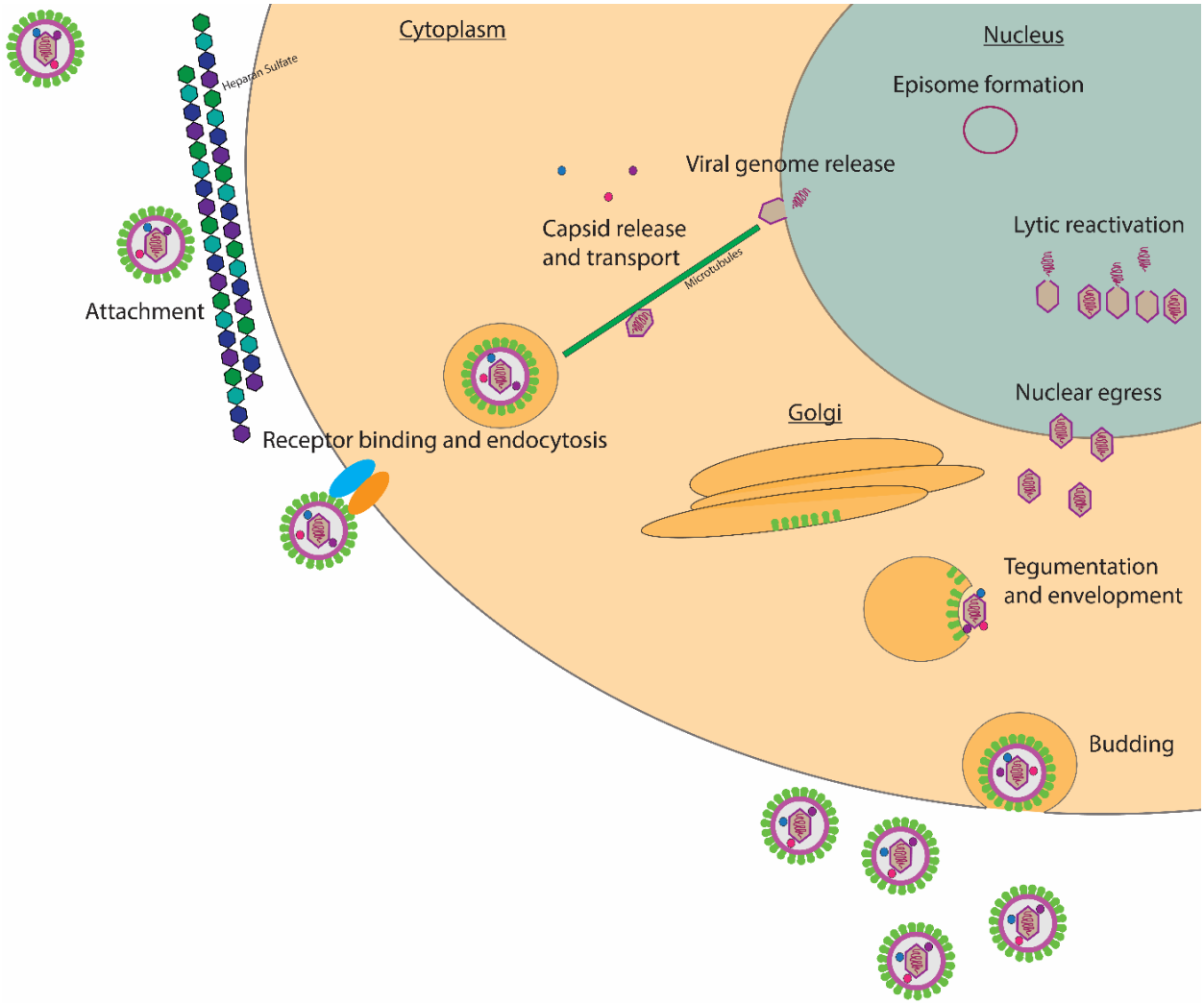


Figure 1.1. KSHV Life Cycle. Schematic of the KSHV life cycle upon infection of a cell, including attachment by interactions with cell-surface heparan sulfate, receptor binding and endocytosis, capsid escape from the endosome and transport to the nucleus along microtubules, viral genome release into the nucleus. The latency cycle involves episome formation and persistence in the nucleus, while the lytic cycle involves viral lytic gene expression, viral DNA replication and packaging into newly-formed capsids, nuclear egress, tegumentation and envelopment, and budding of newly formed virions from the cell.

The KSHV envelope glycoproteins include those conserved among other herpesvirus - gB, gH, gL, gM, and gN - and those specific to KSHV – K8.1A, ORF4, ORF28, ORF47, and ORF68. gH and gL are known to bind to certain ephrin receptors while gB is known to bind to certain integrins and DC-SIGN on the cell surface[24,25]. Binding of glycoproteins to cell-surface receptors plays a role in KSHV entry by endocytic pathways including macropinocytosis, clathrin-mediated endocytosis, and caveolin-mediated endocytosis[26] (Fig. 1.1). The specific glycoprotein-receptor interactions required for entry vary based on cell type, and binding to heparan sulfate proetoglycans plays an important role in virus attachment to the cell surface[27]. After entry, KSHV initiates membrane fusion with the endosomal membrane, although the exact mechanism by which this occurs is unknown. The capsid is then trafficked to the nucleus using the microtubule network, and the KSHV genome is released into the nucleus[28].

Viral life cycle

A distinguishing characteristic of the herpesvirus family is the ability for these viruses to exist in a latent state in infected cells, or to undergo lytic replication. Latency is a quiescent state where the viral DNA is maintained in the nucleus and few viral genes are expressed. Certain stimuli can induce reactivation from this state and cause the virus to undergo lytic replication, which includes the highly regulated temporal cascade of lytic genes expression, viral DNA replication, and the assembly and release of infectious virions from the cell.

In KSHV, the genome typically enters latency upon nuclear entry, where it circularizes and is maintained as an extrachromosomal episome. Latency Associated Nuclear Antigen (LANA) is a key viral regulator of latency. It tethers to the terminal repeat region of the viral genome and recruits cellular machinery to facilitate viral DNA replication, as well as tethers to host nucleosomal proteins to facilitate appropriate segregation of KSHV genomes to daughter cells during mitosis[29]. KSHV episomes contain histones which have specific patterns of activating and repressing markers during either latency or lytic reactivation. Rearrangement of histone modification between these states appears to be mediated by Polycomb group proteins[30]. Reactivation can be caused by various stimuli, including hypoxia, reactive oxygen species, or changes to cellular metabolism[31–33]. Regardless of the stimulus, reactivation entails increased expression of KSHV ORF50, which serves to drive the subsequent expression of lytic genes[34]. The temporal cascade of lytic gene expression starts with expression of immediate early genes, followed by early genes, and finally late genes[35,36]. The immediate early genes predominantly play a role in the transition for latency to lytic replication, while the early genes typically play a role in gene expression and DNA replication, and late proteins are frequently structural proteins for virion generation. This lytic cascade ultimately produces capsids which are packaged with the viral genome in the nucleus and are transported to the cytoplasm. Here, the capsids associate with the tegument proteins in a process whic³⁷³⁷h is poorly understood, before budding into vesicles derived from the trans-Golgi network which contain viral envelope membrane proteins. These viral particles bud from the plasma membrane of the cell as fully-formed viruses and are capable of infecting new cells to restart the cycle[38,39].

Notably, the balance between latent and lytic replication plays a key role in KSHV-associated disease. In KS, most infected cells are in latency, but a small number appear to enter lytic replication and express lytic genes. It has been shown that higher CD4 counts and a longer elapsed time since the initiation of HAART were shown to decrease KS incidence, indicating that control of KSHV infection by the immune system reduced pathogenesis[40]. Reactivation of KSHV in infected cells appears to play an important role in the development of Kaposi's sarcoma, both as a mechanism for infection of naïve cells and through oncogenic effects of certain lytic cycle proteins [41,42]. In primary effusion lymphoma, virus in predominantly in latency, and Epstein-Barr virus coinfection likely plays a role in oncogenesis. Expression of vIL-6, typically considered a lytic gene, in PEL cells may be due to spontaneous reactivation of a small number of cells, or low-level expression during latency[43]. Interestingly, multicentric Castleman disease is characterized by a higher degree of lytic gene expression[44,45]. However, the extent to which lytic replication facilitates Kaposi's sarcoma and other KSHV-associated diseases, and the mechanisms and roles of viral genes in this process is still not fully elucidated.

Oncogenesis

KSHV is one of only a few oncoviruses, however the mechanism by which it causes cancer is still not clear. Cancer typically involves genetic mutation in a cell, which ultimately lead to increased proliferation and immortalization of that cell. These characteristics allow the cell to divide uncontrollably and form tumors. However when spindle cells, the putative cancer cells of KS, were isolated from biopsies and grown in culture, they could not grow indefinitely in culture, and would eventually lose the KSHV episome[42,46]. This seemed to contradict the logic behind traditional tumorigenesis, which led two theories to emerge about the mechanism of carcinogenesis of KS - the abortive lytic and the paracrine oncogenesis hypotheses. The abortive lytic hypothesis postulates that some KSHV-infected cells will express a subset of lytic genes, but then will reenter latency without completing the full lytic cycle. The paracrine oncogenesis hypothesis states that some KSHV-infected cells will enter and complete the lytic cycle. Lytic gene expression will induce expression of angiogenic factors and growth factors which will stimulate growth of latent KSHV-infected cells in a paracrine manner to drive tumorigenesis[4748].

Several KSHV genes have already been implicated as oncogenes. LANA can upregulate the telomerase promoter, inactive Rb to promote cell cycle progression, inhibit glycogen synthase kinase-3β to increase c-Myc stability, inhibit P53, and cleave AURKB to promote the metaphase-to-telophase transition in cells undergoing mitosis[49–52]. vCyclin counters P27 and P21 CDK inhibitors to promote entry into the cell cycle[53]. vFLIP activates NFkB which inhibits apoptosis[54]. vGPCR activates NFkB, the PI3K-AKT-mTOR pathway, and the MAP Kinase pathway[55,56]. Despite these apparent oncogenic capabilities from latent genes (only vGPCR is a lytic gene), it is striking that latently infected cells are not immortalized in vitro.

Experiments in vGPCR-expressing transgenic mice have shown that vGPCR expression is sufficient to induce KS-like tumor formation[57,58]. Another group injected SV40 large T antigen immortalized murine endothelial cells transduced with various latent and lytic genes into mice.

They found that tumor formation was potent with only vGPCR-expressing SVEC, but was most potent when injecting vGPCR-expressing SVEC as well as vCyc and vFLIP-expressing SVEC[58].

There has been very little evidence to recapitulate these experiments in vitro with human cells. Bais et al showed that transduction of vGPCR into HUVEC was sufficient for immortalization[56]. Ciufo et al were able to show that culture of KSHV-infected DMEVC could be propagated indefinitely if they were passaged with fresh DMVEC. They also showed that a small percentage of their culture were expressing lytic genes[59]. Flore et al infected BMECs and HUVECs with KSHV, and saw increased proliferation compared to mock infected cells. These cells also did not reach senescence after 12-14 months, though mock infected cells reached senescence after about 3 weeks. In these experiments about 1-5% of cells were infected, which shows the importance of uninfected cells for tumor formation. Soft agar assays showed that these cells could support anchorage-independent growth, and that the percentage of cells that formed colonies in soft agar seemed to correlate with the percentage of KSHV-infected cells[60].

The results from these experiments suggest that paracrine factors from KSHV-infected cells undergoing lytic replication play a significant role in inducing oncogenesis. vGPCR is the best understood of these factors and appears to have high transforming potential, although most studies involving vGPCR have been performed using mice or murine cells and have not established the potential paracrine mechanism behind this interaction. Also, most KSHV lytic genes are uncharacterized and unstudied, and may also play significant roles in paracrine oncogenesis.

Recombinant KSHV and mutagenesis

Studying KSHV ORF disruptions in the context of the full viral genome was not practical until the development of the first BAC clone of KSHV, the BAC36, in 2002[61]. This clone was generated from the KSHV genome sequence in the primary effusion cell line, BCBL-1. To replace the BAC36, the BAC16 was developed in 2012, generated form rKSHV.219 derived from JSC1 cells[62–64]. This BAC addressed the issue of the 9 kb duplication found in the terminal repeats of a region of the genome containing ORFs 18, 17, 16, K7, K6, K5, part of ORF19, and the BAC cassette[65]. In tandem with the iSLK cell line, which displays tight latency upon infection and robust reactivation upon chemical induction, this quickly became the de facto system for generating KSHV mutants, and iSLK cells were often used for infection studies, despite the discovery that their parent cell line is indistinguishable from Caki-1 cells a known renal carcinoma cell line [66,67]. Mutants in the BAC16 are typically generated using the two-step red-mediated recombination method, which allows for insertion of long or short sequences, insertion of point mutations, and scarless deletions[68,69]. At the time of writing, only 18 ORFs have been studied by other labs through generating and analyzing single ORF disruption mutants in the BAC16[64,70,79–88,71,89–92,72–78]. Therefore, a large part of the KSHV genome has not been analyzed by disruption in the context of authentic KSHV infection.
In this study, we generated 90 mutants in the BAC16, each with a disruption of a single ORF, to cover all of the primary ORFs and some short and upstream ORFs. Seventy-two of our mutants have not been previously generated and most of the ORFs involved in our study have also not

been characterized except by bioinformatics analyses. We classified each of the ORF-disruption mutant based on their ability to generate infectious progeny upon doxycycline and sodium butyrate-mediated induction of lytic replication after transfection into iSLK cells. Mutants for ORFs that were nonessential for this process were assayed for infectious virus generation by de novo infection in iSLK cells and maintenance in conditions to induce either lytic replication, reactivation, or latency. Finally, nonessential mutants were also assayed for lytic antigen expression under conditions inducing latency or reactivation after de novo infection of iSLK cells. These experiments identified viral genes that play important roles in promoting or restricting lytic replication, reactivation, and spontaneous reactivation.

Chapter 2: Generation of a BAC16 mutant library and determination of viral gene essentiality

Introduction

Genetic screens have been an important tool in the field of biology to understand gene function in the context of an organism. An early example of this is the work of Nurse et al., who were studying the cell cycle in the fission yeast Schizosaccharomyces pombe[93,94]. They mutagenized the yeast and isolated temperature sensitive mutants which were then analyzed for the inability to complete the cell cycle under the restrictive temperature to identify genes which played a key role in cell cycle progression. Studies taking similar approaches were performed in other model organisms to identify genes responsible for various processes from circadian rhythm to neurotransmitter release[95,96]. As new techniques were developed, systematic genome-wide genetic screens could be performed to better understand the functionality of all genes in an organism, and were performed with some model organisms including E. coli, S. cerevisiae, and S. pombe[97–100]. Techniques to make targeted deletions in multicellular organisms were developed as well, including in organisms such as C. elegans and Drosophila[101,102].

In the field of virology, global deletion studies are especially useful in viruses with large genomes that are poorly studied. In some herpesviruses, the coding potential of the viruses is very large, so typically only a few viral genes are studied in detail. Consequently, a systemwide study can shed light on the functionality and biological relevance of poorly studied genes. Additionally, studying the disruption of a single gene in the context of the entire organism and its complete infection cycle can elucidate the roles of each open reading frame (ORF) in the viral life cycle and in the virus' pathogenesis, yielding avenues for new studies and targets for new vaccine or therapies. Dunn et al. used a bacterial artificial chromosome of the human cytomegalovirus (HMCV) genome, a DNA virus with a genome greater than 230 kb and with 162 ORFs, and a homologous recombination system in bacteria to generate a collection of 162 HCMV mutants, each with a deletion in a single ORF[103]. This mutant library, along with a cell culture system to propagate and study virus, allowed them to determine ORF essentiality or nonessentiality for virus replication, identify tropism factors for different cell types, and identify temperance factors which inhibited growth.

Kaposi's sarcoma-associated herpesvirus (KSHV) is another pathogenic herpesvirus, this one belonging to the gammaherpesvirus subfamily. KSHV causes two types of cancers - Kaposi's sarcoma and primary effusion lymphoma - and is associated with the plasmablastic variant of multicentric Castleman's disease, and KSHV inflammatory cytokine syndrome[2–5]. It is a DNA virus with a large coding capacity – the coding region is about 140.5 kb and contains at least 70 primary ORFs as well as upstream ORFs, small ORFs, and noncoding RNAs[13,14].

KSHV has been studied since its discovery in 1994, but the genome was so large that genetic manipulations were initially intractable. Early studies focused on only a handful viral genes and frequently cloned these genes into expression plasmids in order to study them in

isolation[104,105]. In 2002, a bacterial artificial chromosome model of the virus, BAC36, was first generated by the recombination of BAC vector sequences with the viral genome in BCBL-1 cells[61]. It was later discovered that the BAC36 contained a 9 kb duplication of a region of the genome containing ORFs 18, 17, 16, K7, K6, K5, part of ORF19, and the BAC cassette, and this duplication was found in the terminal repeats[65]. Consequently, this model was superseded in 2012 by the BAC16, which was generated from rKSHV.219. rKSHV.219 was produced from JSC1 cells, a primary effusion cell line coinfected with KSHV and Epstein-Barr virus[62–64]. In tandem with the iSLK cell line, this is a highly tractable model for generating and studying mutant viruses. The iSLK cell line has been modified to contain the viral transactivator (ORF50) under a tet-on system[66]. Ectopic expression of the transactivator is sufficient to induce the full cascade of viral gene expression. It should be noted that the iSLK cell line was originally thought to be of endothelial origin, however deep sequencing has revealed it is a contaminant of a known renal carcinoma line[67]. Nonetheless, the tight latency and inducibility of iSLK cells make it not only a useful cell line for virus generation, but also for studying KSHV.

Mutants in the BAC16 can be generated using a two-step red-mediated recombination method, which allows for insertion of long or short sequences, insertion of point mutations, and scarless deletions[68,69]. The BAC is maintained and manipulated in an E. coli clone, GS1783, which contains the requisite I-SceI and Red proteins encoded in the genome. After the two-step red-mediated recombination, the modified BAC DNA can be purified and analyzed to confirm its integrity. To reconstitute infectious virus, the BAC DNA can be transfected in permissive cells, typically either 293 or iSLK cells. Since the BAC sequences contain a hygromycin b resistance gene and GFP gene under control of the EF-1α promoter, hygromycin b treatment can be used to enrich for KSHV-infected GFP-positive cells[64]. In iSLK cells, doxycycline and sodium butyrate treatment is used to induce lytic replication, and after 72-96 hours, virus-containing supernatant can be collected. The GFP gene is especially important since it helps facilitate quantification of infectious viral particles by titration on permissive cells. Before the generation of recombinant KSHV, quantifying the amount of infectious virus was difficult – plaque assays were a standard technique in virology but since KSHV entered latency in most host cells in culture, there was no plaque formation[106]. Some labs used qPCR-based techniques to quantify viral DNA but this did not measure the infection potential of these genomes[107]. Another group noticed that infection of DMVEC with KSHV caused conversion of the normal cell morphology to spindle cells and that, as long as the titer of the inoculum was low enough, discrete spindle cell colonies could be counted to calculate the titer of the inoculum[59]. However, it was unknown if all infectious cells yielded spindle cell colonies, and the assays was neither particularly tractable, rigorous, or reproducible, especially since it required the use of primary cells. In 2001, a recombinant KSHV, rKSHV.152, was generated which constitutively expressed GFP under the EF-1α promoter. This allowed the quantification of infectious virus by infecting permissive cells and counting the number of GFP+ cells under the microscope one day after infection[108]. This technique eventually evolved to incorporate the use of flow cytometry to quickly and accurately count the proportion of GFP+ cells in a sample, which allowed the calculation of the viral titer[64].

Despite these advancements, until now there has not been a mutagenesis-based global analysis of the KSHV genome. While the number of ORFs studied has increased, only 18 ORFs have been studied by other labs through generating and analyzing single ORF disruption mutants in the BAC16[64,70,79–88,71,89–92,72–78]. Other labs have also generated mutants containing deletions for each miRNA[109]. We have made 90 mutants using the BAC16, each with a disruption of a single ORF, to cover all the canonical ORFs and some short and upstream ORFs. Seventy-two of our mutants have not been previously generated. Additionally, most of the ORFs involved in our study have also not been characterized except by bioinformatic analyses. This is the first global study on KSHV focusing on the functionality of KSHV ORFs.

In this study, we generated and classified each of the 90 ORF-disruption mutants based on their ability to generate infectious progeny upon doxycycline and sodium butyrate-mediated induction of lytic replication of mutant and WT BAC16-transfected iSLK cells. Mutants for ORFs that were nonessential for this process were assayed for infectious virus generation by de novo infection in iSLK cells and maintenance in conditions to induce either lytic replication, reactivation, or latency. Finally, nonessential mutants were also assayed for lytic antigen expression under conditions inducing latency or reactivation after de novo infection of iSLK cells. These experiments identified viral genes that play important roles in promoting or restricting lytic replication, reactivation, and spontaneous reactivation.

Materials and Methods

Cells, Viruses, Infection, and Antibodies

iSLK cells, the BAC16, and pEPkan-S were generous gifts from Britt Glaunsinger. 293T cells were purchased from ATCC (Manassas, VA). iSLK and 293T cells were cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Hyclone, GE Healthcare, Chicago, IL) and 1% PenStrep (Gibco, Thermo Fisher Scientific, Waltham, MA). BAC16-transfected cells were maintained in DMEM supplemented with 10% FBS, 1% PenStrep, and 1.2mg/ml Hygromycin B (Invitrogen, Carlsbad, CA). To reactivate transfected cells, cell lines were cultured in DMEM supplemented with 10% FBS, 1% PenStrep, 1ug/ml doxycycline (MilliporeSigma, Burlington, MA) and 1mM sodium butyrate (MilliporeSigma, Burlington, MA).

For infection, iSLK or 293T cells were seeded in plates 16-18 hours before infection. Appropriate concentrations of virus dilutions were made in the appropriate media and added to cells. Cells were spun in plates at 2000g for 1hr at 30°C, then put in a 5% CO_2 37°C incubator for an hour. The viral inoculum was removed and replaced with the appropriate media and this time point was designated t=0.

The following antibodies were used in this study: anti-ORF45 (2D4A5, Promab 20016), anti-K8α (8C12G10G1, Promab 20015), anti-LANA (LNA-1, Advanced Biotechnologies 13-210-100), Anti-K8.1A/B (4A4, Santa Cruz Biotechnology sc-65446), Goat anti-Rat IgG Alexa Fluor 568 (Life Technologies A11077), goat anti-rat AlexaFluor 568 (Abcam ab175710), goat anti-mouse

superclonal AlexaFluor647 (Life Technologies A28181), Goat anti-Mouse Alexa Fluor 568 (Invitrogen A-11004)

Primers

Primers were purchased from IDT (San Jose, CA), Invitrogen (Carlsbad, CA), and GenScript (Piscataway, NJ) (Table 2.1).

Sequences for primers used to generate revertants are as follows:
BAC16RevORF59
step 1 for: GAGTCCAAGCTTATGTCGCACACTTCCACC,
step 1 rev: GAGTCCGAATTCAACGAGTACAGGGCCTTG,
step 2 for:
GAGTCCACGCGTGAGCTATTCGGTGCGAATGTACTCGACGCTGGCATAGCCTTTTATCGAAAAGGATGACGACGATAAGTAGGG, step 2 rev: GAGTCCACGCGTAACCAATTAACCAATTCTGATTAG,
step 3 for: TCGTCTCCAGAACACCCAG,
step 3 rev: CAGATAACTGAAGAGCGACAGAG,
BAC16RevORF62
step 1 for: GAGTCCGCATGCTTCCATCAACAGCTTTGTCTGG,
step 1 rev: GAGTCCGAATTCTGAGAGATTGGGCACACATA,
step 2 for:
GAGTCCCCATGGTCCAGCGCCGGGAACCGGCAGGCCTAAACGTTACTATTTATGCCTCGTTGAGGATGACGACGATAAGTAGGG,
step 2 rev: GAGTCCCCATGGAACCAATTAACCAATTCTGATTAG,
step 3 for: TTGTCTGGTGAAGGCTCC,
step 3 rev: GGGACAGCTCCCAAGTGAA

