Title
The Role of ORF10 in Gammaherpesvirus Pathogenesis

Author
Pardamean, Carissa Ikka

Publication Date
2022

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

The Role of ORF10 in Gammaherpesvirus Pathogenesis

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular and Medical Pharmacology

by

Carissa Ikka Pardamean

2022

ABSTRACT OF THE DISSERTATION

The Role of ORF10 in Gammaherpesvirus Pathogenesis

by

Carissa Ikka Pardamean

Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2022

Professor Ting-Ting Wu, Chair

Gammaherpesviruses comprise the human Kaposi's Sarcoma-associated herpesvirus and Epstein-Barr virus, as well as the rodent virus, murine gammaherpesvirus-68 (MHV-68). Since KSHV lacks a small, immunocompetent animal model due to the restricted host range, MHV-68, which resembles human gammaherpesviruses at the genetic and pathological level, is used to investigate viral-host interaction.

Gammaherpesvirus ORF10 is known to inhibit host transcript export selectively through its interaction with the cellular export factor, Rae1. The ORF10-Rae1 interaction is conserved across KSHV and MHV-68 ORF10s. However, the impact of cellular transcript manipulation by ORF10 on both the host and the virus remains unclear. Therefore, we

aimed to investigate the role of ORF10 in gammaherpesvirus pathogenesis through three angles.

The first angle involved an investigation on the molecular mechanism by which the KSHV ORF10-Rae1 complex performs its function in selective host transcript export inhibition. The second angle involved the possibility that the absence of ORF10 leads to an accelerated expression of other KSHV viral genes, implying that KSHV ORF10 could hold a role in controlling the tightly timed and sequential expression of KSHV genes required during lytic replication.

The main study of the dissertation is the third angle, which involves MHV-68 ORF10 as studied within an in vivo system. The initial direction was to verify the potential antagonistic role of MHV-68 ORF10 against host type I interferon signaling, a first-line of defense against viral infection by the mammalian host. Despite the finding that ORF10 is not required for host interferon response evasion, the in vivo study yielded a surprising phenotype that became the center point of this dissertation. We followed-up on these phenotypes with in vitro experiments to confirm the role of ORF10 in cell-to-cell spread and viral dissemination within peripheral organs. Complementary investigations in vitro determined that MHV-68 infection without ORF10 was more significantly impacted when cell-free viral dissemination was impaired. Despite the ambiguous results from MHV-68 ORF10 complementation, we demonstrated that KSHV ORF10 complementation was capable of rescuing the MHV-68 ORF10S viral infection in a forced cell-to-cell spread

setting, highlighting the conserved nature and importance of ORF10's role in cell-to-cell spread and viral dissemination.

The dissertation of Carissa Ikka Pardamean is approved.

Douglas L. Black

Guillaume Chanfreau

Samson A. Chow

James Akira Wohlschlegel

Ting-Ting Wu, Committee Chair

University of California, Los Angeles

2022

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Ting-Ting Wu. I had zero knowledge on virology when I joined the lab and had not held a pipette in five years. The lab was also at a cross roads that added to the arduous nature of earning a PhD degree. It was often quite daunting to be in the lab, not knowing if I'd ever be able to learn any techniques, let alone be able to run a project. But through thick and thin, your unwavering support and your caring nature beyond "where is the data?" made me reach this point. Through all the problems and the plot twists of the projects I was in, you always reminded me that things will be fine in the end. And you were right. Thank you for being the most understanding PIs out there, and the easiest advisor to communicate with, no matter the topic. You are a rarity that shouldn't be rare in a PhD program.

Next, I would like to thank all of the lab peeps. Alex Lam, you are next on the block of the Wu Lab alumni. Thank you for all your support, and thank you for taking on the "sin" of injecting the mice for my project. Thank you to Drs. Gurpreet Brar, Nisar Farhat, and Danyang Gong for being amazing senior lab mates. Watching all of you soar in your careers is inspirational. Thank you, Shirley Zhang, my undergraduate researcher, for working with me despite the fact that the project you worked so hard on ended up being the one that did not work out for my PhD. Thank you to Pu-Lin Teng, Art Catapang, Leibo Zhou, Alex Bollinger, and Tian-hao Zhang for all of your help, advice, and support.

I would also like to thank the faculty team who agreed to be in my committee: Drs. Samson Chow, Douglas Black, Guillaume Chanfreau, and James Wohlschlegel. The project I proposed to do would have benefited immensely from all of your specific expertise. It is unfortunate that I had to change when things did not work out, but thank

you for remaining in my committee to investigate the experimental designs in the field of virology as well as giving me significant ideas on how to connect puzzling phenotypes together. Especially to Dr. Samson Chow, you were the faculty who convinced me to join the program, and you were also the one who took in our lab when we needed space to continue our research. Your kindness and swift response meant a lot to not just me, but to the entire lab as well. Outside of my committee, I would like to thank Dr. David Nathanson. Thank you for being that outside advocate to whom I do not answer. I probably would have entered massive arguments with Dr. Ting-Ting Wu if I had not rehearsed some wordings and conversations with you. Also thank you for having the same mental age as me; still debatable whether we are forever 5- or 8-year olds.

To my friends for over a decade (or two) now, Yi Yu, Dr. Hitomi Nakamura, and Sophia Susanto. Thank you for actually finding my loudness and incessant random knowledge endearing and for being one of the strongest support networks during this endeavor. To all of my cohort mates in the program, thank you as well; we made great memories together. Especially Lisa Ta, who used to feed me and slap me on the arm when I said something funny.

Last but definitely not the least, my family. Thank you to my aunt and uncle for being relatives with whom I actually get along to go on trips and eat lots of good food. Thank you to Christine Ellisa, my cousin but actually quasi sister who is usually the first-line in absorbing all of my frustration during this time. And, I was born to my best friends, aka my parents. Thank you for supporting me to do whatever I want as long I was happy. I am lucky to be a child who actually respects, loves, and likes her parents.

DEDICATION

Thank you for the inordinate amount of chaos that prompts laughter
So that I could decipher the chaos that is science

VITA

EDUCATION

University of California, Los Angeles
September 2016 – present
PhD Candidate
Molecular & Medical Pharmacology Program

Boston University School of Medicine
September 2012 – May 2014
Master of Science in Medical Sciences

University of California, Berkeley
August 2007 – May 2011
Bachelor of Arts in Molecular & Cell Biology; Linguistics

RESEARCH AND WORK EXPERIENCE

University of California, Los Angeles
September 2017 – present
Graduate Student Researcher
Laboratory of Ting-Ting Wu
Molecular & Medical Pharmacology Department

BioRealm Research
January 2015 – September 2016
Statistical Computing and Data Analyst

Boston Children's Hospital
June 2013 – May 2016
Affiliated Clinical Researcher
Division of the Thyroid Program
Department of Endocrinology

Bina Nusantara University
September 2011 – September 2016
Scientific Manuscript Editor (bi-lingual between English and Indonesian)

PUBLICATIONS

Pardamean, CI & Wu, TT. *Inhibition of host gene expression by KSHV: sabotaging mRNA stability and nuclear export.* Frontiers in Cellular and Infection Microbiology. 2021. DOI: 10.3389/fcimb.2021.648055.

Yusuf I, Pardamean B, Baurley JW, Budiarto A, Miskad UA, Lusikooy RE, Arsyad A, Irwan A, Mathew G, Suriapranata I, Kusuma R, Kacamarga MF, Cenggoro TW, McMahan C, Joyner C, **Pardamean CI.** *Genetic risk factors for colorectal cancer in multiethnic indonesians.* Scientific Reports. 2021;11, 9988. DOI: 10.1038/s41598-021-88805-4.

McMahan C, Baurley JB, Joyner C, Bridges W, Kacamarga MF, Lund R, **Pardamean CI**, Pardamean B. *A Bayesian Hierarchical Mixed Model for Identifying Significant Polygenic Effects while Controlling for Confounding and Repeated Measures*. Statistical Applications in Genetics and Molecular Biology. 2017;16(5-6):407-419. DOI: 10.1515/sagmb-2017-0044.