Table 2.1. Primer Sequences

ORF	Stop codon or deletion	Insert Primers	cPCR Primers
K1	D	CCTGTCTTTCAGACCTTGTTGGACATCCCGTACAATCAAGCATTCAGGTAAGATAATCTAAGGATGACGACGATAAGTAGGG ATTATGTTATAGAGAATATTTAGATTATCTTACCTGAATGCTTGATTGTACGGGATGTCCAACCAATTAACCAATTCTGATTAG	CGGCCCTTGTGTAAACCTGTC GCACGGTTATACAATGTCCT
4	D	GTACATTAAAAGGACATTGTATAACCGTGCTACTTACAGCCCTAGACTTGCTCCAGTGTTAGGATGACGACGATAAGTAGGG AAGCAATCATAGCCCTGTCTAACACTGGAGCAAGTCTAGGGCTGTAAGTAGCACGGTTATAACCAATTAACCAATTCTGATTAG	AGGACATTGTATAACCGTGC GTTGTCTTGTATTGGTCGGT
6	D	TACACACGGGTTTTTTGTTGTCTTGGCCAATCGTGTCTCCTTGTGTACCCGTAACGATGGAGGATGACGACGATAAGTAGGG GACCGCCGCCAGTTCCTTTGCCATCGTTACGGGTACACAAGGAGACACGATTGGCCAAGAAACCAATTAACCAATTCTGATTAG	TATAGTGCGCGGTGTGGCAG GAGTAGTGTGCCGTGAAGGCT
7	SC	GACCTGGATTTGTAGTTGTGTACCCGTAACGATGGCAAAGTAGGTAGATAGGGAACTGGCGGCGGTCTATGCAGGATGACGACGATAAGTAGGG TGGCTAGGGCTGACACATCGGCATAGACCGCCGCCAGTTCCCTATCTACCTACTTTGCCATCGTTACGGGTAAACCAATTAACCAATTCTGATTAG	ACAGTCGGTAGTGGAGGAGC GCTCTGAAACTTCCCTGTAGTGA
8	SC	CTGTACCACCACCTGCAATTGAGCAACCACAATGACTCCCTAGGTAGATAGGAGGTCTAGATTGGCCACCCTAGGATGACGACGATAAGTAGGG CCAACAGGATGACAGTCCCCAGGGTGGCCAATCTAGACCTCCTATCTACCTAGGGAGTCATTGTGGTTGCTCAACCAATTAACCAATTCTGATTAG	GAGGAACCGAAACCCGCAGG CCACACTCTTAGGACCAGATGCTT
9	D	CTACTCGTTACACACAGACACAAATTACCGTCCGCAGATCTGACTCAGACGCGGAAACAGAGGATGACGACGATAAGTAGGG AAGAGGAAACTTTCTAGGCGCTGTTTCCGCGTCTGAGTCAGATCTGCGGACGGTAATTTGAACCAATTAACCAATTCTGATTAG	CAATCGCTAGACATCAGTCC CGTTATCTCCCAGTCACCTA
10.1	SC	CTTGCGCTATGTGGGACAACTAGAGTCCAACCTGGCAAGCTAGGTAGATAGGAGTGGAGCAAGACGCCAGACAGGATGACGACGATAAGTAGGG TATTTTTTTCGAGATCGGCTGTCTGGCGTCTTGCTCCACTCCTATCTACCTAGCTTGCCAGGTTGGACTCTAAACCAATTAACCAATTCTGATTAG	AGAACAACACGTCGGCAACC GGATTCTTAGCCGCGTGTAGT
10	SC	AACGTTCATCCTAGGTGACTGGGAGATAACGGTGTCTAACTAGGTAGATAGGTGCCGGTTTACTTGCAGCAGAGGATGACGACGATAAGTAGGG AAAGGGGGCCACATGTTAGGCTGCTGCAAGTAAACCGGCACCTATCTACCTAGTTAGACACCGTTATCTCCCAACCAATTAACCAATTCTGATTAG	CCACACTCTTAGGACCAGATGCTT CCACACTCTTAGGACCAGATGCTT
11AA	SC	CCACGTAGCGATTAGGGCCGACCGCCACGAGGAACCCATGTAGGTAGATAGGCAATCGTGACTGTCCGAGCAAGGATGACGACGATAAGTAGGG TCTGACTCCTGCGCCATATGTGCTCGGACAGTCACGATTGCCTATCTACCTACATGGGTTCCTCGTGGCGGTAACCAATTAACCAATTCTGATTAG	GCCAGGCACCATACAGCTTC GATTAGAGATGACGATGTGGCTCGG
11	D	GCCACGAGGAACCCATGCAATCGTGACTGTCCGAGCACATGTGTCCGGTTCCCACCCACAAGGATGACGACGATAAGTAGGG GAAAGCAATAAAGACAAATGTGTGGGTGGGAACCGGACACATGTGCTCGGACAGTCACGAAACCAATTAACCAATTCTGATTAG	CAGCTTCTACGACTGCAAGG GTGTGTGTTATGTATTCGGGTGG
K2	D	GTATATTAGTGTTATAAGAAATTTTATGTCACGTCGCTCTGGCTGCTAACGCGGCATACAAGGATGACGACGATAAGTAGGG GCTCGGGCACCACCGGCTAGTGTATGCCGCGTTAGCAGCCAGAGCGACGTGACATAAAATAACCAATTAACCAATTCTGATTAG	TGTTCCCATACGCGCCTGTC CTGGCGAGCAAGAGAGGGTTT
2	D	CTAACGCGGCATACACTAGCCGGTGGTGCCCGAGCGGGAGGCCGCGAGGGTATAGGTAAAAGGATGACGACGATAAGTAGGG ACACTGTGTGGTTGGTGGTGTTTACCTATACCCTCGCGGCCTCCCGCTCGGGCACCACCGAACCAATTAACCAATTCTGATTAG	CGCGTTCCAGATACCAGCAG GCTGGACCCTCCTCTCTAGTT
K3	D	GGTAAACACCACCAACCACACAGTGTGCTCTTATATACTTATCCTGAGAGAGAACCCACAAGGATGACGACGATAAGTAGGG GGGTTAATGCCATGTTTTATTGTGGGTTCTCTCTCAGGATAAGTATATAAGAGCACACTGAACCAATTAACCAATTCTGATTAG	TGGTATCAACCGCAACTACACAG GTGAGTGGCTGTAGCATTACC
70	D	GCCCACCACTCTGACCGCACGCTAAACATCGCCCTACCTGGATAGATCCTGGAAGTTTGTAGGATGACGACGATAAGTAGGG CTCTCCGGGCACAGGGCTTCACAAACTTCCAGGATCTATCCAGGTAGGGCGATGTTTAGCAACCAATTAACCAATTCTGATTAG	GATTCATGTAGACAACCCGCTC CGTGGGAACTGTGAGTAATGTG
K4	D	AGAGATCCGTCGCGTAAATGCGCAGCTGGCAAAGCATTCTAACTCCCCTCGTGTGTCCTCAGGATGACGACGATAAGTAGGG TCGCCGTTTCGCATTTACACGAGGACACACGAGGGGAGTTAGAATGCTTTGCCAGCTGCGAACCAATTAACCAATTCTGATTAG	CGCTGTTCTCCTGTAATTGG GGTGTTGGCCTTCGTAATAA
K4.1	D	TATGTCACAGACTCAACACACACGGGCCGTTACGCAACGGACAGTTCTGGCGCCACAACGAGGATGACGACGATAAGTAGGG TGCACCCCTTTGCGCATCATCGTTGTGGCGCCAGAACTGTCCGTTGCGTAACGGCCCGTGAACCAATTAACCAATTCTGATTAG	ACGACGGTTACAGGTCCCTC CATTTGGCAACGCTGGTCTT
K4.2	SC	ACATAATTTATGCACATAAAAGGATTAGCGCATGCAAATTTAGGTAGATAGGAGCTTTGCCGAAGTTCTCGGAGGATGACGACGATAAGTAGGG CAGCGCGCCCCACCGGCTTTCCGAGAACTTCGGCAAAGCTCCTATCTACCTAAATTTGCATGCGCTAATCCTAACCAATTAACCAATTCTGATTAG	CTGGCCGCAATAGCTCAATC CCGCTAACAGCACCAAATCCAC
K5	D	GGGCGTCACGTCACATATCTCTGTGCACCCAAGTGGTTGTCTCTGCAGCTGGGGTGGAAGAGGATGACGACGATAAGTAGGG TCCCCTTTCCCTTTTTCAGACTTCCACCCCAGCTGCAGAGACAACCACTTGGGTGCACAGAACCAATTAACCAATTCTGATTAG	AGGACAGATTTGGGCACAGG CAGGGTGGGTTGTGACAGTT
K6	D	GAGCAGTTGGGCCGCAGTGATATCTTCAACTTTCGACCGTCTGGAGGTGCCAAGTTCGCAAGGATGACGACGATAAGTAGGG TACGGTTTTCTTTAGACTGTTGCGAACTTGGCACCTCCAGACGGTCGAAAGTTGAAGATAAACCAATTAACCAATTCTGATTAG	CATTTCTTCCTCGACAGGTCTTC GCATGTAAGCTGGCGGTTAG
K7	SC	TCCAAAAATGGGTGGCTAACCTGTCCAAAATATGGGAACATAGGTAGATAGGCTGGAGATAAAAGGGGCCAGAGGATGACGACGATAAGTAGGG CAGTGCTAAACTGACTCAAGCTGGCCCCTTTTATCTCCAGCCTATCTACCTATGTTCCCATATTTTGGACAGAACCAATTAACCAATTCTGATTAG	TTATGGATTATTAAGGGTCAGCTTG CGACGCAATCAACCCACAAT
16	D	GGTGCTGTGCGCGTGCTATGTTCCCTGGTGACCGTCCACACGCGTAATTCGAGGTCCCCGAGGATGACGACGATAAGTAGGG	GGAACCAGCTTGGTGATGTG

		CATGCAACCATCTACTCTTCCGGGGACCTCGAATTACGCGTGTGGACGGTCACCAGGGAAAACCAATTAACCAATTCTGATTAG	CAAGCAGTAGCGAACAGTTACG
17	SC	CTCGGTCTCACACACGTATTTTCCGAGCATGGCACAGGGCTAGGTAGATAGGCTGTACGTCGGAGGGTTTGTAGGATGACGACGATAAGTAGGG	GGCAGGACACAACATCTACAAAC
		TGGGGCAGGACACAACATCTACAAACCCTCCGACGTACAGCCTATCTACCTAGCCCTGTGCCATGCTCGGAAAACCAATTAACCAATTCTGATTAG	CATTGGCAGTAGCCTCCCTTAA
18	SC	GCCGCTGAGCCCGGGGCTTAGGAGGCTCATGTGGCGCTTTTAGGTAGATAGGTTGCAAAATAAGAATTTAAAAGGATGACGACGATAAGTAGGG	TACCGTGGGACACTTGAAATAGAC
		GCTCTTGGGCGTGGAATGTATTTAAATTCTTATTTTGCAACCTATCTACCTAAAAGCGCCACATGAGCCTCCAACCAATTAACCAATTCTGATTAG	GCTCTTGGGCGTGGAATGTA
19	SC	GGACCGGCTTGTTCAGGTCCATGACTCACGCGTCCGCGTCTAGGTAGATAGGATTAACGCAGATATCGACGCAGGATGACGACGATAAGTAGGG	CATTACATCGCTCACTCGGCCC
		ATCTTCGGTTTTTTGGGTACCTTAGATAGGACCTTTCTGACCTATCTACCTATGTCAGCATTTTCTCTAGCAAACCAATTAACCAATTCTGATTAG	CGGTGAAGTTACGGTGGCTG
20	SC	CGAGTCCGCTCCAAAACCGCCTTCTGCCATGGTACGTCCATAGGTAGATAGGACCGAGGCCGAGGTTAAGAAAGGATGACGACGATAAGTAGGG	GATCCATTGGCGCGTAGTCTC
		CTGGAAGCCTGCTCAGGGATTTCTTAACCTCGGCCTCGGTCCTATCTACCTATGGACGTACCATGGCAGAAGAACCAATTAACCAATTCTGATTAG	AGCTCGGACGACGAATCAAG
21	SC	TTTCTTAACCTCGGCCTCGGTTGGACGTACCATGGCAGAATAGGTAGATAGGGGCGGTTTTGGAGCGGACTCAGGATGACGACGATAAGTAGGG	CGGCAATTCTGTGCCCTAGAG
		TTTCTCCGCCGCGCCCCACCGAGTCCGCTCCAAAACCGCCCCTATCTACCTATTCTGCCATGGTACGTCCAAAACCAATTAACCAATTCTGATTAG	GTGCTTGATTCGTCGTCCGAG
22	SC	AGTCTAAAGCAGTTAATCACCTAGAGGAGACATGCAGGGTTAGGTAGATAGGCTAGCCTTCTTGGCGGCCCTAGGATGACGACGATAAGTAGGG	CCCGTCGTGATCGAGCTTTG
		ATATGCATCGCCAGCATGCAAGGGCCGCCAAGAAGGCTAGCCTATCTACCTAACCCTGCATGTCTCCTCTAGAACCAATTAACCAATTCTGATTAG	GGAGCGGGTTATTGTCGTCG
23	SC	CGTACGTTGCGTCCGTCCGCTGGTCTAAGCTATGTTACGATAGGTAGATAGGGTTCCGGACGTGAAGGCTAGAGGATGACGACGATAAGTAGGG	GTCATCGGTTCGCCGCTCTA
		GCGCCGCGCCCTCTACTAGACTAGCCTTCACGTCCGGAACCCTATCTACCTATCGTAACATAGCTTAGACCAAACCAATTAACCAATTCTGATTAG	GAACAACAGTGGCATCGGGAC
24	SC	CGGGAAAGGTCGTTGCTCCAAGGTCGCCTCCATGGCAGCGTAGGTAGATAGGCTCGAGGGCCCCCTACTACTAGGATGACGACGATAAGTAGGG	GGACACTGCCTTCTCTGGCG
		TCAGGGAGGCGCTCGGTGGCAGTAGTAGGGGGCCCTCGAGCCTATCTACCTACGCTGCCATGGAGGCGACCTAACCAATTAACCAATTCTGATTAG	CTTCTAAGGTCAGCTCTGCCTGC
25	SC	TGGCAGTAGTAGGGGGCCCTCGAGCGCTGCCATGGAGGCGTAGGTAGATAGGACCTTGGAGCAACGACCTTTAGGATGACGACGATAAGTAGGG	AGTAGTCTGCGTATCGCTCTGC
		CCTCCGTGGCGAGGTACGGGAAAGGTCGTTGCTCCAAGGTCCTATCTACCTACGCCTCCATGGCAGCGCTCGAACCAATTAACCAATTCTGATTAG	CCTTGCCGAGCAATAGCTGAAA
26	SC	TATTAGCTAACCCTTCTAGCGTTGGCTAGTCATGGCACTCTAGGTAGATAGGGACAAGAGTATAGTGGTTAAAGGATGACGACGATAAGTAGGG	CGTCGTGGTCAACGGTACAG
		CGAAGAGTCTGGAGGTGAAGTTAACCACTATACTCTTGTCCCTATCTACCTAGTTGGCTAGTCATGGCACTCAACCAATTAACCAATTCTGATTAG	CAATCTCCGAGCGGCAGTAC
27	SC	GAGACTTTGGCGGCCTCCTGTTGGTATTCCCCACGCTAACTAGGTAGATAGGGATTTGAAGCGGGGGGGGGGGAGGATGACGACGATAAGTAGGG	TCTGGACGTAGACAACACGGATC
		GAATATCAGATGACGCCATACCCCCCCCCCGCTTCAAATCCCTATCTACCTAGTTAGCGTGGGGAATACCAAAACCAATTAACCAATTCTGATTAG	GAGCCGTCATCCGTCCTTGC
28	D	CTCAGTTGAGAGTCAGAGAATACAGTGCTAATCAGGGTAGAACGGGGTGTGTGCTATAATAGGATGACGACGATAAGTAGGG	TTTACGCTAAGAGTTGGGTGCTTG
		TACAGTGCTAATCAGGGTAGAGCCCCCCCCATAGCCATCCATTATAGCACACACCCCGTTAACCAATTAACCAATTCTGATTAG	GGACGACTTACTTGTGGCAGT
29b	SC	AAAGGATGCACTGCCGGCTATTCTGGGTTTCATGCTTCAGTAGGTAGATAGGAAAGACGCCAAGCTTATATTAGGATGACGACGATAAGTAGGG	GTTCGCCTCCTCTCCTTTACTGTTA
		ACGAGTTCACGGATGATATAAATATAAGCTTGGCGTCTTTCCTATCTACCTACTGAAGCATGAAACCCAGAAAACCAATTAACCAATTCTGATTAG	GGTGGAAATCTTCGCGGTGG
30.1	SC	TACGTAGAGCAGGTTAAAGGTCTGTCCCCGAATGCTCTGCTAGGTAGATAGGAGACACGGAAAGACACAAAAAGGATGACGACGATAAGTAGGG	TGGGCGTTCCTGAGGTTAAG
		TAGCCGCTTATGAGCCCCTCTTTTGTGTCTTTCCGTGTCTCCTATCTACCTAGCAGAGCATTCGGGGACAGAAACCAATTAACCAATTCTGATTAG	CCAAGAACAAGAGCCACGCA
30	SC	GGGAATAAAAGGGGGCGTGTGTGCCGATCGTATGGGTGAGTAGGTAGATAGGCCAGTGGATCCTGGACATGTAGGATGACGACGATAAGTAGGG	TCTGAAGCATGAAACCCAGAATAG
		CAAAATCTTTCTCATTCACCACATGTCCAGGATCCACTGGCCTATCTACCTAGTGCCGATCGTATGGGTGAGAACCAATTAACCAATTCTGATTAG	CGACCACTTGCTCCCTAAGG
31	SC	TGTGCCGCCTAGACACCGGTGCGAAATGAAGAGTGTGGCGTAGGTAGATAGGAGTCCCTTATGTCAGTTCCAAGGATGACGACGATAAGTAGGG	CAGTAAAGGAGAGGAGGCGAAC
		GGTACAGGCAAAACACGCCGTGGAACTGACATAAGGGACTCCTATCTACCTACGCCACACTCTTCATTTCGCAACCAATTAACCAATTCTGATTAG	GGACTCGCCACACTCTTCATT
32	SC	ACGACTGCATTGCCAAGCGGGTGCGGACAAAATGGATGCGTAGGTAGATAGGCATGCTATCAACGAAAGATAAGGATGACGACGATAAGTAGGG	CGTGCAGGAAATAGCCCTGG
		GGTGGCAGCGAGGACCTACGTATCTTTCGTTGATAGCATGCCTATCTACCTAGTGCGGACAAAATGGATGCGAACCAATTAACCAATTCTGATTAG	GTGATGCAGCAGAAAGGTCCT
33	SC	AAGGAGGATCTGGTGTTCATTCGAGGCCGCTATGGCTAGCTAGGTAGATAGGCGGAGGCGCAAACTTCGGAAAGGATGACGACGATAAGTAGGG	ACGGACGGTGACTGGTTAGAG
		TGCATTCCTTGTTTAGGAAATTCCGAAGTTTGCGCCTCCGCCTATCTACCTAGCTAGCCATAGCGGCCTCGAAACCAATTAACCAATTCTGATTAG	GAGATAGAGGTGCAGGCGTTAAAG
29a	SC	ACCCTCGGACACGAGCGAGCTCAAAGCAAACATGCTGCTCTAGGTAGATAGGAGCCGTCACAGGGAGCGCCTAGGATGACGACGATAAGTAGGG	GCAGATTGGCTGGACCTCGG
		TCTCCTGCAGGTTGGCGGCAAGGCGCTCCCTGTGACGGCTCCTATCTACCTAGAGCAGCATGTTTGCTTTGAAACCAATTAACCAATTCTGATTAG	GCACTCAGTTCCCACCTCTCTC
34	SC	ATCCGTGCCGTTTTGGGACAGTGTCGCGTGAATGTCGGGGTAGGTAGATAGGCACTCAGTTCCCACCTCTCTAGGATGACGACGATAAGTAGGG	CGTTGTCCTCGGACGGTCTG
		GAGACCGCCAAAGACGCCGGAGAGAGGTGGGAACTGAGTGCCTATCTACCTACCCCGACATTCACGCGACACAACCAATTAACCAATTCTGATTAG	CTACTGGTCACCTCCGGGTCA
34	SC	GGCAAGGCGCTCCCTGTGACGGCTGAGCAGCATGTTTGCTTAGGTAGATAGGTTGAGCTCGCTCGTGTCCGAAGGATGACGACGATAAGTAGGG	GATTTGCTGACGTGGGCGTG
		TGGTCACCTCCGGGTCACCCTCGGACACGAGCGAGCTCAACCTATCTACCTAAGCAAACATGCTGCTCAGCCAACCAATTAACCAATTCTGATTAG	CCAGGTGTGTTCTCGCTAAGGT
35	SC	ATACCGGCGATCATCACCATGATCAAGGAGAATGGACTCATAGGTAGATAGGACCAACTCTAAAAGAGAGTTAGGATGACGACGATAAGTAGGG	TTACATTTCCCACACCTGCCTC
		CCTCCAGAGCCGACTTAATAAACTCTCTTTTAGAGTTGGTCCTATCTACCTATGAGTCCATTCTCCTTGATCAACCAATTAACCAATTCTGATTAG	CTCAAACACGGCGCTGCTAC
36	SC	TTGCCCCCGGTGTGCCCTGAAACTCCCTAAGGCTACCCGGTAGGTAGATAGGATTTCAGAGAGACCCTGGGCAGGATGACGACGATAAGTAGGG	AGTTTCAGGACGCACAGCATG
		GATTCAGCTGCCATGTGGACGCCCAGGGTCTCTCTGAAATCCTATCTACCTACCGGGTAGCCTTAGGGAGTTAACCAATTAACCAATTCTGATTAG	GCGCTTTAAGATACGCGGTGG

37	SC	AGAAAGCTACTAGAGCGAGACTTTTTCAACCATGGAGGCCTAGGTAGATAGGACCCCCACACCCGCGGACTTAGGATGACGACGATAAGTAGGG	GATTCCCATGCGACAGGAGC
		CCAGATAGTCTTCAGAAAACAAGTCCGCGGGTGTGGGGGTCCTATCTACCTAGGCCTCCATGGTTGAAAAAGAACCAATTAACCAATTCTGATTAG	CAGTCTCGAACCTTGGCGTG
38	SC	GAAGTGCGAAGGACACCTTTCCATATATCAAATGGGATTTTAGGTAGATAGGCTCCTATCTATCTGCAAACGAGGATGACGACGATAAGTAGGG	CCTGTCAACTGTACCATCGGTG
		CGTCTACGGGCTGTGAGGGACGTTTGCAGATAGATAGGAGCCTATCTACCTAAAATCCCATTTGATATATGGAACCAATTAACCAATTCTGATTAG	GGATTGCGATTGCTCAAGCA
39	D	ATGGAGGAAGAGGGATGGGTTTATAATGCCAATATATCAGGTTTCTCGGTCTTTTTAACTAGGATGACGACGATAAGTAGGG	GGGATGGAGGAAGAGGGATG
		CCGCAGCGCCGCCTGGCGAAAGTTAAAAAGACCGAGAAACCTGATATATTGGCATTATAAAACCAATTAACCAATTCTGATTAG	GGACGTGAACGCTGTGAAAG
40	D	GCCCCGGGCAGAAGCCAGAGGTAGTCGACTCATTGACTCAAGCGGAGAGGGGGTGGTGCGAGGATGACGACGATAAGTAGGG	GCGGGAGAGCCAATCTGATG
		AAACCCGTCAACTGCCAACTCGCACCACCCCCTCTCCGCTTGAGTCAATGAGTCGACTACAACCAATTAACCAATTCTGATTAG	CCTTTAGAGTAAACCCGGCCATC
41	SC	AGAATTCAAGGATCTCAAAAGGGCCTGCCAGATGGCCGGGTAGGTAGATAGGTTTACTCTAAAGGGGGGGACAGGATGACGACGATAAGTAGGG	AGGTGAGACCTACTGTCCCTG
		AGAATACAAGATCCCCCGAAGTCCCCCCCTTTAGAGTAAACCTATCTACCTACCCGGCCATCTGGCAGGCCCAACCAATTAACCAATTCTGATTAG	GGTGATGTGACGTGGGTTAGG
42	SC	AAATACTGTCTAGTTACACCACCCTTCGAGAATGTCCCTGTAGGTAGATAGGGAAAGGGCCCTGGCGAGACTAGGATGACGACGATAAGTAGGG	GATCGCGGACCTGCTTCAGATG
		TACTCATTGGCACTCCAGTCAGTCTCGCCAGGGCCCTTTCCCTATCTACCTACAGGGACATTCTCGAAGGGTAACCAATTAACCAATTCTGATTAG	TCTAAGTGGCCCATCACGGAC
43	SC	CACTACGCTCCTGACTTTGGCATCCGATGTCATGTTGAGGTAGGTAGATAGGATGAACCCGGGGCTGGGCTCAGGATGACGACGATAAGTAGGG	CTTCGTCTTCCAGTGGATCGA
		AAGGGTGCACTGATATGGACGAGCCCAGCCCCGGGTTCATCCTATCTACCTACCTCAACATGACATCGGATGAACCAATTAACCAATTCTGATTAG	ATAAGAATACTTGCCTTGCAGGATC
44	SC	ATACTTGCCTTGCAGGATCTCAAAGAGGGAGATGGACAGCTAGGTAGATAGGTCGGAAGGGTGCACTGATATAGGATGACGACGATAAGTAGGG	ACTCGTATGTCCTCCAGTCG
		ACCCGGGGCTGGGCTCGTCCATATCAGTGCACCCTTCCGACCTATCTACCTAGCTGTCCATCTCCCTCTTTGAACCAATTAACCAATTCTGATTAG	CAACCTGTCAATCTGTTCCACTAC
45	D	ACACCTATAATGGTCTGTATTGACACCATTCTTTTATTTAGGCCTTGTACGGGGTTGACCAGGATGACGACGATAAGTAGGG	ACTCTGATCTACTGACCCGTACC
		TGTAAATTTCCGCCCCTAGCGGTCAACCCCGTACAAGGCCTAAATAAAAGAATGGTGTCAAACCAATTAACCAATTCTGATTAG	CCAACGACTATTTGACTCGCCAC
46	D	GCTAGGGGCGGAAATTTACAAAGCACACGAGTTATTGCCTGTTGAACTTATTTTCCCTTTAGGATGACGACGATAAGTAGGG	TGGAAGCATTCTCTCTTCATCGTG
		CGGAGAGCGCACGCAGGTCAAAAGGGAAAATAAGTTCAACAGGCAATAACTCGTGTGCTTAACCAATTAACCAATTCTGATTAG	CGAAGTTTGACGGCCTATACTGTA
47	D	CCTAAAGACCGTCTGTTGCAACCATGCGTCCATGTTGAACGGGGCAAATCCGGGTTTCACAGGATGACGACGATAAGTAGGG	CTGAGCAGCGAGAGCAGTTTC
		CCGAACCAGGCAACACAAGGGTGAAACCCGGATTTGCCCCGTTCAACATGGACGCATGGTAACCAATTAACCAATTCTGATTAG	CCTCATTAGTCGGGACTCGC
48	D	GGAAGACGATGGGGGAAATGTGGCATTACCTGACACGGTTGTTCAGTCACATGTACGCTAAGGATGACGACGATAAGTAGGG	GCGAGTCCCGACTAATGAGG
		GGGGTTGGGTGGGGAGACCCTAGCGTACATGTGACTGAACAACCGTGTCAGGTAATGCCAAACCAATTAACCAATTCTGATTAG	GTCATTGCCACCCAGCTACT
49	D	ACAAAAATGGCGCAAGATGACAAGGTAAAGATCGACCTTTTGTAAAAACTATGACACGCCAGGATGACGACGATAAGTAGGG	CAGTAGCTGGGTGGCAATGAC
		TCTTACCCTAAGGAGAGAGTGGCGTGTCATAGTTTTTACAAAAGGTCGATCTTTACCTTGAACCAATTAACCAATTCTGATTAG	GGTCACTGGGATCGTAGATTGTTTC
50	D	ATGTCATTGTAAAAACTATGACACGCCACTCTCTCCTTAGAGTGTTCGCAAGGGCGTCTGAGGATGACGACGATAAGTAGGG	TCTGGATTGAGGGTGGCGAC
		CTGGGAAGTTAACGCAGGCACAGACGCCCTTGCGAACACTCTAAGGAGAGAGTGGCGTGTAACCAATTAACCAATTCTGATTAG	TCGACAACGGAGGAAATACCAC
K8	D	GTCGACTATAACCTGGCGTGTAAACGTGTAACCCTGCCAAACGGGAAACAGGTGTCTATCAGGATGACGACGATAAGTAGGG	CACGAATCTGGTTGATTGTGAC
		TTGAGTAACCAGCCGGCCAAGATAGACACCTGTTTCCCGTTTGGCAGGGTTACACGTTTAAACCAATTAACCAATTCTGATTAG	CTGTTCCTTATGTGCCTCCA
K8.1	D	GGACCGAAGTTAATCCCTTAATCCTCTGGGATTAATAACCTGGTGCTAGTAACCGTGTGCAGGATGACGACGATAAGTAGGG	GGGAGAACCATGCCAGACTTTG
		TGTAGTGGTGGCAGAAAATGGCACACGGTTACTAGCACCAGGTTATTAATCCCAGAGGATAACCAATTAACCAATTCTGATTAG	CATCGTGGAACGCACAGGTAA
52	D	GTTTGGGGGTTGGGTTGTGGCGTGGTGGCTGGTCCGCGGTGTCAGGTACGCGTAGATGTAAGGATGACGACGATAAGTAGGG	GCGTAAGAAACCCTACATAGTG
		TTGTGAGCATCACCAACACGTACATCTACGCGTACCTGACACCGCGGACCAGCCACCACGAACCAATTAACCAATTCTGATTAG	GCATAGACTGGCATGTGATT
53	D	TAGCTTTCGTCAGCGCTTGTGCGAGTAATCACATGCCAGTTATGAACAACCGCCGAGGCTAGGATGACGACGATAAGTAGGG	GTAGATGTACGTGTTGGTGATGCTC
		ACGTTGGATAGACGGCTTGGAGCCTCGGCGGTTGTTCATAACTGGCATGTGATTACTCGCAACCAATTAACCAATTCTGATTAG	GTCTGGCTTCATTTGCTCTCGA
54	D	GGCCACAAATAAAGCCAGGGCCACCGTGGACGCTGTCATTAGCCGCCGCCAAATGCGGCCAGGATGACGACGATAAGTAGGG	TACTGGGACTAGAACGCTCTG
		TCGAATCGCCCTAATAAACTGGCCGCATTTGGCGGCGGCTAATGACAGCGTCCACGGTGGAACCAATTAACCAATTCTGATTAG	GATAGGTTCGACATAGGTTGGCT
55	SC	GCCTCGCGGCGGTATGTCGTCTCCATGGTACACCTGGACGTAGGTAGATAGGTGTTGCGGTATAAACCTTTTAGGATGACGACGATAAGTAGGG	GCTGTCGCCACTCGTACAAA
		AAGCGTGGTTGCCGCGTCCAAAAAGGTTTATACCGCAACACCTATCTACCTACGTCCAGGTGTACCATGGAGAACCAATTAACCAATTCTGATTAG	GTGCCAAGGTTCGACTGGAC
56	SC	CACGTCCAGGTGTACCATGGAGACGACATACCGCCGCGAGTAGGTAGATAGGGCGCTGACAGTAAGGGTTATAGGATGACGACGATAAGTAGGG	ACGTCACCCAGACACACTCC
		TGTCGCCACTCGTACAAAAAATAACCCTTACTGTCAGCGCCCTATCTACCTACTCGCGGCGGTATGTCGTCTAACCAATTAACCAATTCTGATTAG	GCTGTCGCCACTCGTACAAA
57	D	AATATAAGAACCAAAGGACATGGTACAAGCAATGATAGACGGATTGCCAAACCCCATGGCAGGATGACGACGATAAGTAGGG	AACCAAAGGACATGGTACAAGC
		TGGAATACGGGAGACACTCTGCCATGGGGTTTGGCAATCCGTCTATCATTGCTTGTACCAAACCAATTAACCAATTCTGATTAG	GGTGCGATTCACGGTAGAGAAG
K9	D	CTCCCTCCCATAACAATACGGTGTAGGCATTTTGTATTATTGTCCCGCAACCAGACTAGCAGGATGACGACGATAAGTAGGG	CACCTTAAACACAACACCAGACC
		CACTGGACATTGCGGCGCGAGCTAGTCTGGTTGCGGGACAATAATACAAAATGCCTACACAACCAATTAACCAATTCTGATTAG	CCAGGCAATTCTGCGGCTAG
K10	D	ACTACAAGATTACATCCGGTTTTATAATTCACATATATGAACCTGAGGTAGATGCGCCCTAGGATGACGACGATAAGTAGGG	TGAATGGTAACTGTCTGGACAC