Baurley JW, Edlund CK, **Pardamean CI**, Conti DV, Krasnow R, Javitz HS, Hops H, Swan GE, Benowitz NL, Bergen AW. *Genome-Wide Association of the Laboratory-Based Nicotine Metabolite Ratio in Three Ancestries.* Nicotine & Tobacco Research. 2016;18(9):1837–1844. DOI: 10.1093/ntr/ntw117.

Baurley JW, Edlund CK, **Pardamean CI**, Conti DV, Bergen AW. *Smokescreen: a targeted genotyping array for addiction research.* BMC Genomics. 2016;17:145-156. DOI: 10.1186/s12864-016-2495-7.

Pardamean B, Baurley JW, **Pardamean CI**, Figueiredo JC. *Changing colorectal cancer trends in Asians.* International Journal of Colorectal Diseases. 2016;31(8):1537-8. DOI: 10.1007/s00384-016-2564-z.

AWARDS AND HONORS

August 2020 – Present	NIH-NIDCR R01 Diversity Supplement
July 2017 – June 2019	NIH T32 Cellular & Molecular Biology
September 2016 – August 2018	UCLA Graduate Dean's Scholarship
Academic calendar 2016 & 2020	UCLA Eugene V. Cota-Robles

CHAPTER 1

INTRODUCTION

Herpesviruses

The biphasic life cycle is a hallmark of the herpesvirus family[1]. Two stages of the life cycle, the latent and lytic state, refer to the level of viral production. During the latent state, most of the viral gene expression is dormant with the viral genome maintained in the nucleus of cells as a closed episome that in general, is not integrated into the host genome. With few viral genes expressed, the production of new viruses is halted leading to no cytopathology or apparent disease states in the host. The key to maintaining chronic herpesviral infection is through the ability of the virus to infect cells latently then replenish the latent reservoir in the host by triggering the expression of the rest of the genome in a process called reactivation. Latency is established after acute, lytic replication during which most of the genome is expressed and new infectious virions are produced.

The herpesvirus has a large and enveloped DNA genome. The viral genes contained within the genome are divided into immediate early (IE), early (E), and late (L) genes[2–4]. The division is based on a sequential cascade during which the viral gene expressions occur during a productive infection as well as the requirement for de novo protein synthesis for the viral gene expression. Immediate early genes can be expressed without de novo protein synthesis and they encode for regulatory proteins that control the expression of early viral genes. Early genes require de novo protein synthesis and control the viral genome replication as well as the expression of viral late genes. Late genes encode for the structural proteins required for containing the newly synthesized viral genome as well as structures that allow for viral transmission to a new host. Despite the initial categorization to be based on a sequential cascade, recents studies have shown

that overlaps in the expression of IE, E, and L genes as well as certain viral IE genes being expressed only after the expression of particular L genes.

In humans, the herpesvirus family is divided into three subfamilies based on the cell types in which the herpesvirus establishes latency[5,6]: alpha-, beta-, and gamma-herpesviruses. The alpha-herpesviruses establish latency in neuronal cells and the sensory nerve ganglia. These include the Herpes simplex virus-1 and -2 (HSV-1/HHV-1 and HSV-2/HHV-2) as well as the Varicella zoster virus (VZV/HHV-3). The beta-herpesviruses possess the largest viral genome that can be as long as 250,000 base pairs. This sub-family establishes latency in the monocytes, macrophages, and lymphocytes. Cytomegaloviruses (HCMV/HHV-5) is the most extensively characterized human betaherpesvirus. The gammaherpesviruses establish latency primarily in B-cells but can also establish latency in dendritic cells, macrophages, and the lung epithelial cells.

Gammaherpesviruses: the tumor-associated herpesviruses

Gammaherpesviruses comprise the human Kaposi's Sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV)[7,8]. Unlike the alpha- and beta-herpesviruses, all members of the gammaherpesviruses are tumor associated. Primarily, the diseases are lymphotropic, causing a variety of lymphoid cancers such as and B-cell lymphomas. EBV is associated with Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and lymphoproliferative diseases, particularly in individuals who are immunocompromised. KSHV is associated with Kaposi's sarcoma, primary effusion lymphoma, multicentric Castleman's disease, and KSHV inflammatory cytokine syndrome

(KICS)[9]. Infection by EBV or KSHV leads to a latent infection in most of the infected individuals. This latency state is what is associated with diseases caused by the human gammaherpesviruses.