		CGTGTGGATACCAGTGAATGAGGGCGCATCTACCTCAGGTTCATATATGTGAATTATAAAAACCAATTAACCAATTCTGATTAG	GAGAACAAAGCTACGAGGAGG
K10.5	D	CCACAGCCCGTCAAACCACAGGGACCCTGTTGGCTGACTACAATGCACATGCAGATTCTTAGGATGACGACGATAAGTAGGG	CTCTTGACCTGGTAACCCTGG
		TGACCTCACACTGCTTGATAAAGAATCTGCATGTGCATTGTAGTCAGCCAACAGGGTCCCAACCAATTAACCAATTCTGATTAG	CAGTTGATGATGCCAATGCCG
K11	SC	GGTGGGGGCTCAGGGTTTTGTAGGGAGGGATATGCACAGTTAGGTAGATAGGTTGTTTTTTGAAGAGCCAGAAGGATGACGACGATAAGTAGGG	GTCGCCACGCCCACAACATC
		ATGACCCAAACCCCGACGGTTCTGGCTCTTCAAAAAACAACCTATCTACCTAACTGTGCATATCCCTCCCTAAACCAATTAACCAATTCTGATTAG	CCCGTTGGCAAACATAGATCCGTC
58	D	TCATGGTCAACAAACCAAGAAAAACACATGTATTATTCAAGGTGTCAAATCAGGGGGTTAAGGATGACGACGATAAGTAGGG	GCACTGTCCACCCTCTAATACAAG
		AAGGTGCCCAAAACCACATTTAACCCCCTGATTTGACACCTTGAATAATACATGTGTTTTAACCAATTAACCAATTCTGATTAG	CTCACACCAGTTGGTCCCTTTG
59	D	GAGCGACAGAGCGCGCTCACTGTCCAGGCGGCACATGGTGGATTGCGGCCGTAGACGCACAGGATGACGACGATAAGTAGGG	GGTAAAGAGTGTGAACGAGTACAGG
		AGCTTTCCTGTGATTTCTCTGTGCGTCTACGGCCGCAATCCACCATGTGCCGCCTGGACAAACCAATTAACCAATTCTGATTAG	GTGTGACTGACGATTTGTGAAGGT
60	D	TTAGGGGAGGTGGAAGTGTGCGACATGGACAGGTTAACCTTGGCCTCACCCGGCTTGCAGAGGATGACGACGATAAGTAGGG	GCCGTAGACGCACAGAGAAATC
		GTCTGTCAGTAGGTAGGTCTCTGCAAGCCGGGTGAGGCCAAGGTTAACCTGTCCATGTCGAACCAATTAACCAATTCTGATTAG	CTTCAGTGCCTGGCAGATCC
61	D	AACTGAATCCATTGGCCTCACCCGGCTTGCAGAGACCTACGACCTTACAGAAACACAGTCAGGATGACGACGATAAGTAGGG	CGTGGGAAACATCAAGGTGC
		GAGGCCGCGTGTGGCCCCTGGACTGTGTTTCTGTAAGGTCGTAGGTCTCTGCAAGCCGGGAACCAATTAACCAATTCTGATTAG	GCACAGTTCCCTTTGATTCTCATC
62	D	TCCCAAGTGAACCTGACAAAATGTCCGGACAGACATGACCATCCACGCCGGCAATGGACGAGGATGACGACGATAAGTAGGG	GTCTATGAGAGATTGGGCACAC
		CTTTTCAAGAGCGTCTGTGCCGTCCATTGCCGGCGTGGATGGTCATGTCTGTCCGGACATAACCAATTAACCAATTCTGATTAG	GCTCTGTTGTGCTGCTGTTTA
63	D	AGCCGCATTTTCAGCCTGCACCTTCATATCCACGCCGGCAAGGCCATGGCAGCCCAGCCTAGGATGACGACGATAAGTAGGG	GTGGACGCCGCATATTTAGAGAG
		GCCATTCCCTCCATGTACAGAGGCTGGGCTGCCATGGCCTTGCCGGCGTGGATATGAAGGAACCAATTAACCAATTCTGATTAG	GGTACATGACGCAGTTGCTGA
64	D	CGCTGGCAGGCCTCCGGAAACTGTTTGTCGAATAGAGGCCCTCCACGGTTGTCCAATCGTAGGATGACGACGATAAGTAGGG	GAAACGTACTCCCGGTCTGC
		CTGGCAAAAAGAAATAGGCAACGATTGGACAACCGTGGAGGGCCTCTATTCGACAAACAGAACCAATTAACCAATTCTGATTAG	GTATAACCACCCTGTCCTCTGGT
65	D	AGAAGTGGTACTTGTGACTCCACGGTTGTCCAATCGTTGCCTTCCACACAGGCGGGCGAAAGGATGACGACGATAAGTAGGG	GGTCACCCATAGTACCCATCAG
		AGTGTTCCTCCTGAGGCTATTTCGCCCGCCTGTGTGGAAGGCAACGATTGGACAACCGTGAACCAATTAACCAATTCTGATTAG	CTGCGAGGCTGCCCTATTAA
66	SC	TAGGCCGTGCGGTCGCGCTGGTGAGAAGGTCATGGCCCTGTAGGTAGATAGGGATCAGCGCTGGGATCGCTTAGGATGACGACGATAAGTAGGG	GTCACAGCGGTATATTGGGC
		AACCAAACCAAGACACAAGAAAGCGATCCCAGCGCTGATCCCTATCTACCTACAGGGCCATGACCTTCTCACAACCAATTAACCAATTCTGATTAG	AAAGCCACATATTCCTCCACTG
67	SC	GGGGCCTTGCCAGCCCCACCCCGCTGTCGCCATGAGTGTCTAGGTAGATAGGGTTGGTAAGCGTGTAGTGGAAGGATGACGACGATAAGTAGGG	GTTGGAGAGCAAGGTGGACACG
		ATACCACCCGACACAGTTCGTCCACTACACGCTTACCAACCCTATCTACCTAGACACTCATGGCGACAGCGGAACCAATTAACCAATTCTGATTAG	ACCGTGCTGCATTCTAACCGTAC
68	SC	GTCTGACCAGCTTCTGCCTCGTGACATGCAAATTTTATTTTAGGTAGATAGGCCCACGATCTATTGTAGATTAGGATGACGACGATAAGTAGGG	GAGAGTTGGAAGAGACGCGGG
		GACAGTAGTTGATGGCGTTCAATCTACAATAGATCGTGGGCCTATCTACCTAAAATAAAATTTGCATGTCACAACCAATTAACCAATTCTGATTAG	CCACCTTGCTCTCCAACACCAG
68	SC	TCTACAATAGATCGTGGGAAATAAAATTTGCATGTCACGATAGGTAGATAGGGGCAGAAGCTGGTCAGACGCAGGATGACGACGATAAGTAGGG	CCACCTTGCTCTCCAACACCAG
		TGGAACCCAACATGGAGTACGCGTCTGACCAGCTTCTGCCCCTATCTACCTATCGTGACATGCAAATTTTATAACCAATTAACCAATTCTGATTAG	GAGAGTTGGAAGAGACGCGGG
69	D	ACGCTTGAGCTGGTCCCGGGCCTTCGCACCCCATCCACCGCCTCACATGTAGCCTGTCACAGGATGACGACGATAAGTAGGG	TTATTCGGGAGCTAACCGCAC
		CAGTTGCAATAGGAGCTGGGGTGACAGGCTACATGTGAGGCGGTGGATGGGGTGCGAAGGAACCAATTAACCAATTCTGATTAG	CACCTTTCATGGCAGTACATTGC
K12	D	ATTTTATTTTACTGACACTCTTTGGGAGGGCACGCTAGCTGCATTGGGATTGGAGTGAGGAGGATGACGACGATAAGTAGGG	CGGCCTTCTGAACTGTGACTG
		AACCTGGTGCCCTCCTCCCTCCTCACTCCAATCCCAATGCAGCTAGCGTGCCCTCCCAAAAACCAATTAACCAATTCTGATTAG	CTCCTCCCTCCTCACTCCAATC
K13	D	CATACATTCTACGGACCAAAAATTAGCAACAGCTTGTTATGGTGCCGGCTTGTATATGTGAGGATGACGACGATAAGTAGGG	TCGCCTTCAAACAGAAGCACG
		TTTTTCCACATCGGTGCCTTCACATATACAAGCCGGCACCATAACAAGCTGTTGCTAATTAACCAATTAACCAATTCTGATTAG	GATGTTTCCGTTCTACAGGCGG
72	D	AAGGAAAATTTATTTTTCCGCCCTAAACAAAATCACAAGCATAGAGTGGCGAGCGTATGTAGGATGACGACGATAAGTAGGG	TGTCATCCGTGCCCAGTTTC
		CCAGGCTCTAGAGGTAGGCCACATACGCTCGCCACTCTATGCTTGTGATTTTGTTTAGGGAACCAATTAACCAATTCTGATTAG	GTTCTCACGACCCATCTACCTC
73	D	GGTGGCTTCTAGGGAGGAAAAAGGGGGAGAGGTGTGGCTTCCTCGGGAAATCTGGTCTGAAGGATGACGACGATAAGTAGGG	ATGGGTCGTGAGAACACTGC
		CCATAATTTTACTTTGGTTGTCAGACCAGATTTCCCGAGGAAGCCACACCTCTCCCCCTTAACCAATTAACCAATTCTGATTAG	CTGCGATCTCCATCCTGTGG
K14	D	TGCTCCCCCGTGGACGACGCCGAGTGCCTCTCGGGGGTCCCTAGATGGACACCCCGTGAAAGGATGACGACGATAAGTAGGG	AAACTGAAGAAGGCGTGTCTGC
		GGGGTGGGTAAGCACGACGGTTCACGGGGTGTCCATCTAGGGACCCCCGAGAGGCACTCGAACCAATTAACCAATTCTGATTAG	GAAGTGACTGCCAAACCACAC
74	D	CGTGGCTAAACAACACCTATACTACTTGTTATTGTAGGCCCCCGCGGATGTCTACGTGCCAGGATGACGACGATAAGTAGGG	TGTGTTGAAGGACGGATCAGG
		AGATTAAATTAAGGGGGAAGGGCACGTAGACATCCGCGGGGGCCTACAATAACAAGTAGTAACCAATTAACCAATTCTGATTAG	CAAGAAGATCAACGACCACCACTA
75	D	TTATGCGATTAAATGAGGGGTCTGATCCCAAAAGCAATGTGCCTAGAGGGTGCCCCGCCCAGGATGACGACGATAAGTAGGG	TCTAGCCTCCCGTTCCCATG
		ACTACAGAGGGTGTCCCCGGGGGCGGGGCACCCTCTAGGCACATTGCTTTTGGGATCAGAAACCAATTAACCAATTCTGATTAG	AAAGCCCTAACCCAAGTCTGACTAC
K15	D	ACAACAACTCTATTGTAAGCCCTGTGGATACCTAGTCAAACCCTCCACGACCACAGACTTAGGATGACGACGATAAGTAGGG	GAGCCTTGTGTCGGGAATACTTAG
		AAAAAGGTATCGATGTCAAAAAGTCTGTGGTCGTGGAGGGTTTGACTAGGTATCCACAGGAACCAATTAACCAATTCTGATTAG	TATTACGCAGGCACAGGTTGCTC

BAC16 Mutagenesis

A two-step red recombination was used to generate mutations in the BAC16, as previously described[69]. In brief, a forward primer containing 40bp upstream and 20bp downstream of the ORF of interest and approximately 20bp of homology to the pEPkan-S target and a reverse primer containing 40bp downstream and 20bp upstream of the ORF of interest and approximately 20bp of homology to the pEPkan-S target were used to PCR amplify an I-SceI meganuclease restriction site and kanamycin resistance gene of pEPkan-S to generate an insert for ORF deletion. For stop codon insertion, similar primers were used except these contained a triple stop codon sequence, one for each reading frame. To facilitate homologous recombination, red recombination proteins were induced by incubation of E. coli GS1783 containing WT-BAC16 at 42°C and the insert was electroporated into these cells. Kanamycin-resistant colonies were screened by PCR amplification using primers that flank the ORF by about 150 bp each or using a flanking primer and a primer with homology to the kanamycin resistance gene, and by performing an NHEI digest for appropriate incorporation of the insert.

E. coli GS1783 contains a gene coding for the I-SceI meganuclease inducible by arabinose. A second homologous recombination was performed by incubating clones at 42°C to induce red recombination proteins and the addition of 1% L-arabinose to induce the I-SceI meganuclease, which would cleave the restriction site in the insert, facilitating homologous recombination to remove the kanamycin resistance gene. Resulting colonies, which were kanamycin sensitive but chloramphenicol resistant, were screened by PCR amplification using primers that flank the ORF by about 150 bp each and NHEI digest. Sanger sequencing was used to confirm the appropriate insertion of a triple stop codon sequence with a stop a codon in each reading frame (TAGGTAGATAGG) for stop codon mutants. This method generated a scarless deletion or stop codon insertion.

Revertants were generated using a modified version of this procedure. First the ORF of interest was amplified by PCR from the WT-BAC16 before being digested and ligated into pUC19. Then the I-SceI meganuclease site and kanamycin resistance gene of pEPkan-S were PCR amplified with a forward primer containing a restriction enzyme site, a 50bp duplication of the region immediately following the restriction enzyme site in the ORF of interest, and 20bp of homology to pEPkan-S and a reverse primer containing the restriction enzyme site and 20bp of homology to pEPkan-S. This construct was digested and ligated into the pUC19-ORF plasmid to generate the universal transfer construct. This newly generated sequence in the universal transfer construct was amplified by PCR using primers as described above for the first step of mutant generation and all subsequent steps followed this described procedure.

Transfection and Construction of Cell Lines Containing the BAC16

BAC16 mutant DNAs were prepared using a NucleoBond BAC100 kit (Machery-Nagel, Bethlehem, PA) according to the manufacturer's instructions. iSLK cells were seeded in 6-well plates and transfected the next day using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 2.5 µg BAC16 DNA, following the manufacturer's instructions. Cells were washed with PBS and

media was changed approximately 6 hours post transfection. Cells were monitored for GFP and switched to media containing 1.2 mg/ml Hygromycin B at 2 days post transfection or when cells reached confluency, and henceforth were monitored and media was changed approximately every 3 to 4 days until pure GFP+ populations were established.

Immunofluorescence

BAC16-transfected iSLK cell lines were seeded onto coverslips. Cells maintained under noninducing conditions, or at 72 hours after addition of 1mM sodium butyrate and 1µg/ml doxycycline to the media were fixed onto coverslips by 10 min incubation in 4% paraformaldehyde. Fixed cells were permeabilized with 0.2% Triton X-100 for ten minutes and blocked in 5% goat serum (Sigma-Aldrich) in PBST. Cells were incubated for 2 hours in primary antibody diluted in 5% goat serum in PBST, washed, and incubated for 1 hr in secondary antibody diluted in 5% goat serum in PBST. Cells were then incubated for 5min in 1ug/ml DAPI at RT, washed, and mounted on slides using Fluoromount G (Invitrogen) and sealed using nail polish. Slides were imaged on a Nikon TE300 microscope.

Virus Generation and Titration

When 6 T175s of a transfected cell line reached about 80% confluency, cells were washed and media was changed to media containing 1 µg/ml doxycycline and 1 mM sodium butyrate to induce lytic replication. Four days post media change, the supernatant was collected and cleared of cell debris by centrifugation at 3800g, 10 min, 4°C and .45 µm filtration. It was then centrifuged at 13,600 rpm, 3 hrs, and 4°C using a Surespin 630 ultracentrifuge rotor in a Sorvall Discovery ultracentrifuge before resuspension of the viral pellet in 750 µl DMEM containing 10% FBS and 1% Pen-Strep and storage at -80°C.

For virus titration, 293T cells were seeded at $5x10^4$ cells/well in 96 well plates 16-18 hrs before infection. Tenfold serial dilutions of virus were made in media containing 1 µg/ml doxycycline and 1 mM sodium butyrate and were added to the cells in duplicate. Plates were spun at 2000g, 1 hr, 30°C and put in the 37°C incubator for one hour before changing media. 48 hours later, cells were fixed and analyzed by flow cytometry to quantify the percentage of GFP+ cells as described below. Virus dilutions that yielded approximately 2-20% of cells GFP+ were used to calculate virus titer. Titration experiments were performed once in duplicate, using the average value of the duplicates.

Infections

iSLK cells were seeded in plates 16-18 hours before infection to reach 70% confluency at infection. Dilutions of viruses were made in media containing or lacking 1µg/ml doxycycline and 1mM sodium butyrate. Cells were washed before adding these virus dilution to the wells. Cells were spun in plates at 2000for 1hr at 30°C, then put in a 5% CO_2 37°C incubator for an hour. The viral inoculum was removed, and cells were washed with PBS before adding media

containing or lacking 1µg/ml doxycycline and 1mM sodium butyrate. Cells were returned to the incubator and this time point was designated t=0 for experiments.

For the long-term infection experiment, 293T cells were infected in 96-well plates with 40ul of concentrated viral supernatant. At 3dpi, media was supplemented with 200ug/ml hygromycin B for selection of BAC16 infected cells. At 77dpi, wells were scored for the presence or absence of GFP+ cells – now visible as large clusters of cells. The presence of GFP+ cells indicated that the disrupted ORF was not essential while the absence of GFP+ cells indicated that the disrupted ORF was essential.

Flow Cytometry

For virus titration, infected cells were collected at 48hpi, washed twice in PBS with 0.1% BSA, fixed in 1% paraformaldehyde for 5 min at RT, and washed twice in PBS with 0.1% BSA. Fixed cells were run on an LSR Fortessa X-20 flow cytometer (Becton Dickinson, Franklin Lakes, NJ) to quantify the percentage of GFP+ cells. BD FACSDiva (Becton Dickinson, Franklin Lakes, NJ) and Flowing Software 2 (Turku Center for Biotechnology) were used for analysis.

Results

Generation of a BAC16 mutant library

To manipulate the KSHV genome and generate mutant virus, many other labs have used a 2-step red recombination with the BAC16, followed by transfection of the recombinant DNA into iSLK cells, establishment of pure transfected cell populations, and induction of lytic replication to generate recombinant KSHV virions [64,70,79–88,71,89–92,72–78]. To assay the functionality of KSHV ORFs, we constructed a mutant library of 90 recombinant viruses from the BAC16, each with a disruption in a single ORF. In brief, we used a 2-step red-mediated recombination[69] to generate mutant BAC16 DNA (Fig. 2.1A). We then reconstituted mutant virus by transfecting BAC DNAs into iSLK cells, selecting for virus infected cells, and inducing lytic replication to collect the virus in the supernatant (Fig. 2.1B). ORFs disrupted in mutants which were unable to establish cell lines post-transfection were considered essential for the maintenance of latency, while ORFs disrupted in mutants unable to generate infectious virus after lytic induction were considered essential for reactivation or infectious virus generation. Finally, nonessential mutants were assayed by de novo infection of iSLK cells, and measuring infectious virus generation under conditions favoring latency, reactivation, or lytic replication and measuring lytic antigen expression under conditions favoring latency and reactivation. This final series of assays allowed us to look at the full replication cycle, including de novo infection, and to dissect the nuances between the roles of each ORF in parts of the viral life cycle.

In making our genetic manipulations, each disruption was either a scarless full deletion of an ORF for nonoverlapping ORFs or a triple stop codon insertion (TAGGTAGATAGG) briefly after the start codon for overlapping ORFs. The triple stop codon insertion is used for overlapping ORFs to prevent modification of the ORF which is not being targeted.

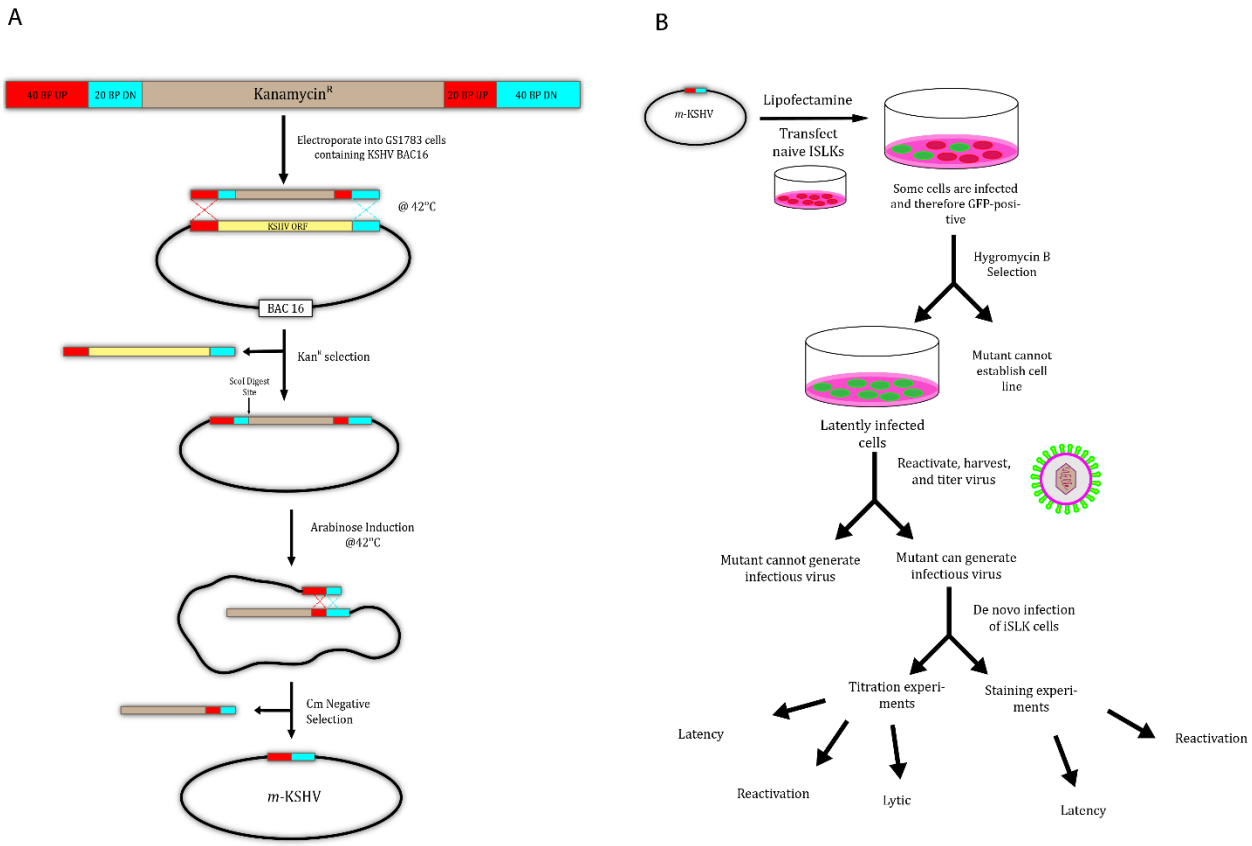


Figure 2.1. Project workflow. (A) Visual representation of the 2-step red recombination cloning process as well as (B) the mutant BAC16 transfections, cell culture, virus production, and downstream experiments used to generate and analyze BAC16 mutants.

ORF deletion primers were designed to contain 3 different sequences – forward primers contain, from 5' to 3', 40bp directly upstream of the targeted ORF, 20 bp directly downstream of the ORF, and 22 bp complementary to pEP-KanS. Reverse primers contain 40 bp directly downstream of the targeted ORF, 20bp directly upstream of the ORF, and 24 bp complementary to pEP-KanS. ORF stop codon insertion primers were designed similarly – forward primers contain, from 5' to 3', 40 bp directly upstream of the stop codon insertion site, a triple stop codon sequence, 20 bp directly downstream from the stop codon insertion site, and 22 bp complementary to pEP-KanS. Reverse primers contain 40 bp directly downstream of the stop codon insertion site, a triple stop codon sequence, 20 bp directly upstream of the stop codon insertion site, and 24 bp complementary to pEP-KanS. The triple stop codon sequence of TAGGTAGATAGG contains 3 stop codons, one in each reading frame, and the insertion site is 9 bp downstream from the start codon for that ORF. The sequences of all primers used for mutant generation are included in Table 2.1.

These primers amplify a 994 bp sequence from pEP-KanS containing an I-SceI restriction site as well as a kanamycin resistance gene, which combined with the 60 bp (deletion) or 72 bp (stop codon) overhangs in each primer, would generate a PCR product of 1114 bp or 1138 bp respectively. PCR products were treated with DpnI to degrade remaining template DNA and were run on 1% agarose gels to confirm the appropriate band size. The PCR product was extracted from the gel and purified.

For the first of two homologous recombinations, GS1783 bacteria containing the BAC16 (generously donated by Professor Britt Glaunsinger) were placed at 42°C to induce expression of the red proteins before being made electrocompetent and electroporated using the purified PCR product before plating on LB kanamycin plates. BAC DNA was purified from colonies which grew on these plates was screened for appropriate incorporation of the insert using PCR or an NheI digest before running the products on an agarose gel. For PCR, purified BAC DNA was amplified using primers which flank the ORF (deletion) or stop codon insertion site (stop codon) by approximately 150 bp in each direction, thereby yielding an amplicon of about 1414 bp (deletion) or 1438 bp (stop codon). Purified BAC DNA was also subjected to NheI digestion to check that the restriction patterns matched the expected results to ensure that no other rearrangements had occurred during this process.

For the second homologous recombination, clones were placed incubated with 1% L-arabinose, placed at 42°C to induce expression of the red proteins, then further incubated with 1% L-arabinose before plating on LB chloramphenicol plates. Clones which grew on LB chloramphenicol were replica plated on LB kanamycin plates and only clones which did not grow on LB kanamycin, indicating that the insert containing the kanamycin resistance gene was removed, were selected for further screening. BAC DNA was purified from these clones and assayed to confirm appropriate removal of the insert using PCR and NheI digestion (Fig. 2.2A, 2.2B). However, appropriate PCR product sizes in this case were about 300 bp (deletion) or 312 bp (stop codon). Additionally, the region of stop codon insertion was sequenced by Sanger

sequencing for stop codon insertion mutants to ensure appropriate incorporation and integrity of the triple stop codon sequence. Expected band patterns from NheI digestion could be calculated from the known BAC16 reference sequence or the mutated sequence by determining the NheI restriction sites and consequent band sizes after digestion (Fig. 2.2C). Due to the large size of some of the bands, certain rearrangements, for example those which removed a restriction site between a very long and very short DNA sequence or which deleted an ORF fully within a high molecular weight band, visualization of the changes caused by some mutations was not possible without techniques yielding higher resolution. In generating the ΔORF62 mutant (and its revertant as described below), the deletion of the ORF yielded a 996 bp deletion in a 7788bp band, which caused a clear shift down the gel of the 7788bp band to 6792bp (Fig. 2.2B).