It is estimated that gammaherpesvirus infections contribute 250,000 new cases of cancer per year[10,11]. The highest concentration of disease incidence are in sub-Saharan Africa and Mediterranean areas where some cases are endemic and occur independently. Another significant contributor of gammaherpesvirus-related cancer cases are from immunocompromised patients that present opportunistic, secondary infections of EBV or KSHV. The high-risk population for opportunistic infections of gammaherpesviruses include patients with HIV who have developed AIDS.

Murine herpesvirus 68 as an in vivo model for gammaherpesviruses

KSHV lacks a small, immunocompetent animal model because of the restricted host range. MHV-68, which resembles human gammaherpesviruses at the genetic and pathological level, is used to investigate viral-host interaction[1,12]. As the human gammaherpesviruses do, MHV-68 also establishes chronic infection through latency in the B cells, which induces lymphoma and lymphoproliferative diseases. The MHV-68 genome consists of 79 open-reading frames (ORFs). MHV-68 is more genetically homologous to KSHV than EBV, with 64 of the MHV-68 ORFs having homology with KSHV ORFs[2,13].

Laboratory mice are typically infected with MHV-68 intranasally, which introduces the virus into the lung epithelium[14]. The acute lytic replication occurs and usually is cleared within 9-12 days. Latency is then established in peripheral lymphoid compartments such as the spleen, primarily in the B-cells, which peaks at two weeks after infection[15,16]. Pathologically, this leads to significant splenomegaly due to the high levels of induced lymphoproliferation. This latent stage is the basis for chronic herpesviral infection and is how the virus persists and evades host immune surveillance. The B-cell latency is also associated with tumor formation in the host.

Gammaherpesvirus Open Reading Frame 10 (ORF10)

ORF10 is one of the dUTPase-like genes conserved across human gammaherpesviruses and MHV-68. While the ORF10 homologues of KSHV and MHV-68 share only 19% amino acid identity, they both share similar functional phenotype. KSHV and MHV-68 ORF10 have both been determined to hijack the cellular export factor, Rae1, in order to inhibit the transcript export of its host in a selective manner[17,18]. This activity requires an interaction with Rae1, a cellular export factor. ORF10 mutants that have lost the Rae1 interaction also lose their ability to exert selective transport inhibition of host mRNA. The conservation of this function between the KHSV and MHV-68 ORF10 highlights its importance to gammaherpesviruses, although the role within host-virus interaction remains unclear.

To achieve the ORF10-Rae1 interaction, homologues from several members of gammaherpesviruses, including KSHV, human Epstein-Barr virus (EBV), MHV-68, and

saimiri herpesvirus-2 (SaHV-2; a non-human primate gammaherpesvirus) conserve a particular C-terminus motif, namely the EEPM-like motif, that is responsible for the Rae1 interaction[18] (Table 1). Other viral proteins that interact with Rae1 also possess the EEPM motif at their C-termini, particularly the methionine residue that is consistently involved in the interaction of viral proteins with Rae1. This includes the M protein of vesicular stomatitis virus (VSIV)[19,20], and the ORF6 of SARS-COV2[21]. Interestingly, unlike the double-stranded DNA genome of gammaherpesviruses, VSIV and SARS-COV2 both possess single-stranded RNA genome[22,23], which further highlights the importance of Rae1 hijacking by viruses.

Table 1. A conserved EEPM motif at the C-terminal of viral proteins interacting with Rae1

Viral family	Gammaherpes				Rhabdo	Corona	
Viral species	KSHV	MHV68	EBV		VSV	SARS-COV-1	SARS-COV-2
Viral protein	ORF10	ORF10	LF1		M	ORF6	ORF6
KSHV Resid. #							
413	**E**	**E**	**E**		V	**E**	**E**
414	**E**	S	**E**		D	**E**	Q
415	**P**	**P**	**P**		E	**P**	**P**
416	**M**	**M**	**M**		**M**	**M**	**M**
417	Q	E	T		D	E	E
418	S	W	F		T	L	I

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Viruses and Cells. 293T or vero cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% penicillin and streptomycin solution. NIH3T3 cells were cultured in DMEM containing 10% bovine calf serum and 1% penicillin and streptomycin. MHV-68 viruses were amplified in NIH 3T3 cells, concentrated by high-speed centrifugation, resuspended in serum-free DMEM, and titered by plaque assays.