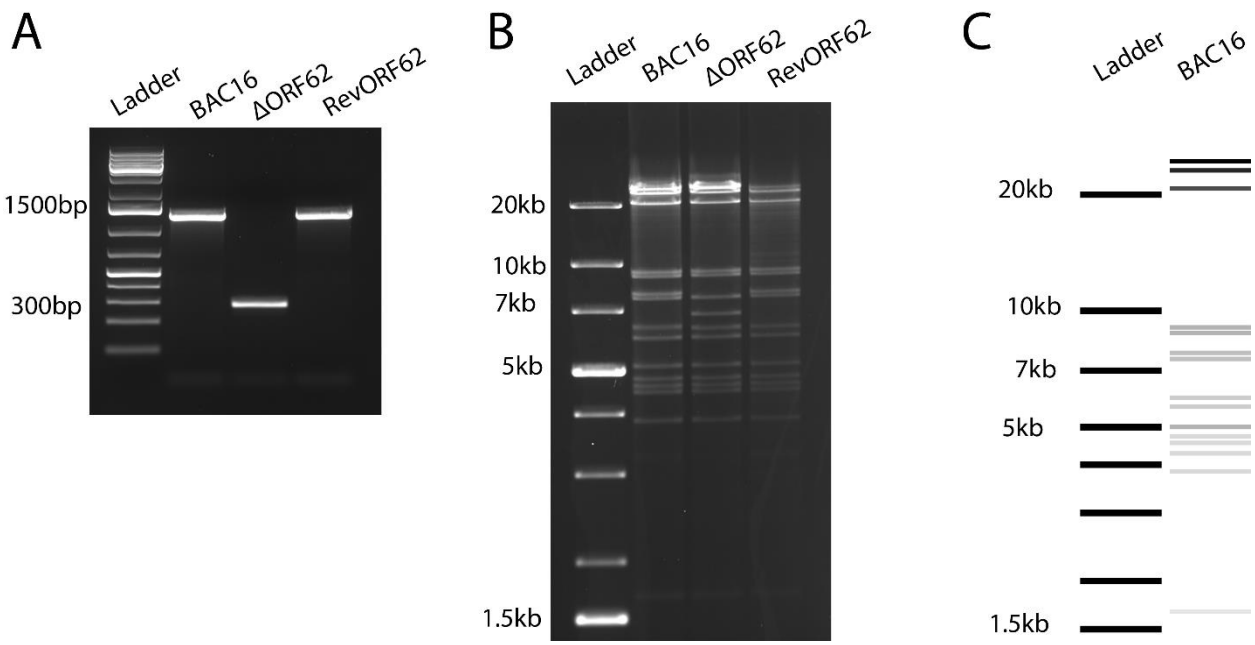


Figure 2.2. Construction of Mutant BAC16s. BAC DNA purified from GS1783 containing BAC16, ΔORF62, or RevORF62 were subject to (A) PCR amplification using primers flanking ORF62 by about 150bp each or (B) NHEI digestion and were run on an agarose gel. (C) A simulated version of the NHEI digest of the BAC16 and its separation on an agarose gel using the BAC16 reference sequence to calculate the location of NHEI restriction sites and consequent band sizes after digestion.

Table 2.2. Mutants Generated

ORF	Orientation	Size (bp)	Deletion or stop codon	HR1 clone	HR2 clone	Stop codon sequencing	Revertant clone
K1	+	855	D	K1D	K1a		
				K1E	K1i		
4	+	1653	D	4E	4E1		
6	+	3399	D	6E	6E1		
				6F	6F1		
7	+	2088	SC	7A1	7a1	x	
				7D	7d	x	
8	+	2538	SC	8B	8a	x	
				8C	8b	x	
9	+	3039	D	9A	9A1		
				9B	9B1		
10.1	+	81	SC	10.1A2	10.1a2-1	x	
				10.1B1	10.1b1	x	
10	+	1257	SC	10E	10a	x	
						x	
11AA	+	12	SC	11AAA1	11AAa1	x	
				11AAC	11AAb1	x	
11	+	1224	D	11E	11E1		
				11F	11F2		
K2	-	615	D	K2A	K2a		
				K2C	K2c		
2	-	633	D	2A	2A1		
				2B	2b1		
K3	-	969	D	K3C	K3a		
				K3D	K3b		
70	-	1014	D	70A	70A1		
				70D	70D5		
K4	-	285	D	K4A	K4b		
				K4C	K4b2		
K4.1	-	345	D	K4.1A	K4.1a		
				K4.1C	K4.1h		
K4.2	+	549	SC	K4.2A	K4.2a	x	
						x	
K5	-	771	D	K5C	K5a		
				K5B2	K5b2-2		
K6	-	288	D	K6C	K6b		
				K6B1	K6b1-1		
K7	+	381	SC	K7A1	K7a1-8	x	
				K7G	K7b	x	
16	+	528	D	16A2	16a1		
				16C	16C5		
17	-	1662	SC	17A1	17a2	x	
				17D	17i	x	
18	+	774	SC	18E	18a	x	
				18G	18c	x	
19	-	1650	SC	19A	19a	x	
				19B5	19b2	x	
20	-	963	SC	20A	20a1	x	
				20C	20o	x	
21	+	1743	SC	21A2	21a1	x	
				21E	21j	x	
22	+	2193	SC	22A1	22a1	x	
				22E	22k	x	
23	-	1215	SC	23A	23a	x	
				23C	23h	x	
24	-	2259	SC	24A	24a	x	
				24C	24b1	x	
25	+	4131	SC	25A	25a	x	
						x	

26	+	918	SC	26B	26a	x	
				26E	26b	x	
27	+	1032	SC	27E	27k	x	
				27B1	27b1	x	
28	+	309	D	28J	28J5		
29b	-	1056	SC	29bA	29ba	x	
						x	
30.1	+	50	SC	30.1B	30.1a	x	
				30.1G	30.1b	x	
30	+	234	SC	30C	30b	x	
				30E	30c	x	
31	+	675	SC	31A	31a	x	
				31C	31i	x	
32	+	1365	SC	32A	32a	x	
				32C	32b	x	
33	+	1005	SC	33B	33a	x	
				33E	33c	x	
29a	-	939	SC	29aA	29aa	x	
				29aC	29ah	x	
34.1	+	87	SC	34.1E	34.1a	x	
				34.1G	34.1b	x	
34	+	984	SC	34D	34k	x	
				34E	34c	x	
35	+	453	SC	35A	35a2	x	
				35B1	35b1	x	
36	+	1335	SC	36A	36a	x	
				36C	36b2	x	
37	+	1461	SC	37B	37a	x	
				37D	37j	x	
38	+	186	SC	38A	38a	x	
				38C	38l	x	
39	-	1203	D	39G	39G1		
				39I	39I9		
40	+	1374	D	40E	40E4		
				40F	40F1		
41	+	618	SC	41A	41a	x	
				41C	41k	x	
42	-	837	SC	42A	42a	x	
				42B2	42b1	x	
43	-	1818	SC	43D	43a2	x	
				43E	43d	x	
44	+	2367	SC	44A	44a	x	
				44C	44h	x	
45	-	1224	D	45 1	45 1-2A4		
				45 1	45 1-3A2		
46	-	768	D	46E	46E1		
				46F	46F5		
47	-	504	D	47A	47A5		
				47F	47F1		
48	-	1209	D	48A	48A7		
				48D	48b1		
49	-	909	D	49D	49a1		
				49E	49E2		
50	+	2058	D	50A1	50a1-12		
				50C	50d		
K8	+	720	D	K8A2	K8a2-2		
K8.1	+	594	D	K8.1B	K8.1a1		
				K8.1B1	K8.1b1-1		
52	-	396	D	52B	52a		
				52E	52e		
53	-	333	D	53A	53b		
				53C	53g		
54	+	957	D	54B	54a		
				54E	54g		

55	-	684	SC	55A	55a	x	
				55C	55e	x	
56	+	2532	SC	56A1	56a1-6	x	
				56B1	56b1	x	
57	+	1389	D	57B	57a		
				57D	57i		
K9	-	1350	D	K9A	K9a		
				K9B2	K9b2-3		
K10	-	2091	D	K10B	K10a		
				K10C	K10e		
K10.5	-	942	D	K10.5F	K10.5F2		
				K10.5I	K10.5I1		
K11	-	1404	SC	K11B	K11a	x	
				K11C	K11e	x	
58	-	1074	D	58A	58A1		
				58B	58B5		
59	-	1191	D	59A	59a		
				59C	59h		r59h-1b
60	-	918	D	60B	60a		
				60F	60b		
61	-	2379	D	61A1	61a2		
				61B4	61b1		
62	-	996	D	62A	62a		r62a-7g & r62a-3h
				62E	62f		
63	+	2787	D	63A	63a		
				63D	63h		
64	+	7908	D	64E	64a		
				64G	64k		
65	-	513	D	65A	65a		
				65D	65h		
66	-	1290	SC	66E	66c	x	
				66F	66d	x	
67	-	816	SC	67A1	67a1	x	
				67C	67g	x	
67.5	-	243	SC	67.5A	67.5a	x	
				67.5C	67.5f	x	
68	+	1638	SC	68A	68a	x	
				68C	68e	x	
69	+	909	D	69A	69a		
K12	-	699	D	K12A	K12a		
				K12B1	K12b1-4		
K13	-	567	D	K13I	K13I1		
				K13J	K13J1		
72	-	774	D	72A	72A2		
				72C	72C4		
73	-	3270	D	73A	73b		
				73C	73g		
K14	+	816	D	K14H	K14H3		
				K14J	K14J2		
74	+	1029	D	74A1	74a1-2		
				74C	74q		r74q-3k
75	-	2816	D	75A	75a		
				75F	75f		
K15	-		D	K15A	K15A3		
				K15D	K15D1		

Two revertants were also generated, for ORFs 58 and 62, which used a modified version of the previous procedure. First the sequence of the ORF of interest was amplified by PCR from the BAC16 before being digested and ligated into pUC19. Then the I-SceI meganuclease site and kanamycin resistance gene of pEPkan-S were PCR amplified with a forward primer containing a restriction enzyme site, a 50bp duplication of the region immediately following the restriction enzyme site in the ORF of interest, and 20bp of homology to pEPkan-S and a reverse primer containing the restriction enzyme site and 20bp of homology to pEPkan-S. This construct was digested and ligated into the pUC19-ORF plasmid to generate the universal transfer construct. This newly generated sequence in the universal transfer construct was amplified by PCR using primers as described above for the first step of mutant generation and all subsequent steps followed this described procedure. A list of all the clones generated is available in Table 2.2.

To generate cell lines harboring the BAC16, mutants, and revertants, we purified these BAC DNAs and transfected them into iSLK cells. Since the BAC16 contains a constitutively-expressed GFP gene driven by the EF-1α promoter, transfected cells could be monitored by fluorescence microscopy. Typically, only a small percentage of cells were successfully transfected as measured at 2 days post transfection, so hygromycin B was added to the cell media at this time point to select for transfected cells since the BAC16 contains a hygromycin B resistance gene driven by the same EF-1α promoter. Transfected cells grew under selective conditions with regular media changes for about 2 months, at which point all remaining cells were GFP+ and formed a monolayer in the larger flasks they had been transferred to, and were subsequently cryopreserved (Fig. 2.3).

Since LANA has been shown to be essential for appropriate maintenance and segregation of the KSHV genome during cell division, we expected to be unable to generate a ΔORF73 cell line[110]. All mutant DNAs could be successfully transfected and grown to a confluent GFP+ monolayer except for BAC16ΔORF73, which matches the observations made by Ye et al with an ORF73 disruption in the BAC36.[72]. Transfection by BAC16-ΔORF73 DNA did yield GFP+ cells, but these were unable to show any significant growth while under selection, even after 62 days (Fig. 2.4). These observations yielded our first categorization: whether an ORF was essential for cell line generation (Table 2.3, Fig. 2.5). These observations suggest that ΔORF73 cannot successfully maintain latency in iSLK cells while all other mutants can.

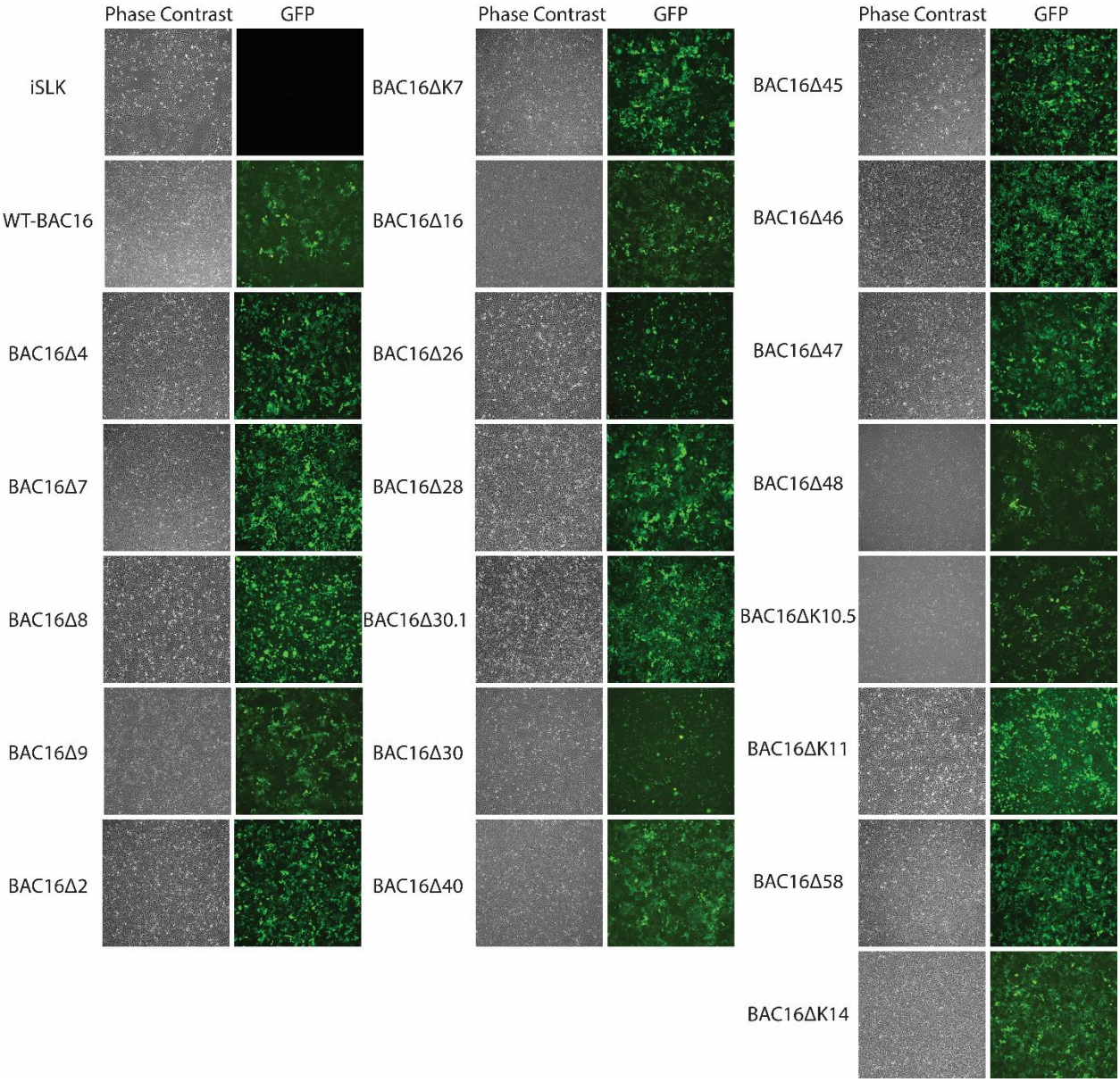


Figure 2.3. Establishment of BAC16 or mutant transfected cell lines. BAC16 or BAC16 mutant DNAs were transfected into naïve iSLK cells and selected for by hygromycin B treatment. Cells were imaged by phase contrast and fluorescence microscopy after pure GFP+ populations were established and reached confluency. Only a few examples of the transfected cell lines generated are shown here.

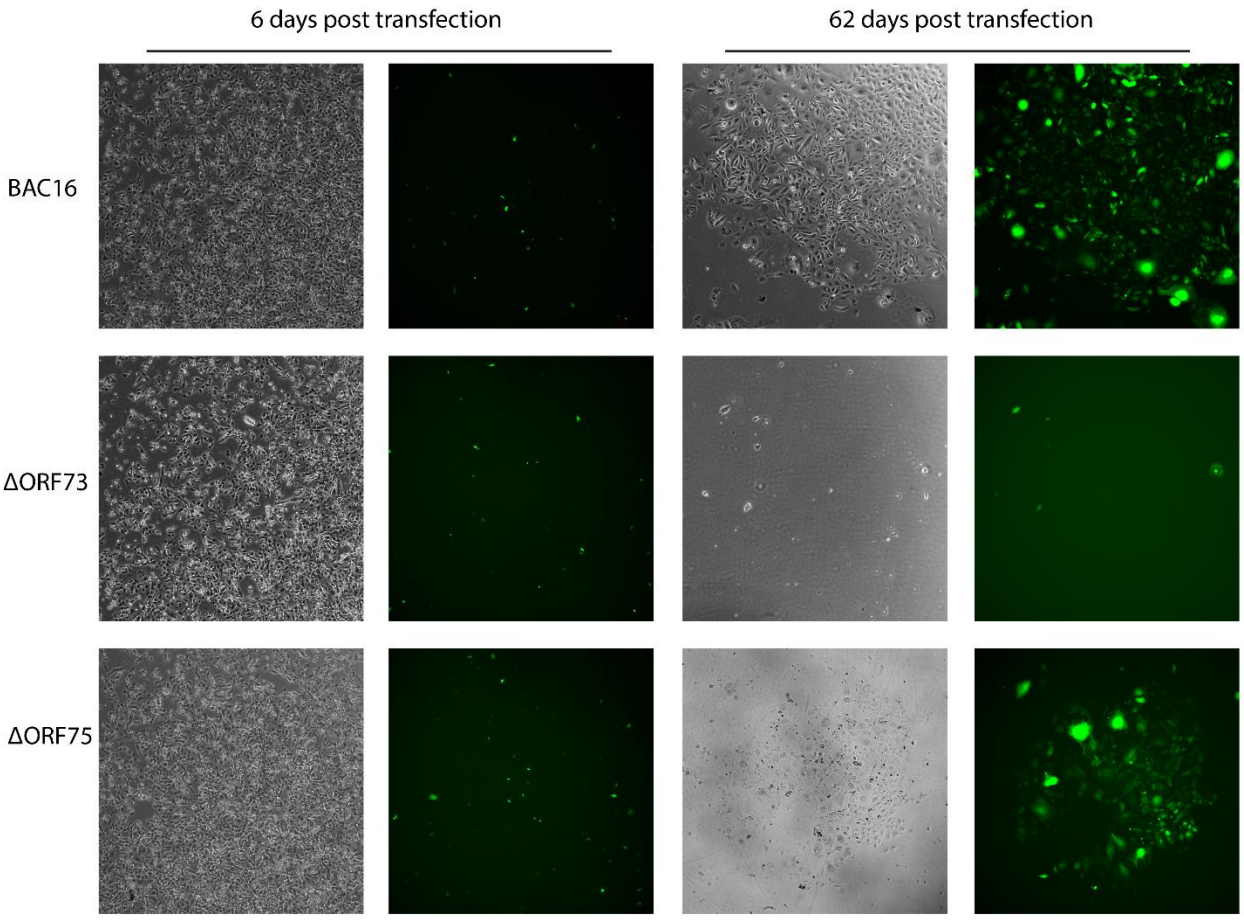


Figure 2.4. Transfection and Growth of BAC16, BAC16ΔORF73, and BAC16ΔORF75. BAC16ΔORF73 Fails to Grow. BAC16, BAC16ΔORF73, and BAC16ΔORF75 DNAs were transfected into naïve iSLK cells. At two days post-transfection, media was supplemented with 1.2 mg/ml hygromycin B. Cells were imaged using fluorescence microscopy, to visualize GFP, and phase contract or brightfield microscopy at 6 and 62 days post transfection. BAC16ΔORF75 is included here as an example of a BAC16 mutant which grew.

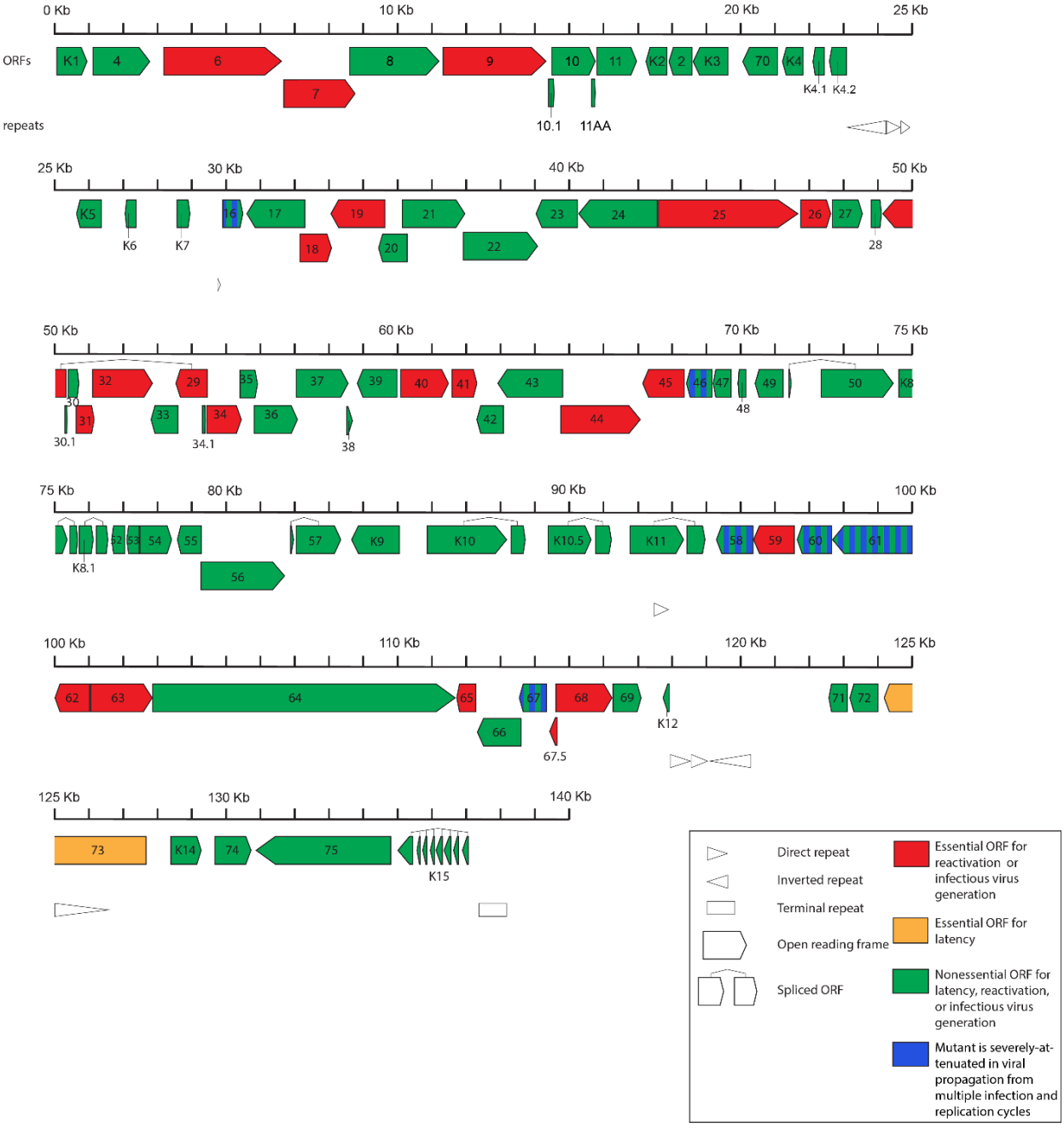


Figure 2.5. ORF Essentiality Map. Map of the KSHV genome with ORFs classified as essential or nonessential for latency, reactivation, or infectious virus generation.

Table 2.3 and Fig 2.5 are meant to provide a reference for the results of the experiments in this chapter as well as all (Table 2.3) or some (Fig 2.5) of the results of chapter 3, either in the form of a table or a map of the genome and including ORF classifications. See Fig. 2.6 for a brief overview of the experiments further discussed in chapter 3 which are included in this table and genome map. Table 2.3 additionally includes information regarding the putative function of each ORF, from the publication by Arias et al., as well as conservation across herpesviruses (from the sixth edition of Fields Virology), and essentiality within the herpesvirus family (from Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis[1,14,43]. It also includes reference to publications where ORF disruption mutants were made and analyzed, most are in the BAC16 but some are in the BAC36. Sections that are blank indicate that either experiments were not performed with that ORF-disruption mutant or there are gaps in the literature regarding ORF annotation.

We also performed immunofluorescent staining of some transfected cell lines for ORF73/LANA, which is constitutively expressed during KSHV infection, to additionally confirm the presence of the BAC16. BAC16, BAC16ΔK9 and BAC16ΔORF25-transfected cell lines were fixed by 4% paraformaldehyde, permeabilized by 0.2% Triton X-100 and stained using a rat anti-LANA antibody and an anti-rat Alexa-Fluor 568 secondary antibody before visualization with confocal microscopy (Fig. 2.7A). These ORFs were chosen since K9 is an immune modulatory protein unlikely to be essential while ORF25 is the major capsid protein which should be essential for infectious virus generation. All tested cell lines showed GFP and LANA expression in all cells, confirming infection by KSHV, and reaffirming that GFP expression should be a reliable proxy for KSHV infection.

To confirm that reactivation under our conditions was faithful to authentic KSHV reactivation, we also stained BAC16-transfected cell lines with antibodies specific for lytic antigens at time points before and after lytic induction (Fig. 2.7A, 2.7B). This allowed us to confirm expected viral antigen expression before and after induction in WT-BAC16. Lytic replication is induced by the addition of sodium butyrate and doxycycline to the media – sodium butyrate is a histone deacetylase inhibitor shown to reactivate KSHV and doxycycline induces expression of the KSHV transactivator RTA encoded in the genome of iSLK cells under the control of a Tet-on promoter.

RTA has been shown to be necessary and sufficient to induce lytic replication[34,64,119–121]. We left transfected cells uninduced or reactivated them by treatment with 1µg/ml doxycycline and 1mM sodium butyrate, and cells were stained using DAPI, as well as antibodies specific for LANA (latent), ORF 45 (early lytic), ORF K8.1A/B (late lytic). We found that BAC16-transfected cells showed robust levels of lytic antigen expression after, but not before, induction, confirming that our model could recapitulate this aspect of authentic KSHV reactivation and lytic infection.