Generation of MHV-68 10S Virus. The MHV-68 10S virus contains a stop codon after the 41st amino acid residue of ORF10 (out of a total of 418 residues). Both 10S and the revertant mutant MHV-68 10R (reintroduction of ORF10 into the 10S mutant, which regains the wild type virus genome again) were constructed based on the BAC mutagenesis method[17,24,25]. The BAC plasmids were screened by EcoRI and HindIII digestions to verify genome integrity, as well as by sequencing of the ORF10 region to confirm the presence of the desired mutation. The viruses were reconstituted from the mutant BAC plasmids through co-transfection with Cre recombinase in 293T cells to remove the BAC sequence that allows the mutant virus to establish latency. A single viral clone was then selected through limiting dilution screening then checked through sequencing for the presence of the desired mutations. The virus is then propagated for use in future experiments in 3T3 cells and titered on vero cells.

Viral quantification/plaque assay. The viral inoculum was serially diluted and incubated on vero cells on 12-well plates as duplicates with 1 hour of absorption period to facilitated

infection. The inoculum was removed and the cells were overlaid with 1% methylcellulose in DMEM containing 10% FBS and 1% penicillin and streptomycin. Six days post-infection, the cells were fixed with 2% crystal violet in 20% ethanol. Viral titers were determined by counting plaque numbers.

In vitro growth curve. The 3T3 cells were plated for 24 hours on 48-well plates prior to infection. Cells were infected at MOI = 0.05 and 5 with MHV-68 WT, 10R, or 10S virus for 1 h at 37 °C. The inoculum was then removed and the cells were washed twice with media before adding fresh media. Infected plates were then collected periodically. Whole plates were subjected to freeze-thaw cycle three times prior to plaque assays on vero cells in 12-well plates to determine the titers of the viruses at each time point.

Infectious center assay. Spleens were treated with the ACK (ammonium-chloride-potassium) lysis buffer for red blood cell removal and processed to yield single-cell splenocytes. The splenocytes were serially diluted and plated on vero cells in 6-well plates and incubated overnight at 37 °C. The splenocytes were removed then washed off by gentle agitation in media. The Vero cells were overlaid with 1% methylcellulose in DMEM containing 10% FBS for 6 days before fixing with 2% crystal violet in 20% ethanol. Infectious centers were determined by counting plaques.

In vivo assays. All animal experiments were done with the approval of the UCLA Institutional Animal Care and Use Committee and the Chancellor's Animal Research Committee. C57Bl/6J and Balb/cJ mice were purchased from the Jackson Laboratory

(Bar Harbor, ME #000664 and #000651). IFN Alpha Ro/o 129/Sv (IFNAR1$^{-/-}$) mice (B&K Universal Ltd.) were a gift from Dr. Genhong Cheng (UCLA). IFNAR1$^{-/-}$ mice were back-crossed to the C57Bl/6 genetic background and maintained by the UCLA Division of Laboratory Animal Management.

Viral Copy Number. Total genomic DNA was harvested from organ homogenates or splenocytes using the Qiagen DNeasy Blood & Tissue kit (Qiagen 69506). To determine the viral copy number of the in vivo samples, the droplet PCR system of the UCLA Virology Core (Bio-Rad QX200™ Droplet Digital™ PCR System) was used. The ddPCRs were run against the ORF6 viral gene of MHV-68.

Fwd: 5'-TGCAGACTCTGAAGTGCTGACT-3'

Rev: 5'-ACGCGACTAGCATGAGGAGAAT-3'

Neutralizing antibody assay. The neutralizing antibody experiments were performed on vero cells seeded at monolayer confluence prior to infection with MHV-68 10R or 10S. The serum used to block cell-free infection were obtained from pooled serum of mice that were infected intraperitoneally and sacrificed 3 months post infection. The working in vitro dilution for the experiments was 1:250.