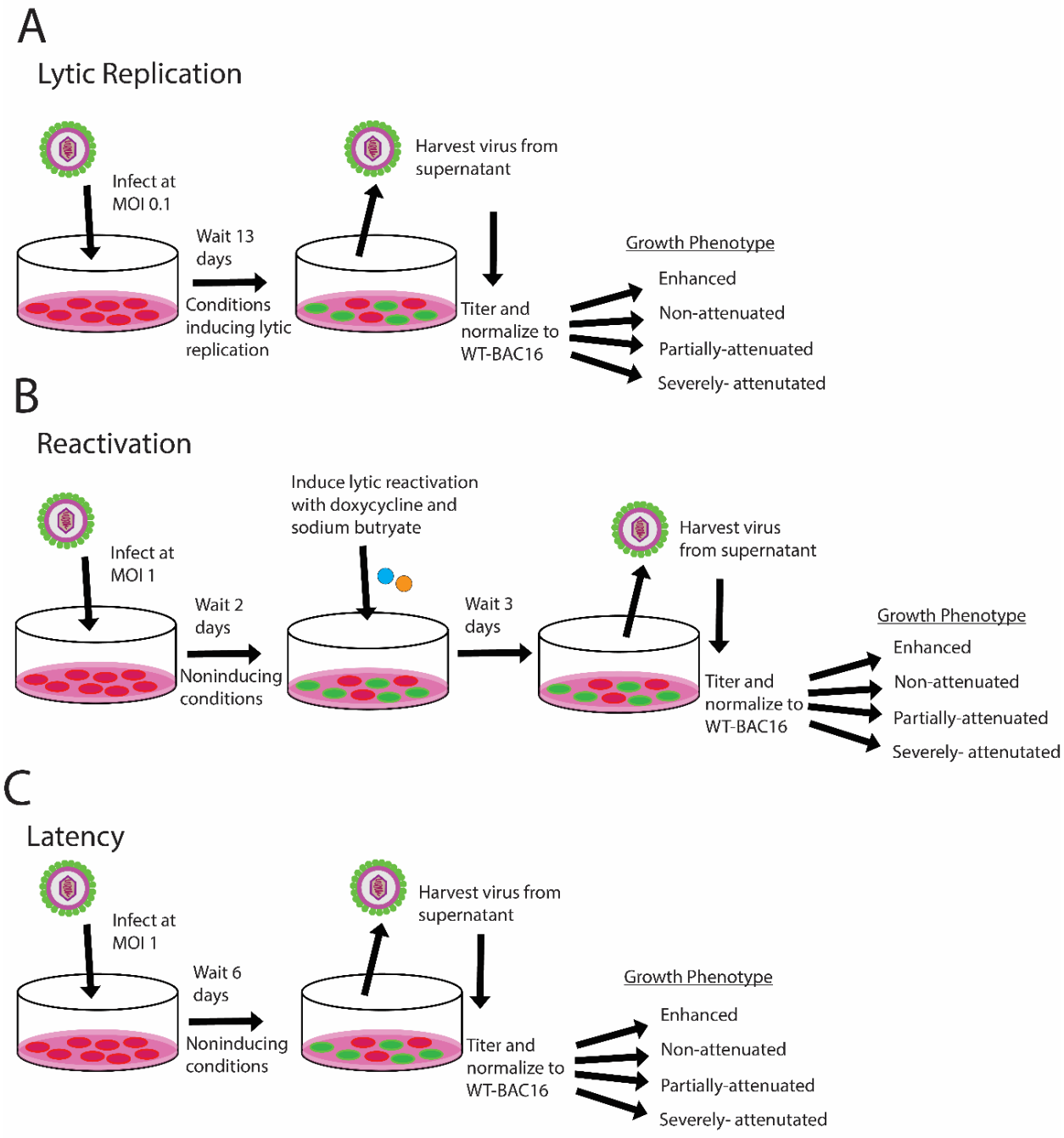


Figure 2.6. Overview of chapter 3 experiments measuring viral progeny generation. iSLK cells were infected with BAC16 or mutants at (A) MOI 0.1 or (B-C) MOI 1. Infected cells were either (A) maintained in the presence of doxycycline and sodium butyrate to induce lytic replication for 13 days, (B) maintained in noninducing conditions for 2 days before addition of doxycycline and sodium butyrate to induce lytic replication for 3 days, or (C) maintained in media lacking doxycycline and sodium butyrate for 6 days. Virus was harvested from the supernatant, titered, and this value was normalized to WT-BAC16 to categorize each mutant by growth phenotype.

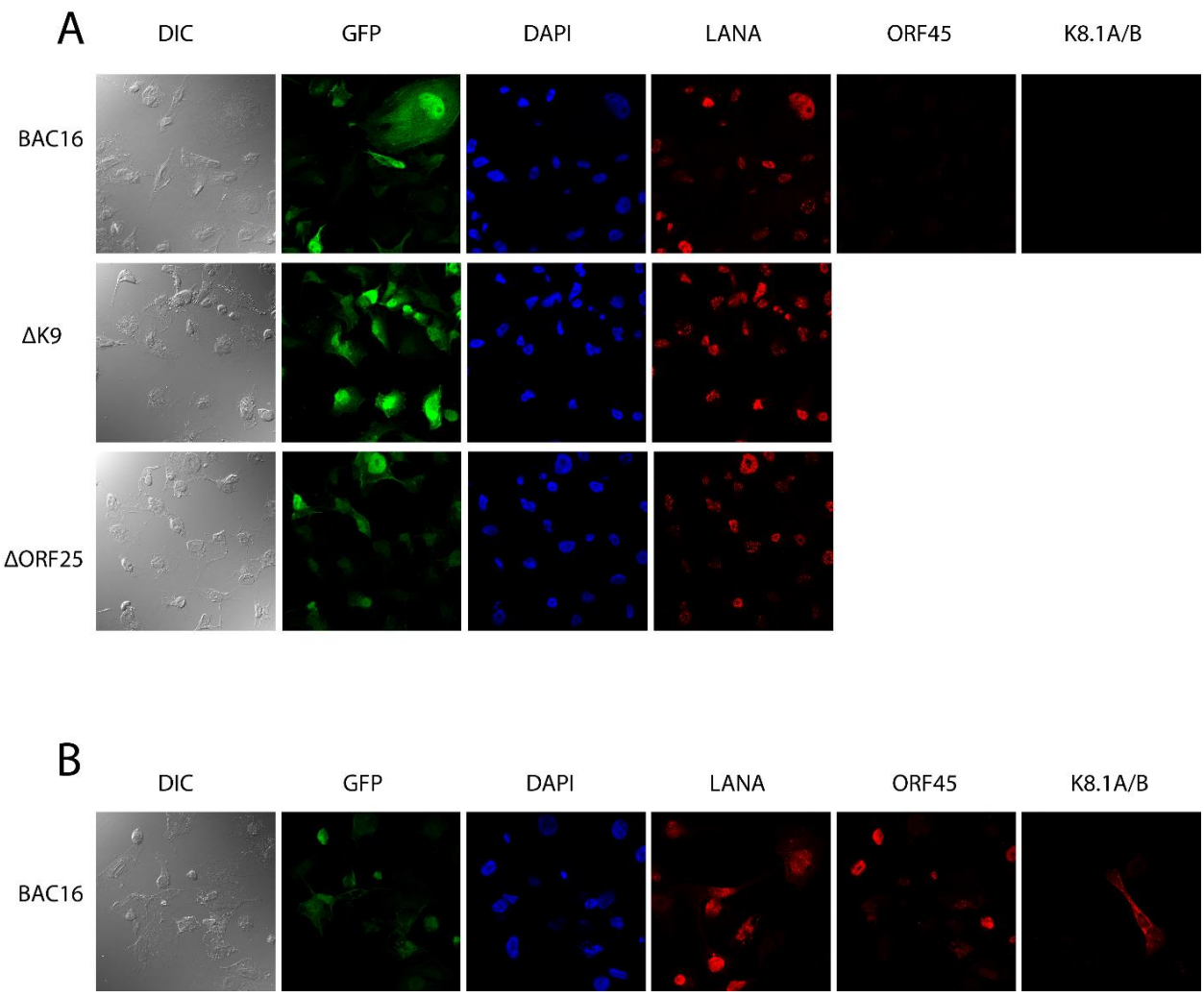
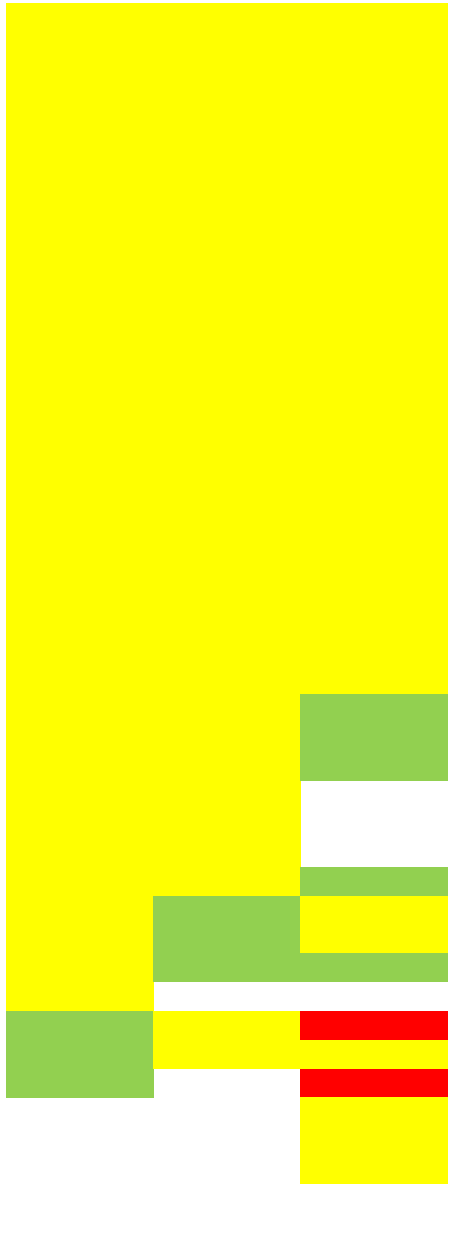


Figure 2.7. Expression of latent and lytic antigens in BAC16 and mutant transfected cell lines. BAC16, ΔORFK9, and ΔORF25 transfected iSLK cells were fixed, stained, and imaged using confocal microscopy to visualize GFP, DAPI, LANA, ORF45, and K8.1A/B.(A) before induction and (B) 72 hours post induction.

Table 2.3. ORF classifications

Cell line & infectious virus generation categorization (ch.2)	ORF	Virus generation in different conditions (ch.3) Lytic	Reactivation	Latency	Timing	Putative Function	Conservation	Essentiality in herpesviruses
Essential for cell line generation	73[72,110,]				latent	LANA	y2 only	
Essential for reactivation and infectious virus generation	6[79]				24-48h	ssDNA binding protein	Core	2
	7				N/A	virion protein	Core	2
	9				48-72h	DNA polymerase	Core	2
	18[87]				24h	late gene regulation (MHV68)	Beta and y	
	19					tegument protein	Core	2
	25				48-72h	Major capsid protein	Core	2
	26				48-72h	Minor capsid protein	Core	2
	31[85]				48-72h	nuclear and cytoplasmic (MHV68)	Core	
	32				48-72h	tegument protein	Core	1
	29				72h	packaging protein	Core	2
	34[73]				24-48h	N/A	Core	1
	40				48-72h	helicase-primase	Core	2
	41				48-72h	helicase-primase	Core	2
	44				48-72h	helicase	Core	2
	45[75]				8h	RSK activator	Beta and y	
	59[84]				24h	Processivity factor	y - only	2
	62				72h	N/A	Core	2
	63				N/A	NLR homolog	Core	1
	65[90,91]				48-72h	capsid	Alpha and y	1
	67.5				48-72h	N/A		
	68				48-72h	glycoprotein	Core	2
Nonessential for cell line generation, reactivation, and Infectious virus generation	67				48-72h	nuclear egress complex	Core	2
	46				24h	Uracil deglycosylase	Core	1
	61				24-48h	Ribonucleoprotein reductase	Core	1
	58				24h	N/A	y - only	
	60				24-48h	Ribonucleoprotein reductase	Alpha and y	
	16[88,89]				8h	Bcl2 homolog	y-2 only	
	38[92]				24-48h	Myristylated protein	Core	1
	33[92]				48-72h	tegument protein (MHV68)	Core	1
	36				24-48h	Serine protein kinase	Core	
	35[78]				24-48h	N/A	Beta and y	
	4				24h	complement binding protein	y-2 only	
	8[70,112]				48-72h	Glycoprotein B	Core	2
	K11				48-72h	vIRF2	unique	
	56[113]				48-72h	DNA replication	Core	1



K2	latent	viral IL-6 homolog	unique	
23	48-72h	Glycoprotein (predicted)	y - only	1
47	24h	glycoprotein L	Core	2
K4.1	8h	N/A	unique	
K1[82]	latent	glycoprotein	unique	
70	8h	thimydlate synthase	y-2 only	
34.1				
37	24-48h	Sox	Core	1
K13[89,11]	latent	vFLIP	unique	
2	N/A	dihydrofolate reductase	y-2 only	
48	N/A	N/A	y - only	
74	24-48h	vGPCR	Beta and y	
10.1				
K9	48-72h	vIRF1	unique	
K10	48-72h	vIRF4	unique	
K12	latent	Kaposin	unique	
K4.2			unique	
20			Core	
21	48-72h	Thymidine kinase	Alpha and y	
K10.5	48-72h	vIRF3	unique	
K14	24-48h	vOX2	unique	
42	48-72h	tegument protein	Core	2
K8.1[71]	48h	glycoprotein	y - only	
11	8h	predicted dUTPase	y-2 only	
K6	8h	vMIP-IA	unique	
11AA				
K7[89]			unique	
55	48-72h	tegument protein	Core	1
K15[80]	N/A	LMP1/2	unique	
17	48h	Protease	Core	2
72	latent	vCyclin	y2 only	
K5[64]	8h	RING-CH E3 Ubiquitin ligase	unique	
K4	8h	vMIP-II	unique	
K3[64]	24h	immune modulator	unique	
30.1				
50[74]	8h	RTA	y - only	
28	48-72h	BDLF3 EBV homolog	Core	
49	N/A	activates JNK/p38	y - only	
75[81]	48-72h	FGARAT	y - only	
27	48-72h	glycoprotein (MHV68)	y - only	
K8[83]	8-24h	bZIP	unique	
10	48-72h	Regulator of interferon function	y-2 only	
24[86,115]	48-72h	Essential for replication (MHV68)	Beta and y	

30[87]	48-72h	late gene regulation (MHV68)	Core	
43[116]	48-72h	portal protein (capsid)	Core	
53	48-72h	Glycoprotein N	y - only	1
57[76,77]	8h	mRNA export/splicing	Core	2
66	48-72h	capsid	Beta and y	2
22[117]	48-72h	Glycoprotein H	Core	2
39	24-48h	Glycoprotein M	Core	1
52[118]	48-72h	tegument protein	y - only	
54	48-72h	dUTPase/Immunomodulator	Core	1
64	N/A	deubiquitinase	Core	2
69	48-72h	BRLF2 nuclear egress	Core	2

Legend

1	required for replication in some viruses or some settings
2	required for replication in all viruses and settings tested

	severely-attenuated virus generation
	partially-attenuated virus generation
	non-attenuated virus generation
	enhanced virus generation

Determining KSHV ORF essentiality for the production of infectious virus

We next sought to understand the essentiality of viral ORFs for reactivation and lytic virus generation. We assayed the presence or absence of infectious virus in the supernatant of induced cell lines – the presence of infectious virus indicates that the ORF is nonessential for successful reactivation and infectious virus production, and the absence of infectious virus indicates essentiality for these processes. We expected WT-BAC16 to robustly generate infectious progeny upon infection, as demonstrated by Brulois et al. who recovered a BAC16 virus stock with a titer of about $5x10^7$ IU/ml following induction of lytic replication by the addition of 1μg/ml doxycycline and 1mM sodium butyrate to the media, supernatant harvest at 96 hours post induction, and concentration of the supernatant about 133-fold by ultracentrifugation[64]. We also expected many mutants to be completely deficient at infectious virus generation, indicating that the disrupted ORFs play essential roles in lytic reactivation, infectious virus generation, or de novo infection[64].

Four days after reactivation of BAC16 and mutant-transfected cell lines by doxycycline and sodium butyrate treatment, the supernatant was collected, centrifuged and .45um filtered to remove cell debris, and concentrated by ultracentrifugation at 25,000g for 3hr at 4°C before resuspension in DMEM at about 1:100 of the original volume. To quantify the amount of infectious virus present, these virus stocks were serially diluted and used to infect 293T cells, a cell line highly permissive to KSHV infection, using spinoculation. After 48 hours, the percentage of GFP-expressing cells was determined by flow cytometry, which represented the percentage of infected cells, and we used this value to quantify the titer of infectious virus in each viral stock, leading us to our second classification: whether the cell lines generated from these mutant viruses could yield infectious virus upon reactivation.

In this analysis, we only assigned ORFs to one of two categories, essential (infectious virus is generated) or nonessential (infectious virus is not generated) for reactivation and infectious virus generation, due to the inconsistent nature of this assay: another group and we have both observed significant variation in the levels of virus generated from the lytic induction of iSLK cells independently transfected with the same BAC DNA[122]. We generated three independent cell lines by transfection of iSLK cells with WT-BAC16 followed by selection with hygromycin B and expansion to larger culture vessels. Induction of lytic reactivation yielded titers of $1.51x10^7$ IU/ml, $2.43x10^6$ IU/ml, and $2.92x10^8$ IU/ml respectively from these three WT-BAC16 transfected cell lines (Fig. 2.8). Despite this inconsistency between replicate cell lines, the titer of infectious virus generated from each cell line remained relatively consistent among different trials, indicating that the assay itself was reliable. Due to this high degree of variability, we used this assay only to assess the presence or absence of infectious virus and not to further categorize mutants by the degree of their virus generation. For cell lines which generated no virus, a second independent transfection with independently generated mutant BAC16 DNA was performed to confirm this phenotype.

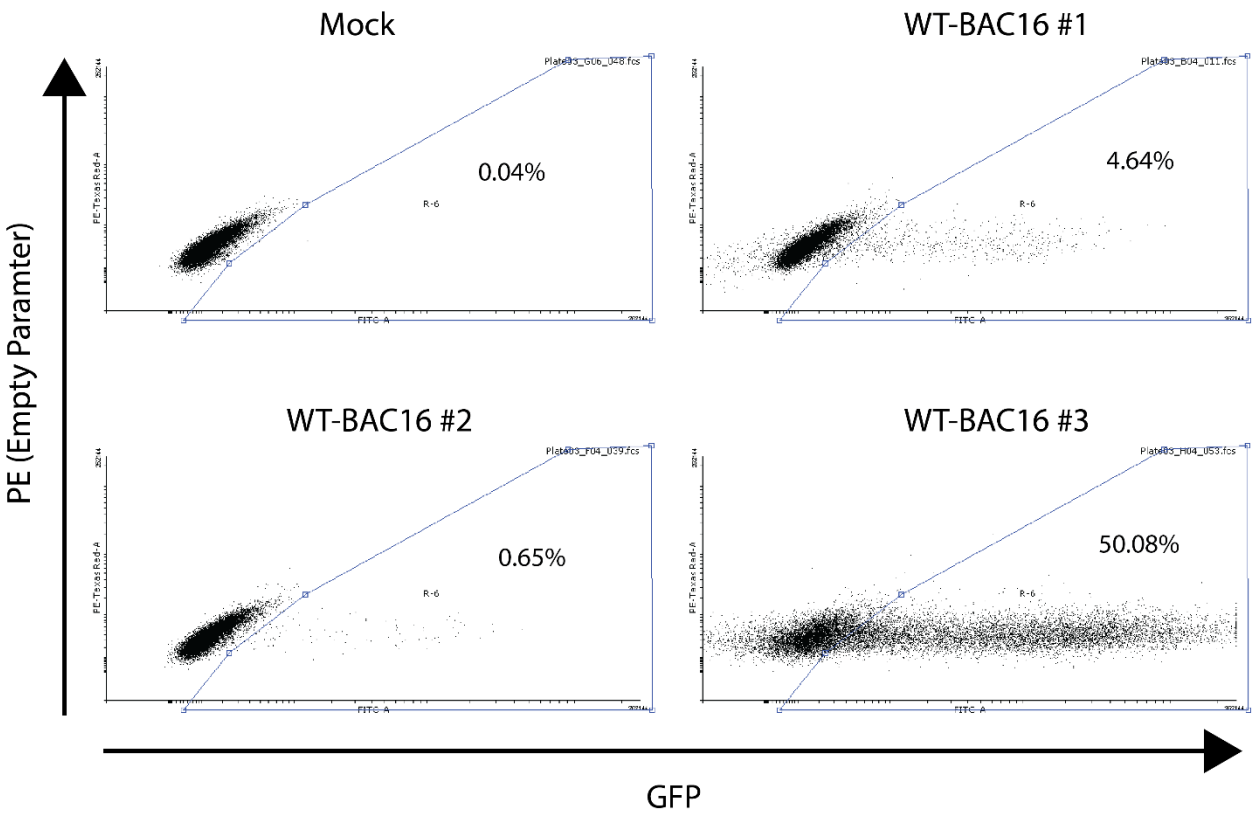


Figure 2.8. Virus titration to quantitate virus generation from three clones of WT-BAC16. WT-BAC16 #1, 2, and 3 transfected cell lines were induced to reactivate, and virus was harvested, purified, and concentrated from the supernatant 96 hours post induction. This viral stock was titered on 293T cells and appropriate dilution wells were harvested, fixed, and analyzed by flow cytometry flow 48 hours post infection. This figure shows the results of the mock-infected sample as well as the 10^{-3} dilution of WT-BAC16 #1, 2, and 3. Calculated titers are as follows - WT-BAC16 #1: 1.51e7 IU/ml, WT-BAC16 #2: 2.43e6 IU/ml, WT-BAC16 #3: 2.92e8 IU/ml.

However, at very low titers of virus this assay could yield false positives due to carry-over of small amounts of GFP-containing cell debris into the virus stock during harvest, purification, and concentration. Additionally, at the low multiplicity of infection correlated with infection by a low titer virus stock, infected cells exhibit a very faint GFP signal, making them especially difficult to distinguish from GFP-negative uninfected cells and reducing the sensitivity of the assay. Therefore, for viruses determined to have a low titer (about ≤ 10^5 IU/ml) by this assay, we used a growth-based assay to judge the presence or absence of low levels of infectious virus. 293T cells were infected with a set volume of virus stock followed by hygromycin B selection for 77 days, after which point the presence or absence of at least one cluster of GFP+ cells, generated from at least 1 infectious unit, would be assessed by fluorescence microscopy. The amplification of signal in this assay allowed us to determine more robustly if any infectious virus was generated. These two assays, in tandem, informed our second classification: whether the cell lines generated from these mutant viruses could yield infectious virus upon reactivation (Table 2.3, Fig. 2.5). If a mutant had a titer ≥ 10^5 IU/ml OR showed outgrowth of GFP+ cell clusters, the ORF was deemed nonessential for reactivation and infectious virus generation. If a mutant had a titer of ≤ 10^5 IU/ml AND there was no observable outgrowth of GFP+ cell clusters, the ORF was deemed essential for reactivation and infectious virus generation.

Here I've included flow cytometry data demonstrating the wide difference in infectious virus generation from essential or nonessential mutants (Fig. 2.9). BAC16Δ63, an essential mutant, shows negligible infectious virus production while BAC16ΔK10 and WT-BAC16 show similar virus levels. Note that the dot plots for BAC16ΔK10 and WT-BAC16 are from infections with viruses diluted by a factor of 10 compared to the BAC16Δ63 virus used. The essentiality of ORF63 was confirmed by a lack of GFP+ cluster formation in the growth-based assay.

Together, these assays ultimately revealed that 1 ORF is essential for cell line generation and 21 ORFs are essential for reactivation and infectious virus generation. Sixty-eight ORFs are not essential for either of these processes and therefore are not essential for the establishment of latency, reactivation, and infectious virus generation in this system.

Of these 22 essential ORFs, 19 are conserved core herpesvirus genes. Six belong to the DNA replication machinery class of herpesvirus core genes (ORF 6, 9 ,40, 41 ,44, 59), six to the processing and packaging of DNA class of genes (ORF 7, 19, 29, 32, 67.5, 68), four to the capsid structural class of genes (ORF 25, 26, 62, 65), two to the tegument class of core genes (ORFs 34,63), and one is unclassified (ORF31)[43]. Of the remaining three ORFs, two (ORF18 and 45) are conserved among beta and gamma herpesviruses, and one (ORF73) is gamma 2 herpesvirus specific[1]. Unsurprisingly most essential ORFs are conserved among herpesviruses and all gamma 2 specific ORFs other than LANA are nonessential.

Nine of these essential ORFs have putative functions which would clearly be deemed as essential. This includes ORF9 (DNA polymerase), ORF25 (major capsid protein), ORF26 (minor capsid protein), ORF40 (helicase-primase), ORF41 (helicase-primase), ORF44 (helicase), ORF65 (small capsid protein), ORF73 (LANA), and ORF 29 (packaging protein).

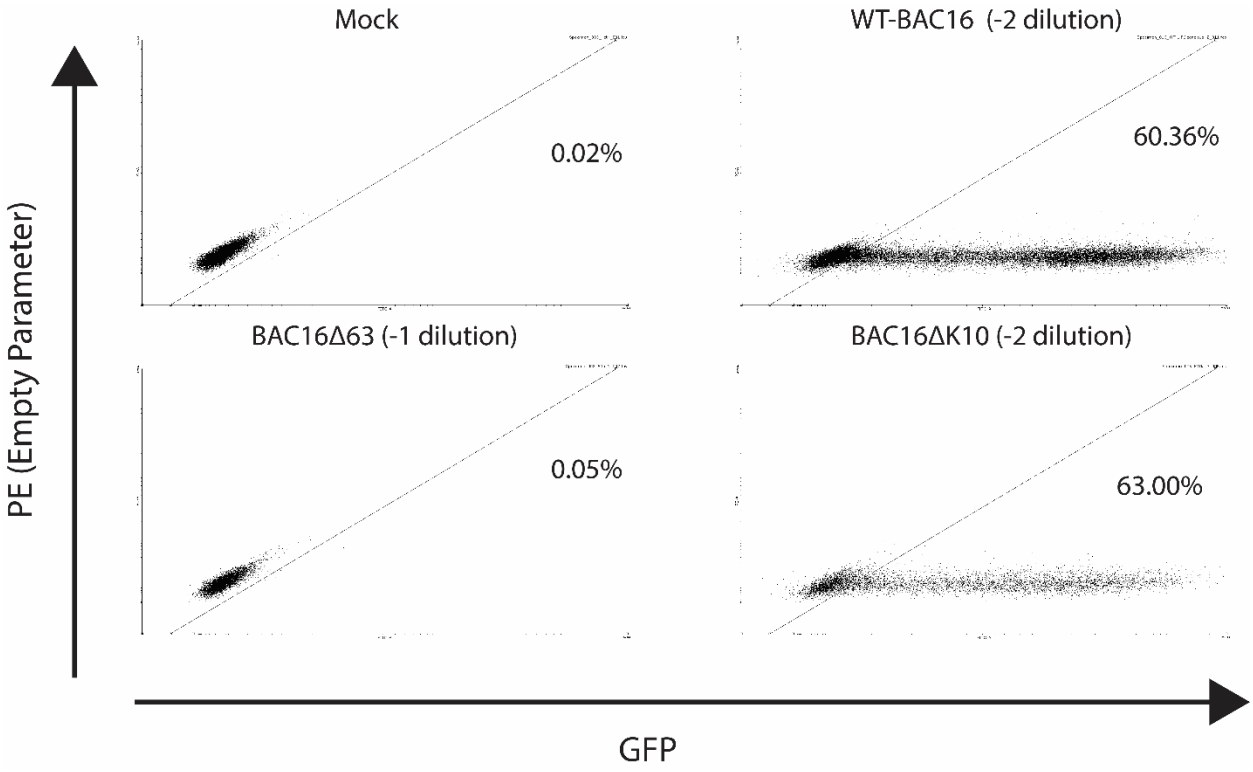


Figure 2.9. Examples of virus titration from essential and nonessential BAC16 mutants. WT-BAC16, BAC16Δ63, and BAC16ΔK10 transfected cell lines were induced to reactivate, and virus was harvested, purified, and concentrated from the supernatant 96 hours post induction. This viral stock was titered on 293T cells and appropriate dilution wells were harvested, fixed, and analyzed by flow cytometry flow 48 hours post infection. The dot plot for BAC16Δ63 shows the -1 dilution while the dot plots for WT-BAC16 and BAC16ΔK10 show the -2 dilution.

Ten of the remaining 13 ORFs have been assigned putative functions that are not specifically known to be essential in the viral life cycle. The 3 remaining ORFs - ORF34, ORF62 and ORF67.5, were not ascribed specific functions in KSHV.

Interestingly, the nonessential genes included all of the herpesvirus conserved envelope glycoproteins – ORF8 (gB), ORF22 (gH), ORF39 (gM), ORF47 (gL), ORF 53 (gN), which means that maintenance of latent infection, reactivation, and the generation of infectious virus do not require any virion glycoproteins. Strikingly, ten herpesvirus core ORFs that were classified as nonessential from our experiments were classified as required for replication in all viruses and settings tested according to Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.[43] These are ORFs 8, 17, 22, 42, 47, 57, 64, 66, 67, and 69. Additionally, ORF43, the putative portal protein, was unexpectedly classified as nonessential in our study.

Discussion

Multiple studies have generated and analyzed one or two single ORF disruption mutants in the BAC16. This is the first study to generate and analyze a library of mutants with genetic disruptions covering all primary ORFs, allowing direct comparison of phenotypes for different mutants to better understand the role of each ORF in the viral life cycle. Additionally, this study involved the generation of 72 novel single ORF disruption mutants which have not been previously generated or studied.

In the process of generating these mutants, we discovered that no single ORF other than ORF73 is essential for the maintenance of latency, since all mutants could be successfully transfected into iSLK cells and these cells maintained GFP expression under extended cultivation under selection. ORF73 has been studied since the initial discovery of KSHV, and it is known that the gene product of ORF73, LANA, can bind to both host nucleosome proteins through its N-terminal chromatin-binding domain as well as a sequence in the terminal repeat region of the KSHV genome through its C-terminal domain[123]. This allows it to recruit cellular factors to the KSHV genome to promote processes such as DNA replication, and it also allows LANA to tether the viral genome to the host chromatin, facilitating the appropriate segregation of the genome to daughter cells during mitosis[29]. LANA mutants lacking internal sequences have been shown to be severely deficient in persistence of episomes containing the KSHV terminal repeat sequence[110,124]. Additionally, LANA can function to inhibit expression of ORF50, the viral protein responsible for the lytic switch[125]. Therefore, it's not surprising that LANA disruption would prevent the normal maintenance of latency, however it is a novel finding that no other viral gene, on its own, is also essential for the normal maintenance of latency. This finding truly underscores the important role of LANA in maintaining latency in infected cells, which is the viral state in most infected cells in KSHV-associated diseases.

In this study we found that 1 ORF is essential for the maintenance of latency and 21 ORFs are essential for reactivation and infectious virus generation. Many of these essential ORFs are conserved among all herpesviruses, and all KSHV-specific ORFs other than LANA are not essential, which is not surprising since we would generally expect the genes with the highest

levels of conservation to be the genes with the most functions that are most general to the viral life cycle. In previous studies on KSHV, three of these essential ORFs (ORFs 18, 31, and 34) have been shown to be essential for the generation of infectious virus in the BAC16[85–87], one (ORF 6) has been shown to be essential for the generation of infectious virus in the BAC36, and one (ORF45) has shown significantly attenuated infectious virus production in the BAC36[75]. Matching our observations, though in the BAC36, it was shown that disruption of ORF73 prevents episome persistence after transfection of the BAC. For two other ORFs (ORFs 59 and 65), studies have been performed on BAC36 mutants with disruptions in these ORFs or on the ORF itself, but infectious virus generation from transfected cell lines was not directly measured. For 14 of these essential ORFs, no mutagenesis studies have been performed.

Herpesvirus envelope glycoproteins typically play a key role in facilitating viral entry into the cell. Of the core herpesvirus glycoproteins, gB, gL, and gH have been shown to be required for replication in all herpesvirus in all settings tested, and the remainder, gN and gM, have been shown to be required in some herpesviruses in some settings tested[43]. Therefore, it was especially striking that each of these envelope glycoproteins was classified as nonessential in our study. This may indicate that KSHV uses a different mechanism from other herpesviruses for viral receptor binding and entry, or perhaps that the envelope glycoproteins have redundant functions in facilitating viral entry. Regardless of the mechanism, these results could have wide-reaching implications for vaccine development targeting the stage of viral entry. However, further research is required to better understand the roles of KSHV ORFs in this process. It is also important to note that we used spinoculation during virus titration, which could potentially cause receptor-independent virus internalization, and thus it would be important to repeat these experiments without the use of spinoculation in the future.

Ten herpesvirus core ORFs that we classified as nonessential, ORFs 8 (gB), 17 (protease), 22 (gH), 42 (tegument protein), 47 (gL), 57 (mRNA export/splicing), 64 (deubiquitinase), 66 (capsid), 67 (nuclear egress complex), and 69 (BRLF2 nuclear egress), were classified as essential from studies of their homologues in other herpesviruses[43]. In other herpesviruses, ORF 42, 64, 66, 67, and 69 are involved in egress and thus it's possible that these genes function slightly differently in KSHV – perhaps there is a higher level of redundancy for these proteins. Our classification of ORF17, the protease, as nonessential is especially surprising given that herpesvirus proteases are typically required for capsid maturation, which should be essential for the production of infectious virus[126].

Also, conflicting with our nonessential designations, other groups have found that ORF24 (essential for replication in MHV68), 30 (late gene regulation in MHV68), and 75 (FGARAT) were found to be essential for virus generation in the BAC16, and ORF57 (mRNA export/splicing) was found to be essential for virus generation in the BAC36, which varies from the status of these as nonessential in our study. One group found that ORF8 is essential for the generation of extracellular DNase I-protected viral DNA upon induction of a BAC36ΔgB transfected cell line. However, it is possible that some of these discrepancies are due to differing methodology. By and large, there is no consistent definition in the field for essentiality. Some measure a 100-fold decrease in infectious virus production after disrupting an ORF and claim that this shows that

the gene is essential. Others would see a similar result and simply state that the ORF is important for efficient production of infectious virus. Additionally, different methods are used to measure infectious virus production, with varying levels of sensitivity, including infection followed by microscopy to count GFP+ cells, infection followed by flow cytometry to measure the percentage of GFP positive cells, or qPCR of encapsidated viral DNA. Each of these methods has different levels of sensitivity and their own advantages and disadvantages. In order to increase sensitivity beyond flow cytometry methods while also maintaining the high-throughput required in a global profiling, we turned to a different assay involving long-term infection in order to analyze mutant viruses which generated very low titers of virus to determine if these ORFs were essential or not. Since this assay relies on segregation of the episome among dividing cells in order to measure the presence of infectious virus, this places an additional constraint that the virus must meet for that ORF to be deemed nonessential. However, it is very unlikely that any mutants would have defects in this aspect of the viral life cycle since all of the mutants assayed were able to segregate the viral episome in BAC16 transfected cell lines. Additionally, it has previously been shown that LANA is necessary and sufficient for the persistence of episomes containing a specific KSHV DNA fragment[127]. This assay allowed a very high sensitivity – we could in theory detect infectious virus down to a concentration of 800,000 times lower than WT-BAC16 and we considered an ORF nonessential if any measurable virus could be generated. This very low threshold for virus detection could impact our essentiality results when compared to other studies.

In determining essentiality for reactivation and infectious virus generation, it is important to note that the process of transfection, selection and cell growth, and reactivation will have substantial differences from a natural infection with a virus. The reconstitution of mutant virus involves transfection, not de novo infection. Viral entry of KSHV is a complex process involving receptor binding, endocytosis, and the release of virion-associated proteins into the cell, all of which can have major effects on host cell biology including immunomodulatory effects. For example, ORF45, a tegument protein, has been shown to interact with and inhibit interferon-regulatory factor 7 (IRF-7) phosphorylation, preventing nuclear translocation of IRF-7 and expression of interferon genes, which are responsible for some of antiviral response in cells[128]. Additionally, a BAC36ΔORF45 mutant showed lower infectivity than WT-BAC36, suggesting that the role of ORF45 as a tegument protein may directly affect the ability of the virus to establish successful infection of a cell[75]. It is also possible that certain steps during viral infection may be important for priming the cell for successful reactivation and lytic replication of KSHV in the future. Consequently, it is not unreasonable to believe that some ORFs we've determined as nonessential for reactivation and infectious virus generation may show significant deficiencies in infectious virus generation upon de novo infection with this mutant virus and subsequent reactivation. Therefore, in the next chapter I will present experiments involving de novo infection to better recapitulate authentic KSHV infection.

In our analysis, ORFs may have shown an essential phenotype for a variety of reasons including deregulated lytic gene expression, inability to properly form and release viral particles, or lack of infectivity of viral particles. We did not seek to dissect the specific functions of ORFs which led to the phenotypes we saw, but solely to better understand the big picture of the roles of

KSHV ORFs with regards to the viral life cycle and infectious virus production, and to provide a framework from which future studies could begin. Future studies to more closely analyze the function of these ORFs will provide a much more thorough understanding of KSHV.

Chapter 3: Generation of viral progeny by BAC16 mutants under conditions favoring latency, reactivation, or lytic replication

Introduction

The Kaposi's sarcoma-associated herpesvirus (KSHV) life cycle consists of 2 main states once inside a host cell: latency and lytic replication. As the switch from latency to lytic replication, reactivation plays an important role in KSHV infection and KSHV-associated disease. Developing a deeper understanding of viral control of these states can help us to better understand the molecular mechanisms of pathogenesis in associated diseases.

Early studies using bacteriophages hoped to better understand their life cycle and how they replicate, since it was known that viruses could multiply within their host organisms. In a 1939 study, Ellis and Delbrück sought to understand the quantitative aspect of the increase in number of bacteriophage after infecting host cells[129]. They mixed a phage with its E. coli host and quantified the number of infectious bacteriophage at various time points by plaque assay. When using a low ratio of phage:host they found that multiple bursts of phage generation would happen, likely due to the similar timing of the phage life cycle – this was akin to a multi-step growth curve. To study only a single-step of growth, they used a very high ratio of phage:host and as expected, only saw a single burst of phage growth. The theoretical underpinnings of these assays remain similar in the assays we have performed, and are critical to derive information about viral growth and its kinetics. Growth curves have still been used to analyze viral growth in more recent publications. In a study on human cytomegalovirus (HCMV), Dunn et al. used growth curves to understand viral growth of HCMV mutants in different cell types, and identify viral genes important for host cell tropism[103].

In this study we wanted to better understand the roles of KSHV viral genes in the different parts of the viral life cycle using a top level approach. Since KSHV typically enters latency in iSLK cells, and KSHV-infected iSLK cells can be induced to reactivate using the chemical agents doxycycline and sodium butyrate, we were able to use growth curve experiments with our KSHV mutant library of 90 single ORF disruption mutants to interrogate the roles of each ORF in latency, reactivation, and lytic replication. Using either multi-step or one-step growth curves was critical to either amplify enhancements or deficiencies in viral progeny generation, or to isolate only the reactivation step involving a single lytic replication cycle and not subsequent cycles. Although this high-level approach only measuring infectious viral progeny generation does not delve deeply into the specific functions of KSHV ORFs and how they facilitate viral life cycle stages, it does provide a general and global framework of ORF functionality from which more focused and targeted experiments can be developed.

Notably, we found that six mutants, in ORF60, 58, 67, 16, 61, and 46, showed severely-attenuated viral progeny generation in a multi-step lytic growth curve, indicating that they likely play an important, possibly essential, role in early infection events to set up the cell for successful lytic replication. Additionally, mutants in ORFs 50, 56, 49, and K11 showed

attenuation in viral progeny generation during latency, but not during reactivation or lytic replication, indicating that these ORFs play an important role in promoting spontaneous reactivation.

Materials and Methods

Cells, Viruses, Infection, and Antibodies

iSLK cells and BAC16 were generous gifts from Britt Glaunsinger. 293T cells were purchased from ATCC. iSLK and 293T cells were cultured in DMEM (Gibco, Thermo Fisher) supplemented with 10% FBS (Hyclone) and 1% PenStrep (Gibco). BAC16-transfected cells were additionally supplemented with 1.2mg/ml Hygromycin B (Invitrogen). To reactivate BAC16-transfected cells, cell media was additionally supplemented with 1µg/ml doxycycline and 1mM sodium butyrate (MilliporeSigma).

Infections

iSLK cells were seeded in plates 16-18 hours before infection to reach 70% confluency at infection. Dilutions of viruses were made in media containing or lacking 1µg/ml doxycycline and 1mM sodium butyrate. Cells were washed before adding these virus dilution to the wells. Cells were spun in plates at 2000for 1hr at 30°C, then put in a 5% CO_2 37°C incubator for an hour. The viral inoculum was removed, and cells were washed with PBS before adding media containing or lacking 1µg/ml doxycycline and 1mM sodium butyrate. Cells were returned to the incubator and this time point was designated t=0 for experiments.

Virus Generation and Titration

For virus titration, 293T cells were seeded at $5x10^4$ cells/well in 96 well plates 16-18 hrs before infection. Tenfold serial dilutions of virus were made in media containing 1µg/ml doxycycline and 1mM sodium butyrate and were added to the cells in duplicate. Plates were spun at 2000g, 1 hr, 30°C and put in the 5% CO_2 37°C incubator for one hour before changing media. 48 hours later, cells were fixed by paraformaldehyde and analyzed by flow cytometry to quantify the percentage of GFP+ cells as described below. Virus dilutions that yielded approximately 2-20% of cells GFP+ were used to calculate virus titer. Titration experiments were performed once in duplicate, using the average value of the duplicates.

Flow Cytometry

For virus titration, infected cells were collected at 48hpi, washed twice in PBS with 0.1% BSA, fixed in 1% paraformaldehyde for 5 min at RT, and washed twice in PBS with 0.1% BSA. Fixed cells were run on an LSR Fortessa X-20 flow cytometer (Becton Dickinson, Franklin Lakes, NJ) to quantify the percentage of GFP+ cells. BD FACSDiva (Becton Dickinson, Franklin Lakes, NJ) and Flowing Software 2 (Turku Center for Biotechnology) were used for analysis.

Results

Assaying infectious viral progeny under conditions favoring lytic replication uncovers mutants with defects in early infection events important for viral progeny generation

We next shifted our focus to studying the effects of ORF disruption on the viral life cycle following de novo infection. Since long-term transfected cell lines may differ in behavior from infected cells due to the long time spent in culture or the lack of authentic early infection events including virion binding and tegument protein release, we used de novo infection of iSLK cells at a fixed multiplicity of infection (MOI) with our mutant BAC16 virus library to study the effects of ORF disruption on the generation of infectious progeny during specific parts of the viral life cycle. This approach ensured authentic and consistent results. This would help us to determine the roles of ORFs in enhancing or disrupting infectious virus generating in these phases of the viral lifecycle.

Using our library of mutant BAC16 viruses, we infected iSLK cells under latent, reactivation, or lytic-inducing conditions and subsequently measured infectious virus production. These analyses were only possible using mutants which generated sufficient virus from lytic reactivation of transfected cell lines, so we only performed these experiments with 55 of the 68 mutants containing disruptions in nonessential genes.

To assay the ability of mutants to undergo multiple cycles of lytic replication and infection, iSLK cells were infected at an MOI of 0.1, to amplify defects or enhancements in growth, and maintained in media containing 1 μg/ml doxycycline and 1 mM sodium butyrate to induce lytic reactivation. Supernatant was collected every 3 days from 1dpi to 19dpi and spun at 2000g for 5 min to remove cell debris before titration on 293T cells as previously mentioned (Fig. 3.1). In general, titer began to plateau around 13dpi, and this was a time point of peak titer for many mutants, so this time point was chosen for comparison among the mutants and WT-BAC16 (Fig. 3.2). Our results showed that mutants could be categorized into four major groups based on infectious virus generation from multiple rounds of lytic replication: those for which viral progeny generation was severely-attenuated (at least 100-fold lower), partially-attenuated (10 to 100-fold lower), non-attenuated (within 10-fold), or enhanced (at least 10-fold higher) compared to WT-BAC16 (Table 2.3). These delineations could be clearly seen when tracking the amount of infectious virus in the supernatant over 13 days. Severely-attenuated mutants such as BAC16ΔORF46 did not accumulate virus in the supernatant over time, and virus levels were near the noise level of the assay. ORFs assigned to other classifications did accumulate virus in the supernatant over time, but the rate at which this occurred, and the virus concentration where a plateau was reached clearly varied. BAC16ΔORF28 is an example of a mutant which showed enhanced viral growth, reaching a plateau at much higher concentration of virus than WT-BAC16.

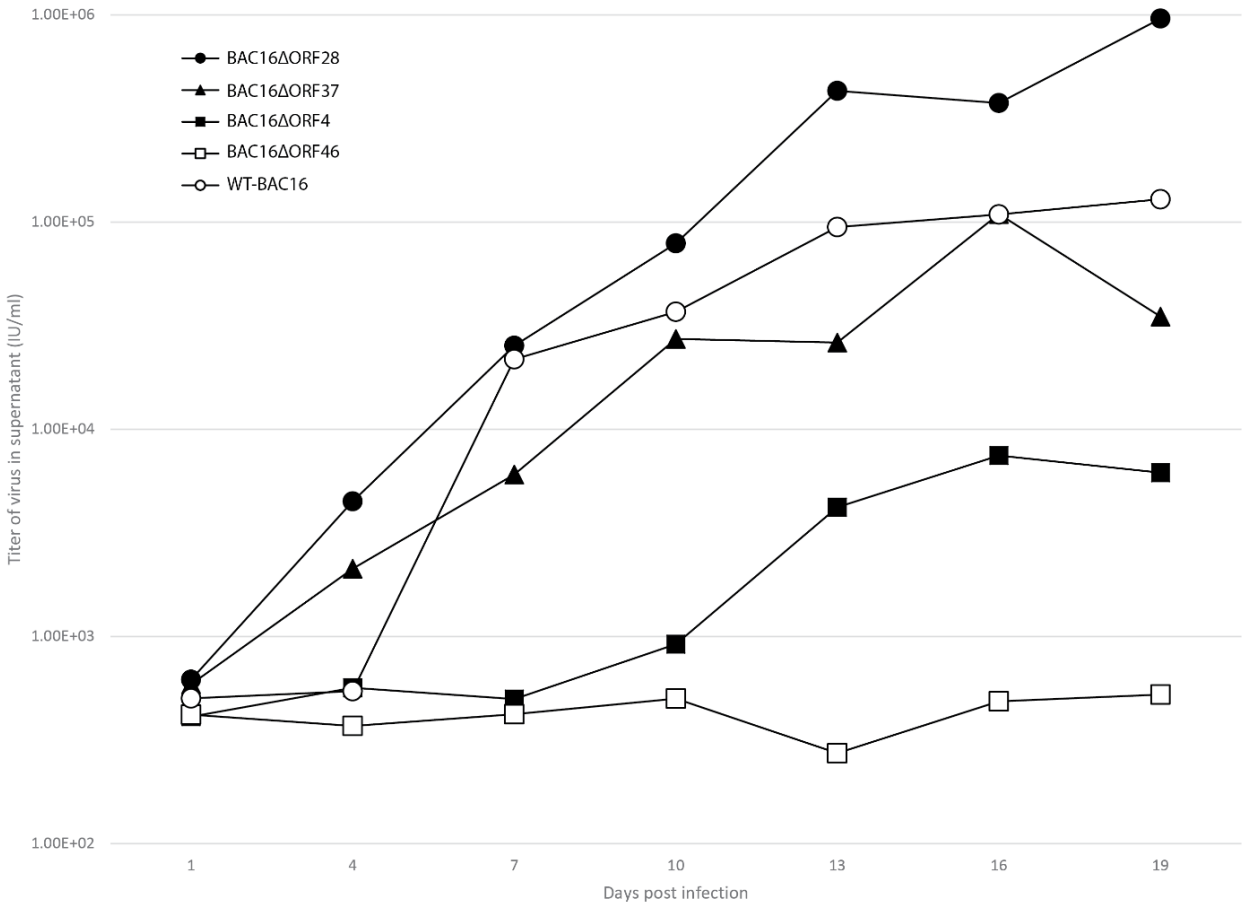


Figure 3.1. Representative examples of lytic growth curve data over 19 days post-infection.
iSLK cells were infected at an MOI of 0.1 under lytic cycle-inducing conditions. Supernatant was harvested and titered every 3 days from 1dpi to 19dpi. BAC16ΔORF28, BAC16ΔORF37, BAC16ΔORF4, and BAC16ΔORF46 are examples of mutants showing enhanced, non-attenuated, partially-attenuated, and severely-attenuated infectious virus generation respectively.

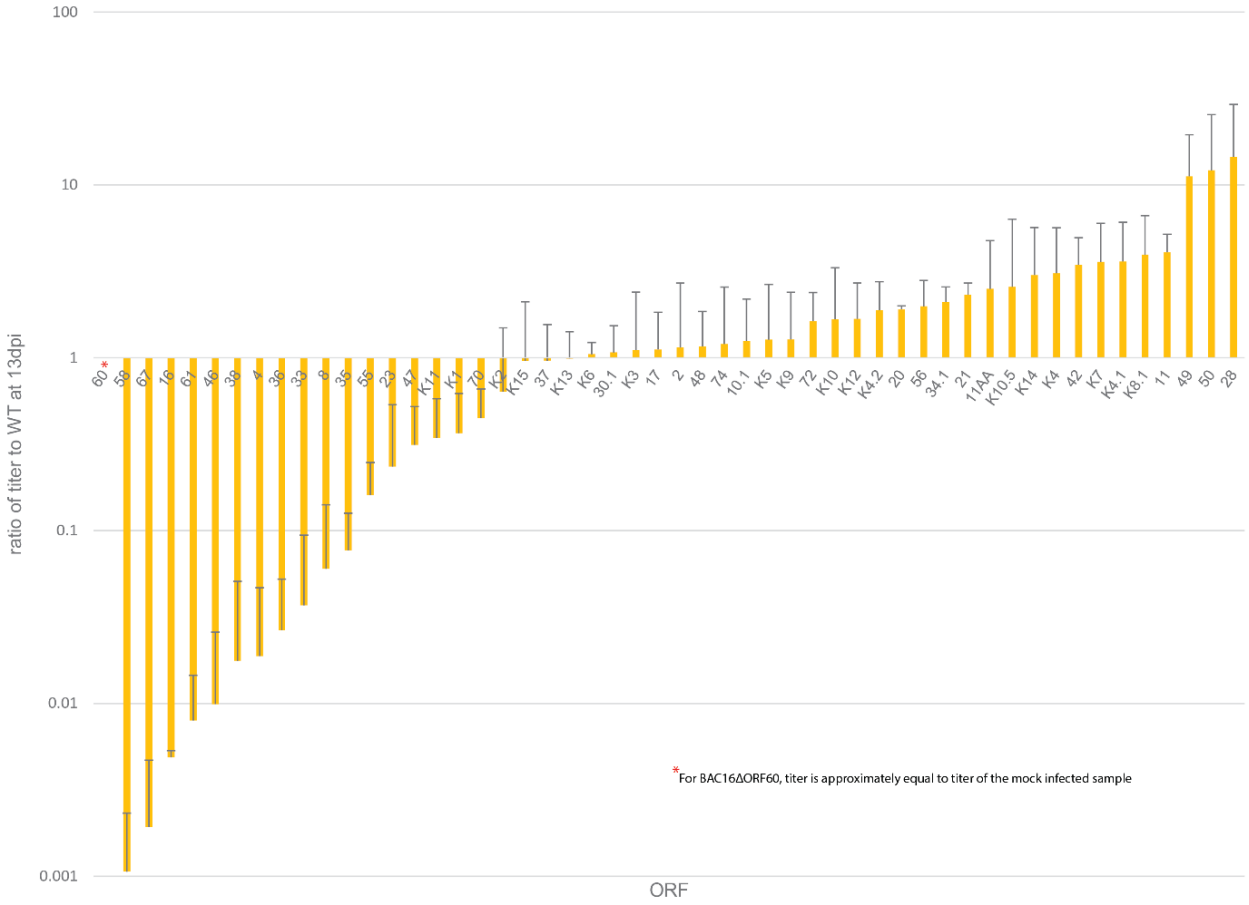


Figure 3.2. Virus generation of BAC16 and mutants under conditions promoting multiple rounds of lytic replication. iSLK cells were infected at an MOI of 0.1 under lytic cycle-inducing conditions. Supernatant was harvested and titered at 13dpi and this value was normalized to BAC16 titers. Error bars represent the standard deviation of 3 independent trials.

In fig 3.3, I show data from some of the most severely-attenuated (BAC16Δ58) and enhanced (BAC16Δ50) growth mutants, as well as a non-attenuated (BAC16Δ37) mutant and WT-BAC16. Although the difference in viral titer is easily apparent, microscopy of the infected iSLK cells at 13dpi shows little visible difference between the number or intensity of GFP+ cells for BAC16Δ58 compared to BAC16Δ37 or WT-BAC16. BAC16Δ50 does appear to show a higher number of GFP+ cells as well as a higher GFP intensity in certain cells, notably those in foci of what appear to be rounded cells undergoing lytic replication.

We expected some mutants, for example those with mutations in suppressors of lytic replication, to generate more virus than WT-BAC16, and others, for example those with mutations in genes facilitating lytic replication or virus entry, to generate little or no virus. Six mutants were severely-attenuated: BAC16ΔORF60, 58, 67, 16, 61, and 46. Six mutants were partially-attenuated: BAC16ΔORF38, 4, 36, 33, 8, and 35. Three mutants were enhanced: BAC16ΔORF28, 49, and 50, indicating that these ORF may function as temperance factors. All 37 other mutants tested showed a similar phenotype to WT-BAC16, indicating ORF disruption caused no attenuation of virus generation from multiple lytic replication cycles. Since our previous assays only assayed infectious virus production after a single replication cycle, the inability for severely-attenuated mutants to propagate under multiple cycles of infection and replication provided further insight into ORF functionality and therefore these observations were included on our genome map of ORF essentiality (Fig. 2.5). This phenotype indicated that the disrupted ORFs likely play a role in early infection events to set up the cell for successful lytic replication, a deficiency which would not be seen from induction of transfected cell lines.

Measuring generation of infectious viral progeny under conditions favoring reactivation finds that K3, K4, and K5 inhibit reactivation

To determine the effects of ORF disruption on lytic reactivation, we next measured the levels of infectious virus generation after de novo infection with BAC16 mutants and subsequent induced lytic reactivation. In this case, we used a higher MOI infection (MOI 1) and a shorter incubation time to simulate a single-step growth curve, so that we would not conflate the ORF disruption effects on lytic reactivation vs effects specific to multiple replication cycles. For this assay, iSLK cells were infected with mutant and WT BAC16 viruses at an MOI of 1 and incubated for 48 hours in the absence of sodium butyrate and doxycycline. At this time point the percentage of WT-BAC16 infected cells expressing ORF45, K8α, or K8.1A/B was determined to be 1.24%, 0.61%, and 0.33% respectively using antibody staining and flow cytometry, showing that most infected cells were entering latency (Table 3.1). At 48 hours after infection, the media was changed and supplemented with 1 mM sodium butyrate and 1 μg/ml doxycycline. At 48 hours after this media change, the percentage of WT-BAC16 infected cells expressing ORF45, K8α, or K8.1A/B was determined to be 34.03%, 26.68%, and 5.44% respectively using antibody staining and flow cytometry (Table 3.1), showing effective reactivation by this time point. At 72 hours after this media change, the supernatant was harvested and titrated on 293T cells to determine the concentration of infectious virus in each sample and this was normalized to WT-BAC16 (Fig. 3.4).