Immunofluorescence assay and imaging. Cell surface staining with MHV-68 infected mouse serum was done to mark infected vero cells with anti-K8.1 antibody (Santa Cruz Biotechnology sc-65445) through overnight incubation at 4ºC. For immunofluorescence imaging, the goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody,

Alexa Fluor 594 was used (ThermoFisher Scientific A-11032). The IFA images were taken and analyzed with the Nikon NIS Basic Research system.

Lentiviral complement assay. Viral genes expressed via lentivirus were cloned into the pLV-EF1a-IRES-Puro backbone (gift from Dr. Koki Morizono) through Gibson cloning with the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs M5520). The lentiviral constructs were transfected into 293T cells with Lipofectamine 3000 (Invitrogen L3000015). The supernatant of the transfection was clarified from cell debris then used to transduce vero cells with 8ug/mL of hexadimethrine bromide. Post-transduction, the vero cells were subjected to puromycin selection at 4ug/mL prior to use for experiments and maintained in the same puromycin concentration post infection.

Statistical analysis. Unless otherwise stated, the in vivo data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA) using Mann-Whitney test. The in vitro data were analyzed with unpaired Student's t-test.

Immunoblot. Cells were harvested on ice then agitated at 4ºC on an inverted shaker for 40 minutes in lysis buffer (50 mM Tris pH 7.5, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 5% glycerol) supplemented with 1 mM PMSF, 1 mM Na3VO4, and 1 mM NaF. Lysates were then centrifuged at 4ºC for 20 minutes at maximum speed. The clarified total protein lysates were incubated at 95ºC for 15 minutes with 4x Laemmli sample buffer (0.25 M Tris pH 6.8, 8% SDS, 40% glycerin, 20% DTT, 0.01% bromophenol blue).

Co-immunoprecipitation. Plasmids were transfected into 293T cells in 10-cm plates at a 1:1 ratio with polyethylenimine and opti-MEM medium. 48 hours post transfection, the cells were lysed and clarified from cell debris then subjected to pre-clarification with G-sepharose beads (Protein G Sepharose 4 Fast Flow GE17- 0618-01). The pre-clarified lysates were then divided equally among pre-conjugated beads for pull down. FLAG (ANTI-FLAG M2 Affinity gel, Sigma A2220) and HA (EZ view Red Anti-HA Affinity Gel, Sigma E6779). The pull down was allowed to take place overnight. Then the beads were washed three times before elution with 4 x Laemmli sample buffer through incubation at 95ºC for 15 minutes.

Table 2. Resources list

Animals	**Source**	**Catalog Number**
BALB/c wild type mouse	Jackson Laboratory	#000651
IFNAR1-/- mouse	B&K Universal Ltd.	
Antibodies and beads		
Anti-HHV-8 K8.1A Antibody (2A3)	Santa Cruz Biotechnology	sc-65445
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	ThermoFisher Scientific	A-11032
Monoclonal ANTI-FLAG® M2-Peroxidase (HRP)	Sigma-Aldrich	A8592
HRP Anti-HA tag antibody	Abcam	ab183884
G-sepharose beads (Protein G Sepharose 4 Fast Flow)	GE	GE17- 0618-01
ANTI-FLAG M2 Affinity gel	Sigma-Aldrich	A2220
EZ view Red Anti-HA Affinity Gel	Sigma-Aldrich	E6779
Cloning		
pLV-EF1a-IRES-Puro	Gift from Dr. Koki Morizono	
NEBuilder® HiFi DNA Assembly Cloning Kit	New England Biolabs	M5520
KOD Hot Start Polymerase	EMD Millipore	71086
In vitro cell culture		
Lipofectamine 3000	Invitrogen	L3000015
Puromycin	Invivogen	ant-pr-1
Polybrene	EMD Millipore	TR-1003-G