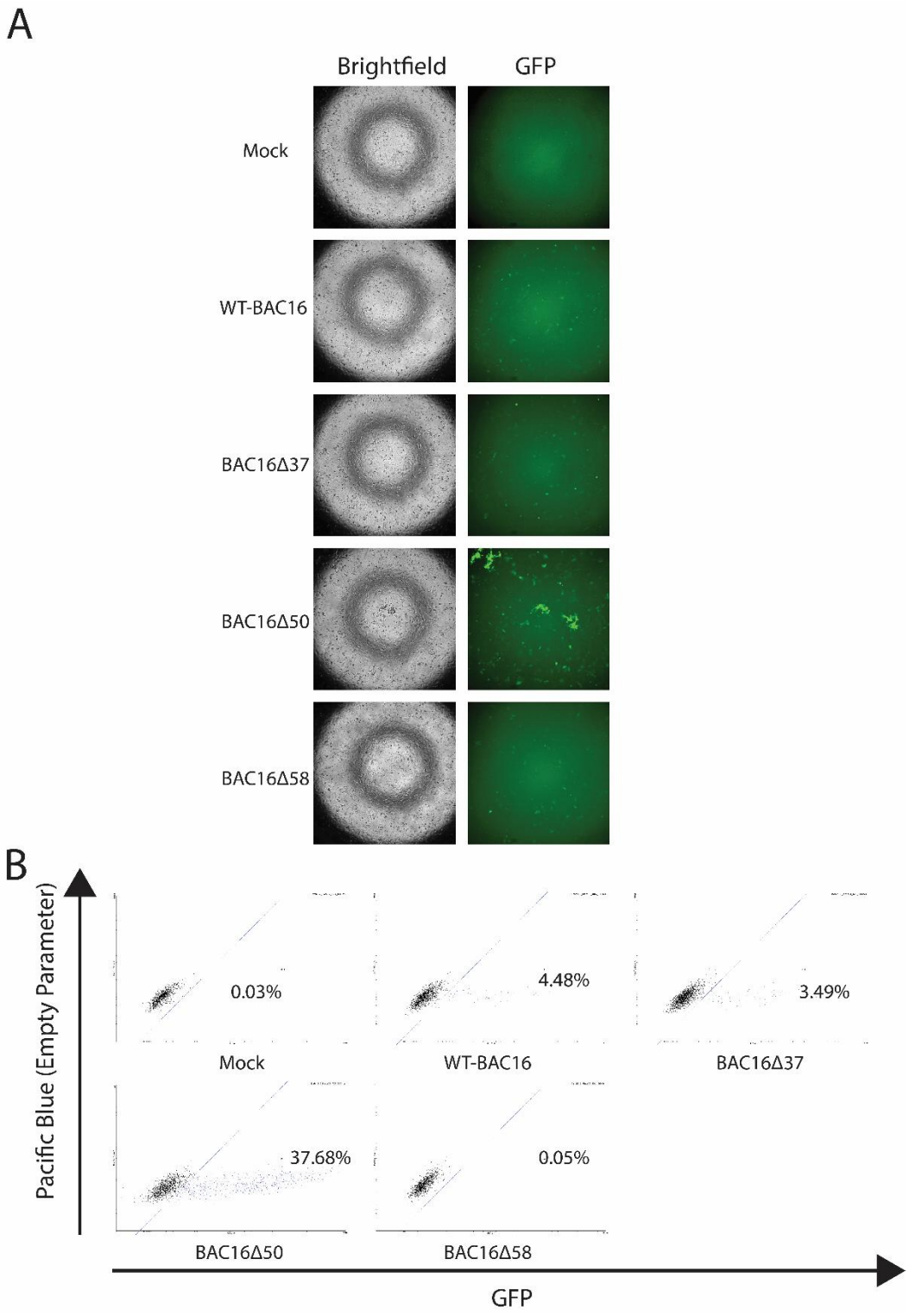


Figure 3.3. Infectious virus generation and microscopy of mutants showing non-attenuated, enhanced, or severely-attenuated growth during multiple rounds of lytic replication. iSLK cells were infected at an MOI of 0.1 under lytic cycle-inducing conditions with BAC16 or mutants. (A) Wells were imaged by brightfield and fluorescence microscopy and (B) supernatant was harvested and titered at 13dpi by 293T infection before flow cytometry analysis. BAC16Δ37, BAC16Δ50, and BAC16Δ58 show non-attenuated, enhanced, and severely-attenuated growth respectively.

Table 3.1. Percentages of WT-BAC16 infected cells expressing ORF45, K8α, or K8.1A/B at different time points and under different conditions after de novo infection of iSLK cells.

	Short-term latency	Reactivation	Long-term latency
ORF45	1.24%	34.03%	0.31%
K8α	0.61%	26.68%	0.25%
K8.1A/B	0.33%	5.44%	0.09%

Since all mutants used for this experiment could be successfully reactivated in transfected iSLK cell lines to generate viral progeny, we expected all mutants to generate a measurable level of infectious virus. this assay would provide a reproducible and accurate method to compare virus levels generated from reactivation to better understand the roles of these ORFs in reactivation and infectious virus generation. We expected that partial deficiencies in infectious virus generation compared to WT-BAC16 in this assay would be due to incomplete deficiencies in either the process of reactivation or infectivity of progeny virus. We also expected that trends in the results would be similar to those of our multi-step lytic growth cure described above, only with smaller deviations from the WT phenotype due to the lack of exponential viral growth, unless the disrupted ORF only plays a role specific to reactivation or to subsequent replication cycles.

We found that the majority of mutants generated a titer within 10-fold above or below that of WT-BAC16, indicating little to no dependence on those ORFs for reactivation and infectious virus generation. However, a few mutants generated titers of about 100-fold or less when compared to WT-BAC16, indicating severe attenuation in infectious virus generation during reactivation (Table 2.3). These mutants are BAC16ΔORF46 (uracil deglycosylase), 67 (nuclear egress complex), 33 (tegument protein in MHV68), and 38 (myristylated protein). BAC16ΔORF46 and 67 were also severely-attenuated in the lytic growth curve while BAC16ΔORF33 and 38 were partially-attenuated. This suggests that ORF 46 and 67, and possibly ORF33 and 38, likely do not play a role specific to either reactivation or multiple rounds of de novo infection and lytic replication. Notably, BAC16ΔK3, K4, and K5 showed a degree of increased viral production over WT-BAC16 in this reactivation assay, yet show a comparable level to WT-BAC16 in the lytic multi-step growth curve, implying that they normally function to specifically inhibit reactivation.

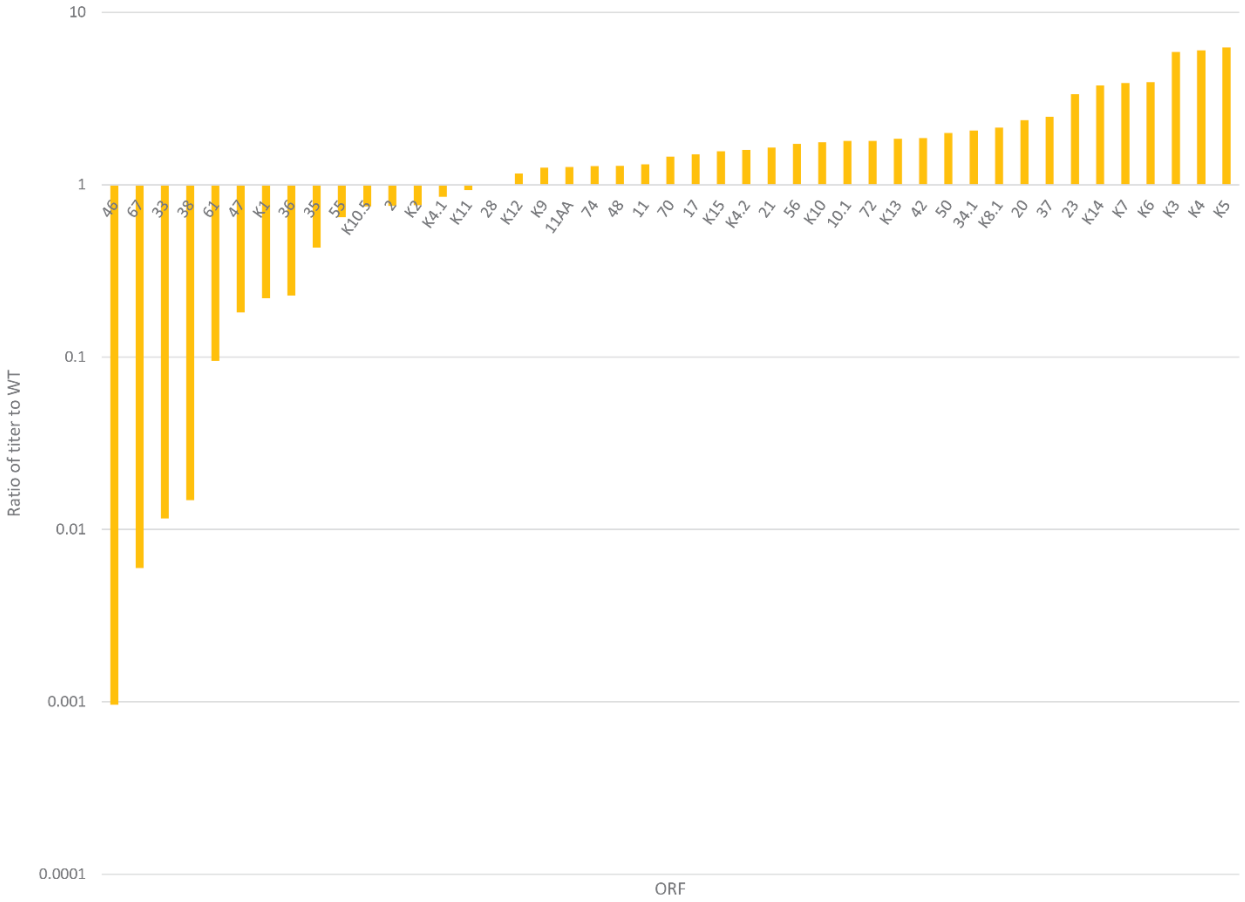


Figure 3.4. Virus generation of BAC16 and mutants under conditions promoting reactivation. iSLK cells were infected at an MOI of 1 under non-inducing conditions. Infected cells were treated with sodium butyrate and doxycycline at 2dpi to induce lytic reactivation and supernatant was harvested at 5dpi. Supernatant was titered and normalized to BAC16 titers.

Infectious viral progeny generation under conditions favoring latency shows that ORFs 49, 50, 56, and K11 appear to play a role in enhancing levels of spontaneous reactivation

Finally, we also chose to assay the levels of virus progeny in the supernatant of iSLK cells after de novo infection with mutant or WT BAC16 and maintenance in the absence of lytic cycle-inducing factors to see if disruption of any genes led to increased levels of spontaneous reactivation and lytic replication. In the absence of lytic induction, virus generation is likely predominantly driven by spontaneous reactivation. iSLK cells were infected with BAC16 or mutants at an MOI of 1 and maintained in media lacking doxycycline or sodium butyrate. At 6dpi the supernatant was collected and titrated on 293T cells to determine the concentration of infectious virus in each sample and this was normalized to WT-BAC16 (Fig. 3.5). At this time point, the percentage of WT-BAC16 infected cells expressing ORF45, K8α, or K8.1A/B was determined to be 0.31%, 0.25%, 0.09% respectively, indicating that most infected cells had entered latency (Table 3.1). We did expect the disruption of some genes, such as ORF50, the protein responsible for the lytic switch, to reduce levels of spontaneous reactivation and viral titer compared to WT-BAC16. We also expected to identify ORFs which normally function to repress spontaneous reactivation, the disruption of which would yield a higher titer than WT BAC16 in this assay. We also expected most ORFs to have little or no role in these processes and yield a similar titer to WT BAC16.

As expected, disruption of many viral genes showed little to no difference in viral titer generated (Table 2.3). However, disruption of 7 genes [ORF50 (RTA), ORF56 (DNA replication), ORF67 (nuclear egress complex), ORF49 (activates JNK/p38), ORF61 (ribonucleoprotein reductase), K11 (vIRF2), ORF58 (N/A)] showed a decrease of approximately 10-fold or more compared to WT-BAC16. Though BAC16ΔORF58, 61, and 67 were classified as severely-attenuated in the multi-step growth curve, and BAC16ΔORF67 was classified as severely-attenuated in the reactivation assay, BAC16ΔORFs50, 56, 49, and K11 did not show any attenuation in these two assays, suggesting that their reduced progeny generation in latency is likely due to the role of these ORFs in specifically enhancing levels of spontaneous reactivation. Also, BAC16ΔORF49 and 50, which show enhanced virus production in the lytic growth curve, may play contrasting roles in infected cells under conditions favoring lytic or latent replication.

Several mutants achieved enhanced viral progeny generation, defined by a lower bound of about 3-fold higher titer than WT. These included BAC16ΔORF11AA (N/A), 72 (vCyclin), K7 (N/A), K6 (vMIP-IA), and K3 (immune modulator). Although BAC16ΔK3 shows enhanced growth under the reactivation assay, the four others show no attenuation in the previous two assays, showing that their role is likely specific to repressing spontaneous reactivation. The presence of viral genes which either enhance or repress spontaneous reactivation demonstrates the biological importance of tight viral control of levels of spontaneous reactivation.

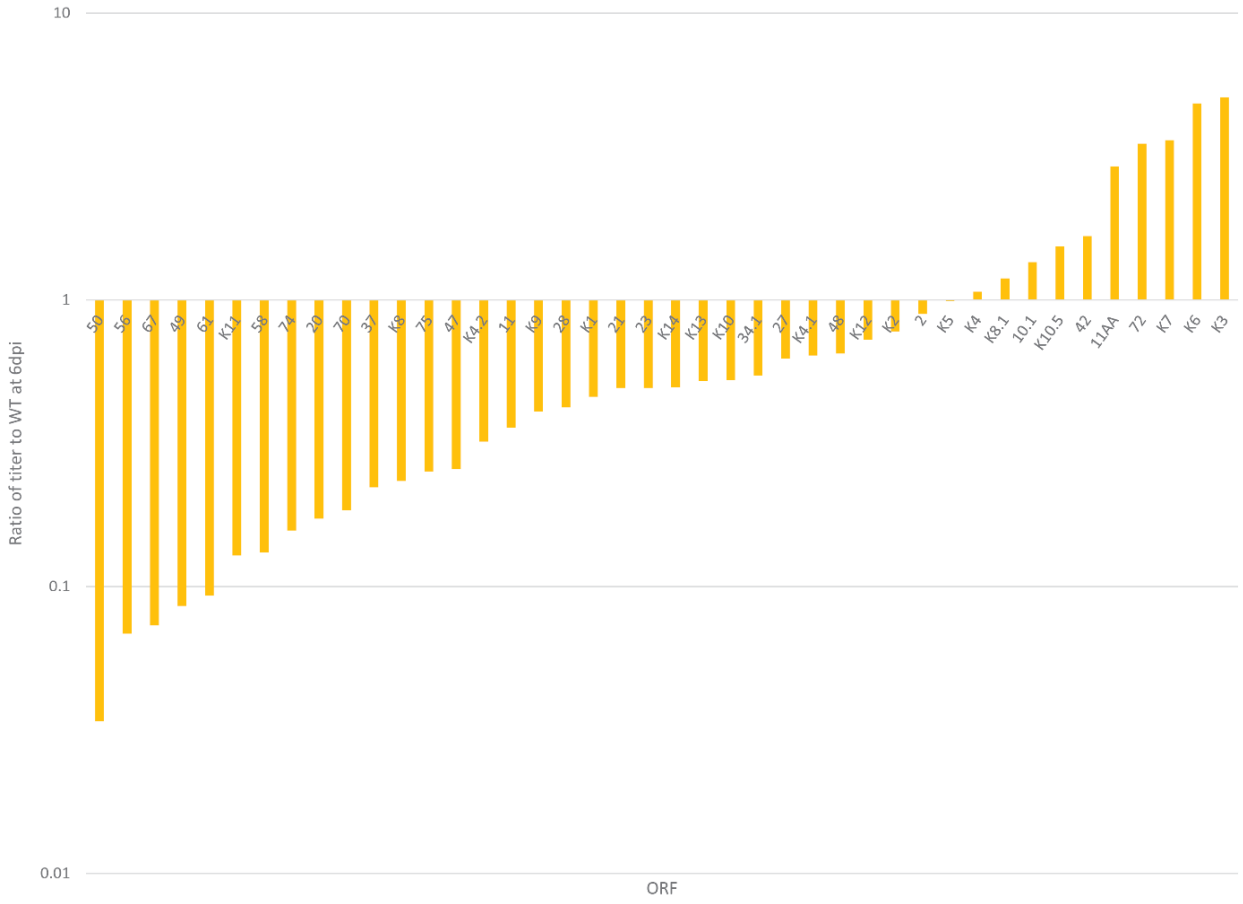


Figure 3.5. Virus generation of BAC16 and mutants under conditions promoting latency. iSLK cells were infected at an MOI of 1 under non-inducing conditions. Infected cells were maintained under non-inducing conditions and supernatant was harvested at 6dpi. Supernatant was titered and normalized to BAC16 titers.

Discussion

By using de novo infection to dissect the effects of ORF disruption on specific parts of the viral life cycle, we found many interesting phenotypes warranting further study. When assaying gene essentiality for multiple cycles of lytic replication, six mutants (BAC16ΔORF16, 46, 58, 60, 61, 67) generated negligible levels of infectious virus compared to WT-BAC16, despite generating significant virus from transfected cell line induction This suggests that these ORFs may play an important role in the early stages of infection after virion attachment in priming the virus to allow successful reactivation, possibly through an imprinting mechanism such as an epigenetic change. It's also possible that some of these mutants are simply so deficient in virus generation during each viral replication cycle that there is almost no virus present when the supernatant was harvested. The putative functions of these genes vary widely - ORF16 is a BCL-2 homolog, ORF 67 functions as part of the nuclear egress complex, ORF46, 60, and 61 play a role in nucleotide metabolism while ORF58 is an Epstein-Barr virus BMRF2 homologue. Analysis of early infection events using these mutants after could be fruitful in elucidating this mechanism, and these ORFs may present valuable therapeutic targets to inhibit KSHV lytic replication. The enhanced virus generation of BAC16ΔORF50, a mutant lacking the lytic switch protein, in this assay was also surprising since the gene product is complemented by the induction of the ORF50 transgene in iSLK cells by doxycycline treatment. This suggest a complex relationship between ORF50 and lytic replication and it is possible the ORF50 locus in the BAC16 genome plays important and distinct regulatory roles in the decision to reactivate under various conditions. LANA is known to bind the ORF50 locus and repress its transmission, and ORF50 is known to autoactivate itself so it's possible that ORF50 as a cis-acting element is the source of the phenotype we see[125,130]. Our observation may also suggest that high expression levels of ORF50 could play an inhibitory role in lytic replication. More studies would help further elucidate the mechanism behind this phenotype and understand its role in KSHV-associated disease.

We also found that BAC16ΔK3, K4, and K5 all showed enhanced virus generation compared to WT-BAC16 under reactivation conditions, although they showed levels comparable to WT-BAC16 in the lytic growth curve, suggesting that these factors play a role specifically during reactivation to restricting virus generation. Since all three are KSHV-specific genes the mechanisms by which these temperance factors inhibit reactivation would have evolved with KSHV for its niche. Interestingly, K3 and K5 are known to be ubiquitin E3 ligases which degrade MHC-I on the cell surface, and K4 is a viral chemokine that inhibits natural killer cell migration[122,131]. Neither of these functions seem that they would be particularly relevant in a cell culture environment, thus these ORFs certainly warrant further study which could uncover unique mechanisms that would help us better understand what drives reactivation and how we can modulate it, which would have significant clinical significance in controlling KSHV-associated disease.

When studying latent infection, we could not definitively determine the mechanisms for increased or decreased virus production without further investigation. However, it is notable that BAC16ΔORF50, 49, 56, and K11 showed severe attenuation and BAC16ΔORF11AA, 72, K6,

and K7 showed enhanced virus generation under conditions favoring latency but not in the lytic or reactivation assays, suggesting that these phenotypes are specifically due to repression or enhancement of spontaneous reactivation. Since other studies show that ORF50 is necessary and sufficient for reactivation, the observation that BAC16ΔORF49, 56, and K11 show comparable phenotypes emphasizes their function in promoting spontaneous reactivation, perhaps acting as important parts of the latent/lytic switch. This uniquely underscores the evolutionary importance of spontaneous reactivation for KSHV, which is logical since previous studies have demonstrated the importance of spontaneous reactivation in pathogenesis[42,58]. Although ORF50 is thoroughly studied for its role in controlling the switch of lytic reactivation from latency it is possible that other lesser-studied viral proteins still play significant roles in this process. Due to the clinical importance and incomplete understanding of spontaneous reactivation, these ORF are especially important to pursue with further study. These ORFs may serve as effective targets for anti-KSHV therapeutics, and further studies could help us better understand viral control of spontaneous reactivation.

Regarding our assay under latency promoting conditions, many of the ORFs tested in this assay typically are only expressed at significant levels during lytic replication, and therefore wouldn't be expressed at high levels in most cells under these conditions. This either suggests that we are only observing the effects of ORF disruption on cells that are already entering spontaneous reactivation, low levels of viral lytic gene expression during latency can play important roles in the decision of entering spontaneous reactivation, or these ORFs function under these conditions as cis-acting elements to maintain levels of spontaneous reactivation. In either case, these ORFs likely play an important role in the latent/lytic switch or act downstream of ORF50 activation to drive completion of the lytic cycle and enhance viral progeny production after spontaneous reactivation, but not during normal lytic replication.

Interestingly, the disruption of ORF49 increased virus generation for the lytic growth curve but decreased virus generation under condition favoring latency. This would suggest that this factor inhibits lytic replication under lytic cycle-inducing conditions but facilitates spontaneous reactivation or completion of the lytic cycle for reactivating cells during noninducing conditions. It is possible that this factor acts as some sort of regulator to prevent excessive virus production and cell death during lytic, while also facilitating necessary levels of spontaneous reactivation and that this regulation is evolutionarily selected for and biologically significant. It is also notable that BAC16ΔORF50 falls into this same category, and while it is known to be essential for lytic reactivation, it is not clear how ORF50 functions to control viral progeny generation under lytic cycle-inducing conditions[132].

It should be noted that we were unable to use these assays to study mutants nonessential for reactivation and infectious virus generation that generated very low amounts of virus upon cell line lytic induction. These mutants would have required huge amounts of transfected cell lines to be grown and for the supernatant to be very highly concentrated, neither of which we had the resources for.

Ultimately, these assays measuring virus generation after de novo infection helped to define the roles of these ORFs in facilitating progression through the viral life cycle. Measuring viral progeny generation shows us a high-level view of the roles of ORFs in parts of the viral life cycle. Future studies based off of the data found in these studies should be performed to better understand the more specific function of these ORFs in different viral life cycle stages. Developing a better understanding of viral control of the viral life cycle will play a key role in understanding and controlling KSHV-associated disease.

Chapter 4: Lytic gene expression of BAC16 mutants under conditions favoring latency and reactivation

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) gene expression is highly regulated. During latency, only a few of the virus' over 70 primary ORFs are expressed, including ORF73 (LANA), K13 (v-FLIP), ORF72 (v-cyclin), and K12 (kaposin), as well as the 25 KSHV miRNAs[133,134]. These genes have diverse functions including the maintenance, replication, and appropriate segregation of the KSHV genome as well as promoting oncogenesis and modulating signaling pathways[135]. ORF50 (RTA) functions as the lytic switch, and when expressed at high levels, induces the full lytic gene cascade[34,121]. Interestingly the RTA protein can auto-activate its own promoter providing a positive feedback loop that pushes the virus into lytic replication[130]. Additionally, LANA and ORF50 function to inhibit each other's expression suggesting a model where ORF50 expression must increase sufficiently to overcome inhibition by LANA in order to reactivate from latency[125]. In lytic replication, viral genes are categorized by the timing of their expression into immediate-early, early, or late lytic genes. Immediate-early gene expression is first detectable by about 4 hours after lytic induction, while early gene expression appears 8-13 hours after lytic induction and peaks at 20 hours. Late gene expression begins at 20-30 hours after lytic induction[35]. By definition, late gene replication requires viral DNA replication is a prerequisite. At the end of this progression, new virions will be assembled in the nucleus and packed with the viral genome before egress from the nucleus, tegumentation, and budding from the cell.

An early tool useful for the study of latency and lytic replication was a recombinant virus, rKSHV.219[63]. This virus was generated from JSC-1 cells, a primary effusion cell line coinfected with KSHV and EBV, and contained a GFP gene under the control of the EF-1α promoter as well as an RFP gene under control of the KSHV Pan promoter, only active during lytic replication[62]. This tool allowed for the determination of the viral state in living cells infected in culture by fluorescence microscopy which greatly facilitated latent/lytic studies. It didn't require the disruption of the cells and the additional complexity of staining cells for latent or lytic antigens. Since KSHV enters latency in most cell types in culture and can be reactivated using compounds such as 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) or sodium butyrate, this system facilitated the approach of manipulating the host cell environment and determining how these manipulations affected viral latency or lytic replication[106,136]. Although the BAC16 unfortunately lacks a fluorescent marker expressed during lytic replication, we can still use immunofluorescent staining identify lytic replication and to more deeply study patterns of lytic gene expression in infected cells.

After our previous high-level studies on infectious virus generation, we wanted to delve deeper by analyzing lytic antigen expression patterns. Using iSLK cells and our mutant library of 90 gene disruption mutants in the BAC16, we analyzed immediate-early, early, and late gene of BAC16 mutant-infected cells under different conditions promoting either latency or reactivation.

Notably, we identified ORF20 as a viral factor which tempers lytic expression upon reactivation. ORF 10.1, 20, 23, 27, 58, and 67 appear to facilitate the burst of lytic gene expression shortly after infection, and ORF 11, 28, K10, K10.5, and K9 promote the establishment of latency after this.

Materials and Methods

Cells, Viruses, Infection, and Antibodies

iSLK cells and the BAC16 were generous gifts from Britt Glaunsinger. iSLK cells were cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Hyclone, GE Healthcare, Chicago, IL) and 1% PenStrep (Gibco, Thermo Fisher Scientific, Waltham, MA). BAC16-transfected cells were maintained in DMEM supplemented with 10% FBS, 1% PenStrep, and 1.2mg/ml Hygromycin B (Invitrogen, Carlsbad, CA). To reactivate transfected cells, cell lines were cultured in DMEM supplemented with 10% FBS, 1% PenStrep, 1ug/ml doxycycline (MilliporeSigma, Burlington, MA) and 1mM sodium butyrate (MilliporeSigma, Burlington, MA).

The following antibodies were used in this study: anti-ORF45 (2D4A5, Promab 20016), anti-K8α (8C12G10G1, Promab 20015), anti-LANA (LNA-1, Advanced Biotechnologies 13-210-100), Anti-K8.1A/B (4A4, Santa Cruz Biotechnology sc-65446), Goat anti-Rat IgG Alexa Fluor 568 (Life Technologies A11077), goat anti-rat AlexaFluor 568 (Abcam ab175710), goat anti-mouse superclonal AlexaFluor647 (Life Technologies A28181), Goat anti-Mouse Alexa Fluor 568 (Invitrogen A-11004)

Infections

iSLK cells were seeded in plates 16-18 hours before infection to reach 70% confluency at infection. Dilutions of viruses were made in media lacking 1µg/ml doxycycline and 1mM sodium butyrate. Cells were washed before adding these virus dilution to the wells. Cells were spun in plates at 2000for 1hr at 30°C, then put in a 5% CO_2 37°C incubator for an hour. The viral inoculum was removed, and cells were washed with PBS before adding media containing or lacking 1µg/ml doxycycline and 1mM sodium butyrate. Cells were returned to the incubator and this time point was designated t=0 for experiments.

Flow Cytometry

For flow cytometry staining experiments, iSLK cells were fixed in 4% paraformaldehyde for 5 min at room temperature, permeabilized with 0.1% Triton X-100 (Thermo Fisher Scientific, Waltham, MA) for 5 min at room temperature, then probed with anti-LANA (LNA-1; Advanced Biotechnologies) and either anti-ORF45 (2D4A5, Promab), anti-K8α (8C12G10G1; Promab), or anti-K8.1A/B (4A4, Santa Cruz Biotechnology). These cells were then reacted with goat anti-mouse IgG conjugated to AlexaFluor647 (A28181, Invitrogen) or goat anti-Rat IgG conjugated to AlexaFluor568 (ab175710, Abcam). Samples were analyzed on a BD LSR Fortessa X-20 flow cytometer. BD FACSDiva and Flowing Software 2 were used for analysis.

Results

Analyzing levels of lytic antigens after infection or after infection and reactivation shows that multiple viral factors play roles in important early viral infection events leading to the establishment of latency

In order to more closely investigate the effects of ORF disruption on the viral life cycle, we decided to analyze viral gene expression during infection under conditions favoring different life cycle stages. This allowed us to quantitatively analyze the ability of BAC16 mutants to establish normal latency and reactivate from latency following de novo infection. After testing a panel of antibodies, we decided to use antibodies targeting ORF45, K8α, and K8.1A/B to assess immediate-early, early, and late lytic gene expression respectively as these antibodies consistently yielded reliable results after optimization. Many commercially available antibodies targeting KSHV antigens were unable to yield reliable results in immunofluorescence or flow cytometry-based assays after undergoing optimization. Additionally, GFP would be used as a marker of infected cells since the BAC16 contains a GFP gene which is constitutively expressed under control of the EF-1α promoter. Using flow cytometry, this allowed us to gate on GFP+ (infected) cells and determine the percentage of GFP+ antigen- and GFP+ antigen+ cells. These values could then be compared to those for WT-BAC16 as the baseline for normal lytic antigen expression. We conducted this assay at three time points to look at short-term latency, long-term latency, and reactivation conditions.

Here I've included representative reactivation data for mock, BAC16, BAC16ΔORF20, and BAC16ΔK9 infected cells to show raw data before processing and analysis (Fig. 4.1). GFP- cells were considered uninfected and were not included in the downstream analysis. Differences in the percentage of lytic-antigen expressing cells for mutants could already be seen at this stage. Compared to BAC16, a higher proportion of BAC16Δ20 GFP+ cells are also positive for ORF45, K8α, or K8.1A/B. Conversely, a lower proportion of BAC16ΔK9 GFP+ cells are positive for ORF45, K8α, or K8.1A/B compared to BAC16. Most mutants did not show such a consistent increase or decrease of lytic antigen-positive cells across all three antigens tested.

For WT-BAC16, we expected a low baseline percentage of lytic antigen-expressing cells in the absence of induction and an elevated percentage after induction. We found that the percentage of infected cells expressing ORF45, K8α, or K8.1A/B at fixation in short-term latency was 1.24%, 0.61%, and 0.33% respectively, which rose to 34.03%, 36.68%, and 5.44% two days after reactivation (Table 3.1). These figures matched our expectations since a small lytic burst of gene expression happens shortly after infection, and we still saw a large increase in lytic antigen-expressing cells, after induction. In long-term latency, these values were 0.31%, 0.25%, 0.09%, which also matched our expectations of the majority of infected cells entering latency.

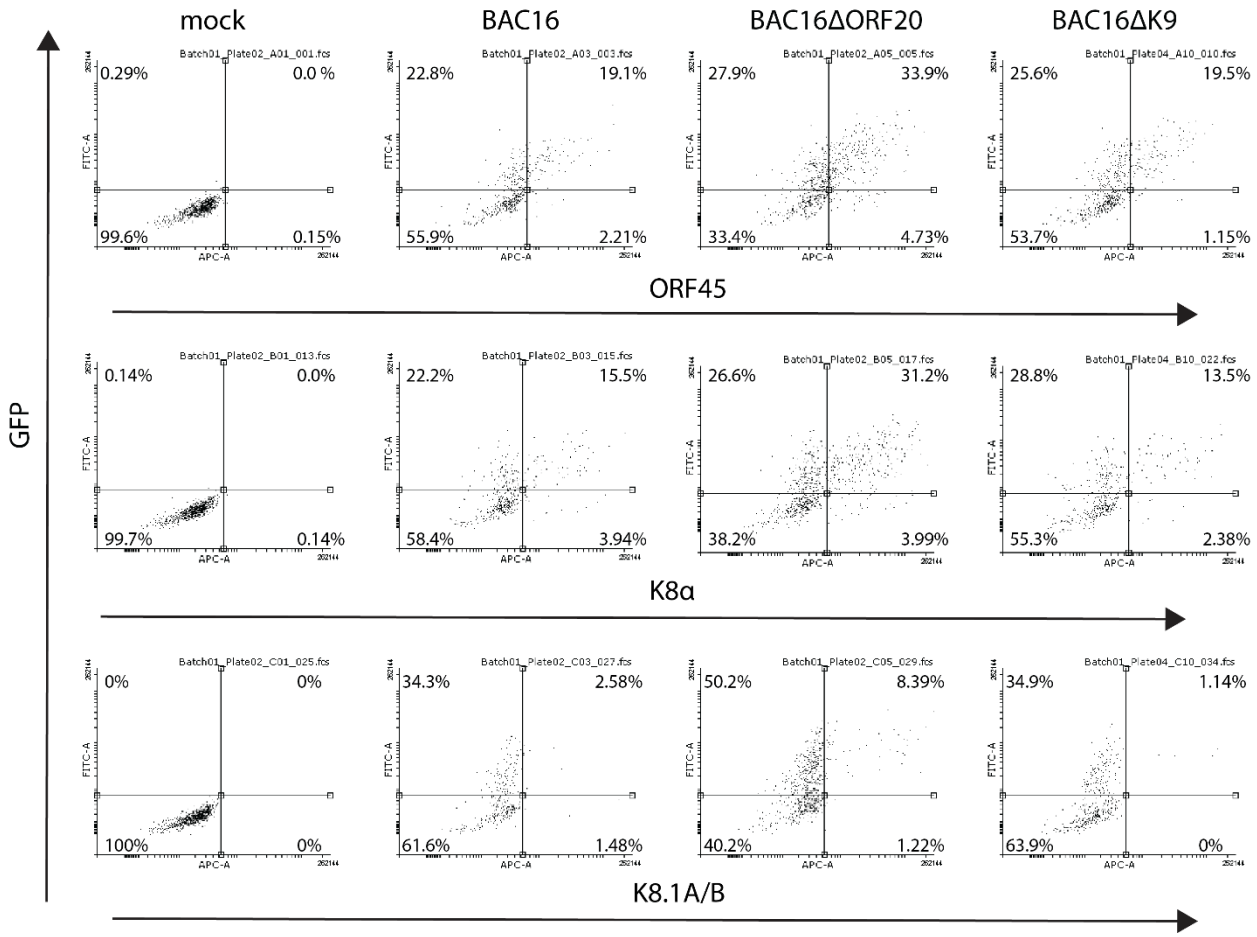


Figure 4.1. Examples of lytic antigen expression of BAC16 and mutant-infected cells by flow cytometry. Representative data of flow cytometry experiments under reactivation conditions showing mock, BAC16, BAC16ΔORF20, and BAC16ΔK9 infected cells stained for lytic antigens ORF45, K8α, or K8.1A/B. Stained cells were analyzed by flow cytometry for GFP and lytic antigen expression. Percentages of cells in each quadrant are noted.

To assay short-term latency, iSLK cells were infected with WT-BAC16 or mutant viruses at an MOI of 1 and fixed with paraformaldehyde at 2dpi. Fixed cells were permeabilized and stained by the aforementioned antibodies targeting ORF45, K8α, and K8.1A/B to study immediate-early, early, and late lytic gene expression and were analyzed by flow cytometry (Fig. 4.2). We did not expect to see mutants with dramatically elevated levels of lytic antigen expression since we knew from our generation of transfected cell lines in chapter 2 that only ORF73 was essential for the maintenance of latency, and increased lytic gene expression in the absence of lytic cycle-inducing conditions likely would have inhibited generation and growth of the transfected cell lines. Since a burst of lytic gene expression occurs shortly post-infection, we expected to see a decrease in lytic gene expression upon deletion of ORFs playing a role in this process and therefore better understand this phenomenon.

We found that no mutant reliably showed significantly increased lytic gene expression and the majority of mutants showed similar or decreased lytic gene expression compared to WT-BAC16. Since it is a late lytic gene, K8.1A/B expression levels should act as a good proxy for the ability of the virus to proceed through all stages of viral expression. Interestingly, mutants in ORF 11, 17, 28, 47, 56, 74, K10, K10.5, and K9 all showed very low levels of K8.1A/B expression, on par with the negative control for this antigen, BAC16ΔK8.1, which could implicate these ORFs as key gene expression regulators that facilitate the lytic burst and establishment of latency.

We next chose to assay a time point longer past the day of infection to further allow for the establishment of latency and hopefully to negate the effect of the early lytic burst on lytic gene expression. Here we infected iSLK cells with WT-BAC16 or mutant viruses at an MOI of 1 and fixed the cells at 6dpi. Fixed cells were stained with antibodies targeting ORF45, K8α, and K8.1A/B and were analyzed by flow cytometry (Fig. 4.3). At this time point, we expected that some of the mutants with a low percentage of K8.1A/B-expressors identified in the short-term latency assay would also have a low percentage of K8.1A/B-expressors in this assay and the others would have a percentage closer to WT-BAC16. This would allow me to distinguish which ORFs functioned specifically in facilitating the lytic burst and which functioned more generally to maintain spontaneous reactivation during latency.

Notably, we saw a greater divergence in lytic gene expression from that of WT-BAC16, as many mutants showed significantly increased or decreased lytic gene expression. Mutants in ORFs 10.1, 20, 23, 27, 58, and 67 showed at least a two-fold increase in the percentage of cells expressing K8.1A/B relative to WT-BAC16. Conversely, we saw significantly repressed K8.1A/B expression in mutants in ORF 47, 49, 56, 70, 74, 75, K1, K11, K13, K2, K4, K4.2, and K8. As mutants in ORF 47, 56, and 74 showed a low percentage of K8.1A/B-expressing cells in both short and long-term latency, it's possible that these ORFs are important for maintaining levels of spontaneous reactivation during latency. Mutants in ORFs 11, 28, K10, K10.5, and K9, which also showed a low percentage of K8.1A/B-expressing cells in short-term latency, generally show levels near WT-BAC16 during long-term latency, implying that the normal function of these ORFs in maintaining lytic-gene expression only applies at times shortly after infection.

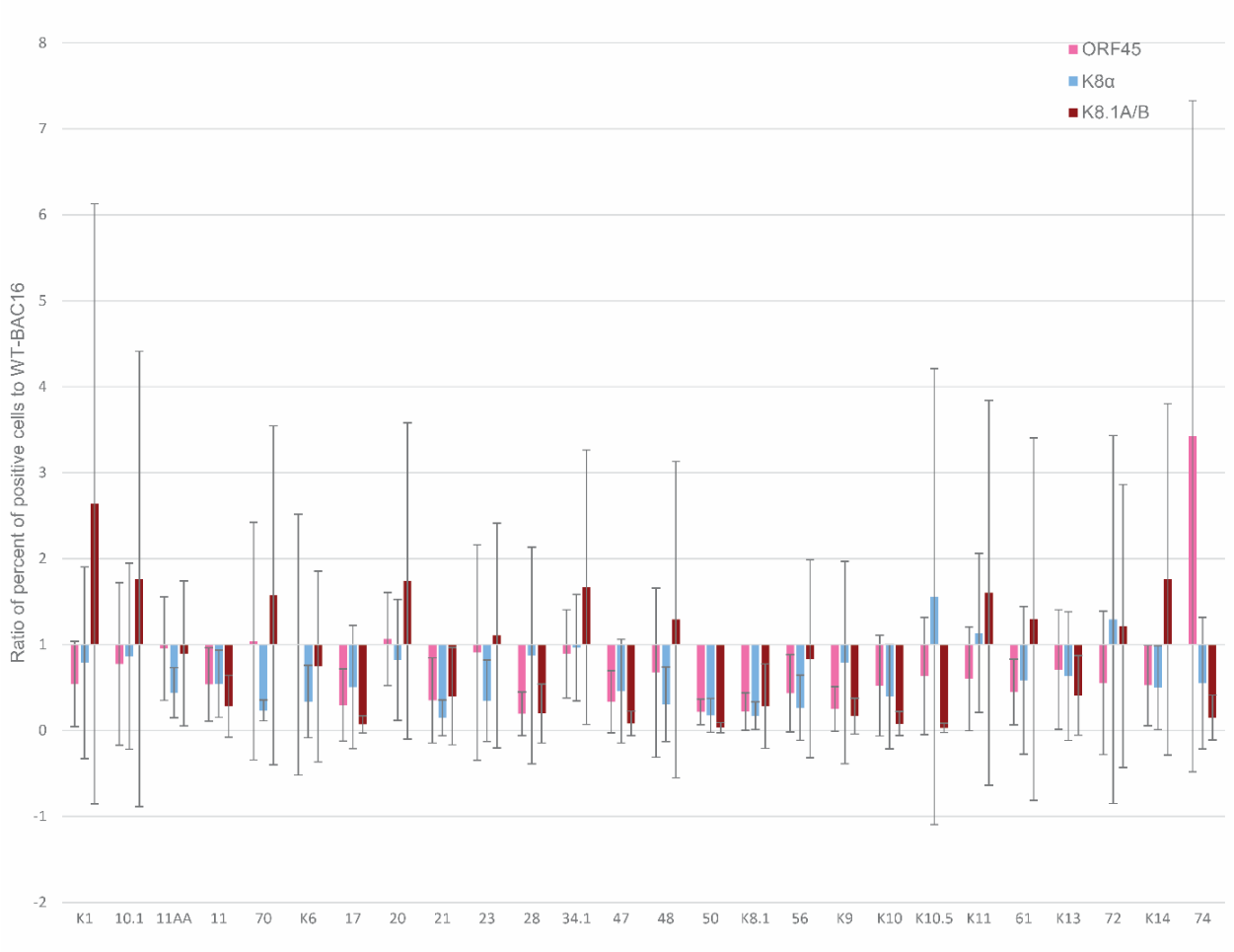


Figure 4.2. Lytic antigen expression during short-term latency after de novo infection with BAC16 and mutants. iSLK cells were infected by BAC16 or mutants at an MOI of 1 under noninducing conditions. Cells were fixed and stained for lytic antigen expression at 2dpi. The percentage of GFP+ cells expressing these lytic antigens was quantified and normalized to BAC16.

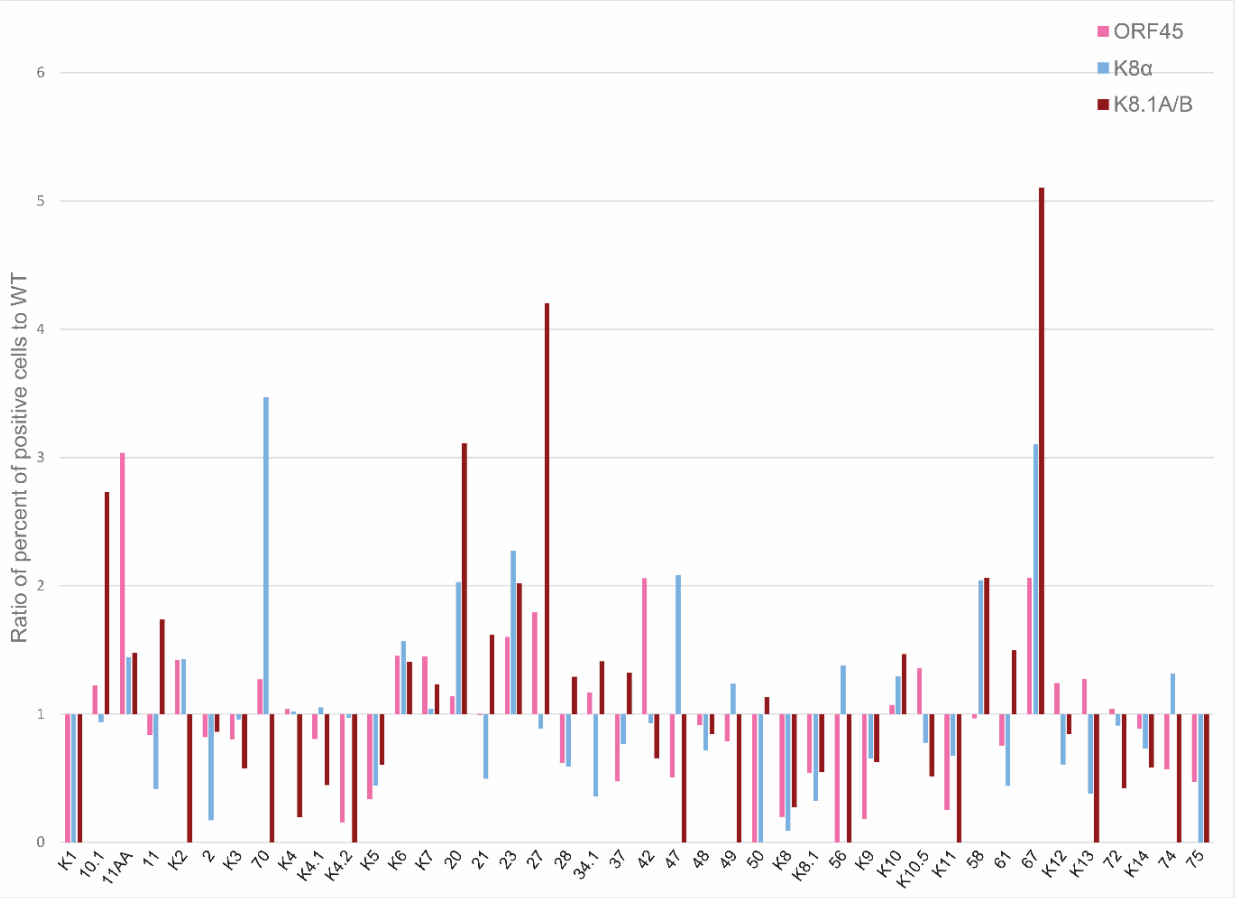


Figure 4.3. Lytic antigen expression during long-term latency after de novo infection with BAC16 and mutants. iSLK cells were infected by BAC16 or mutants at an MOI of 1 under noninducing conditions. Cells were fixed and stained for lytic antigen expression at 6dpi. The percentage of GFP+ cells expressing these lytic antigens was quantified and normalized to BAC16.

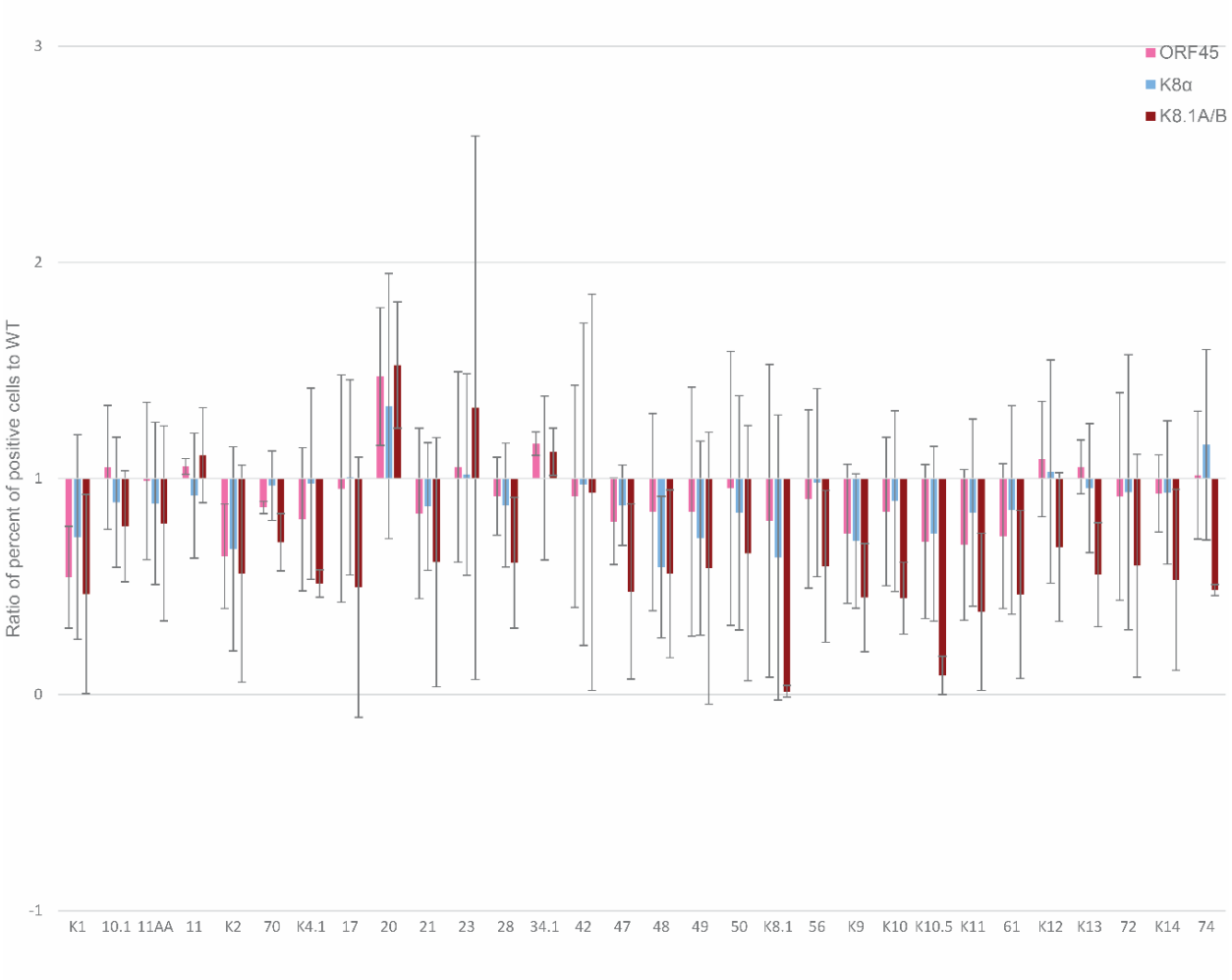


Figure 4.4. Lytic antigen expression during reactivation after de novo infection with BAC16 and mutants. iSLK cells were infected by BAC16 or mutants at an MOI of 1 under noninducing conditions. After 48 hours, cells were treated with doxycycline and sodium butyrate to induce lytic reactivation. 48 hours after this treatment, cells were fixed and stained for lytic antigen expression. The percent of GFP+ cells expressing these lytic antigens was quantified and normalized to BAC16.

Finally, we measured lytic antigen expression after reactivation in the hopes of identifying mutants which cannot appropriately regulate this step of the viral life cycle. iSLK cells were infected with WT-BAC16 or mutant viruses at an MOI of 1. After two days in culture, cells were treated with sodium butyrate and doxycycline to induce ORF50 expression and reactivation. Two days after lytic cycle induction, cells were harvested, fixed, stained for ORF45, K8α, and K8.1A/B, and analyzed by flow cytometry (Fig. 4.4). Since ORF50 expression has been shown to be necessary and sufficient for inducing lytic reactivation in KSHV, we expected the majority of mutants to show similar results as WT-BAC16[132].

Strikingly, we saw that almost all mutants showed at least some degree of decrease in the percentage of cells expressing lytic antigens and this decrease was most dramatic in staining for K8.1A/B. BAC16ΔORF20 was the only mutant showing reliably significantly enhanced numbers of lytic antigen-expressing cells. Mutants in ORFs 10.1, 11, 11AA, 34.1, 42, 70, K12 showed overall percentages of lytic-antigen expressing cells fairly similar to WT, indicating that ORF50 expression was sufficient to drive the full lytic cascade in these cells.

Discussion

Analyzing lytic gene expression in a medium-throughput format has provided a wealth of data regarding the effects of KSHV ORF disruption on gene expression. We were able to identify notable trends among the mutants which have helped us in further understanding the functionality of KSHV ORFs.

Overall, it was clear that enhanced lytic gene expression at levels much higher than that of WT-BAC16 was relatively rare. What this implies is that, out of the ORFs tested, there is no single ORF that has a large effect on inhibiting lytic gene expression. This is perhaps not too surprising. Although ORF50, on the other hand, is required for initiating lytic replication, temperance of lytic replication appears to be shared among multiple viral genes which each have smaller contributions to the overall effect[132]. During reactivation, BAC16ΔORF20 was the only mutant tested showing significantly increased lytic gene expression. ORF20 has previously been shown to enhance transcription of and bind to oligoadenylate synthetase-like (OASL) protein, an interferon-stimulated gene product[137]. Although elevated OASL in cells has been shown to have a proviral effect by increasing infectivity of KSHV, it's possible that it may also act in an antiviral manner to dampen lytic gene expression. It's also possible that ORF20 is acting through different mechanisms for these different effects. Since lytic antigen expression plays a major role in KSHV-associated diseases, developing a better understanding of viral mechanisms of temperance of lytic antigen expression could prove useful for developing treatments against these diseases[138].

The identification of KSHV ORFs which play a role in viral lytic gene expression early after infection is especially intriguing. It is known that KSHV expresses lytic genes for some time shortly after infection, before switching into a canonically latent gene-expression program, and that the timing of this switch varies in different cell types[139]. The mechanism for the establishment of latency by KSHV after infection is not fully understood. Studies have found

that, shortly after de novo infection, there is a euchromatin to heterochromatin transition that occurs in the KSHV genome, where activating H3K4me3 and H3K27ac markers are replaced with repressive H3K27me3 and H2AK119ub markers, corresponding with a decrease in lytic gene expression[140]. Additionally, this epigenetic shift is dependent on Polycomb Repressive Complex 1 and 2, which are recruited to the viral genome by LANA[141]. However cohesins have also been shown to be essential in this process, and their knockdown prevents the reduction of lytic gene expression establishment of latency by KSHV[142]. Our identification of mutants with elevated lytic gene expression in long-term latency may help shed more light on this aspect of the viral life cycle. ORFs 10.1, 20, 23, 27, 58, and 67 may play a role in the switch from the initial lytic burst to establishment of latency since their disruption prevents the reduction of lytic gene expression. In addition to what has already been discovered, there may be additional mechanisms for this switch and it is possible that some of these ORFs could modulate cohesion functions. These ORFs are certainly worthy of further study to better understand the reasons behind their modulation of lytic gene expression.

Equally as interesting is the collection of mutants which only showed reduced K8.1A/B- expression in short-term, but not long-term latency. Mutants in these ORFs (ORF11, 28, K10, K10.5, and K9) therefore normally function to enhance lytic gene expression only shortly after infection. This seems to imply that these genes facilitate the lytic burst after de novo infection that occurs before the establishment of latency. Although previous studies have focused on the repression of lytic gene expression and switch to latency, it is possible that KSHV encodes factors which actually promote this early lytic gene expression, underscoring its possible biological significance. Lytic gene expression plays a significant role in most KSHV-associated disease. For example, in Kaposi's sarcoma the ORF74 gene product, vGPCR, promotes angiogenesis and tumorigenesis through its upregulation of cyclooxygenase-2[143]. In multicentric Castleman disease (MCD), a higher level of the K2 gene product, vIL-6, is associated with a greater severity of disease flares, and vIL-6 expression in mice causes a disease phenotype similar to that seen in humans with MCD[144–146]. Similarly, KSHV inflammatory cytokine syndrome is a disease associated with high levels of vIL-6[5]. Therefore, a better understanding of the role of the lytic burst, and of viral mechanisms behind its control could identify drug targets for controlling KSHV-associated disease. This is an area of KSHV biology would greatly benefit from more research.

Unfortunately, we only performed viral antigen staining assays using mutants with disruptions in ORFs nonessential for cell line and infectious virus generation, and that generated sufficient virus upon lytic induction of the cell line for these assays to be tractable. To achieve the numbers of positive events necessary for statistical significance required a significant amount of virus, especially for a medium-throughput assay, and thus mutants which generated little virus could not be assayed. It would be interesting to expand the scope of an assay of this nature. This might require generating complementing cell lines for the disrupted ORFs so that mutant viruses could be generated at a higher enough titer. In particular, further experimentation could be useful in studying the unique environment generated in the host cell shortly after de novo infection, and it is very possible that some of the essential ORFs play key roles in these steps.

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