Viral Copy Number		
QX200™ ddPCR™ EvaGreen Supermix	BioRad	1864034
Qiagen DNeasy Blood & Tissue Kit	Qiagen	69506
qPCR ORF6-Fwd 5'-TGCAGACTCTGAAGTGCTGACT-3'	IDT	N/A
qPCR ORF6-Rev 5'-ACGCGACTAGCATGAGGAGAAT-3'	IDT	N/A

CHAPTER 3

KSHV ORF10 AND SELECTIVE INHIBITION OF HOST TRANSCRIPT EXPORT

INTRODUCTION

Previously, it was found that the KSHV ORF10 requires an interaction with Rae1, a cellular export factor, in order to perform its role in selectively inhibiting host transcript export. This chapter documents the attempts to follow up on the trans and cis regulatory components potentially involved in the ORF10-Rae1 mechanism of action. The project was halted by inconsistent GFP reporters used as a tool to gauge the ORF10 inhibition level in the study.

RNA-protein interactions are crucial for the process of mRNA translation within eukaryotic gene expression. The RNA transport from nucleus to cytoplasm in eukaryotes is achieved by the interaction of the RNA transcripts with protein complexes comprising export factors, messenger ribonucleoparticles, and nuclear pore complex[26]. Disruption of the host cell gene expression through the prevention of host cell RNA export allows for the virus to more freely gain access to the gene expression machinery of the host cell that viruses require to replicate its own genes[27–29]. Elucidating the mechanism by which viruses manipulate host cell RNA transport garners further knowledge on how viruses have the ability to survive within a host cell. For gammaherpesviruses, the manipulation of host RNA is performed by multiple ORFs, involving an array of mechanisms[27]. One mechanism involves nuclear retention and the induction of RNA instability, as performed by ORF37 and ORF10[30].

Previous published work by our lab[17] has demonstrated that Kaposi's sarcoma-associated herpesvirus (KSHV) performs a disruption of RNA export in a selective manner. This is achieved through the interaction of a KSHV nuclear viral gene, ORF10,

with the export factor Rae1 of the nuclear host cell to bind RNA and prevent its export with specific selectivity based on the 3'UTR of the targeted genes expressed by the host. With the initial assumption that neither ORF10 nor Rae1 is an RNA-binding protein, we hypothesized that the ORF10-Rae1 complex interacts with an RNA-binding protein (RBP) that specifically recognizes an RNA motif.

The 3'UTR of an mRNA contains various *cis* regulatory elements that interact with *trans* regulatory components, such as RNA-binding proteins; these regulatory components are crucial in gene expression regulation[31]. Our preliminary RNA sequencing data have captured several protein candidates with downregulated expression due to ORF10 expression. These candidate proteins included RBL2, RUNX1, and BRCA2. The hypothesis is that there is a common motif contained by transcripts selected by ORF10 inhibition within the 3'UTRs of ORF10-sensitive transcripts.

THE STUDY

The original aims of the project fell into two parts:

1. Investigation of the RNA-binding protein that could convey the ORF10-Rae1 function;
2. Investigation of the 3'UTR motif(s) that convey selectivity to ORF10-Rae1 complex for transcript export inhibition.

To start the investigation on the RNA-binding protein, sequential immunoprecipitation of the ORF10-Rae1 complex was performed. The isolated complex was then subjected to mass spectrometry in order to determine candidate proteins that could be involved in the ORF10-Rae1 action of selectively inhibiting host transcript export (Table 3). We

attempted to assess the importance of these RBPs through siRNA knock down of the RBP of interest then assess the ORF10 ability to downregulate affected GFP reporters. If an RBP is involved in ORF10-Rae1 transcript export inhibition, then we expect a loss of the ability to downregulate affected proteins after the RBP knockdown. Since this is an investigation on transcripts, we also aimed to assess the impact on transcript export through nuclear fractionation to assess affected transcript distribution between the nuclear and cytoplasmic fractions.

Endogenous targets such as RUNX1, RBL2, and IFNAR1 were affected inconsistently during the attempt of the follow-up study. Therefore, to investigate RBP candidate validity in ORF10-Rae1 complex, we relied on the GFP reporters, in which the GFP transcript had its 3'UTR altered based on affected and unaffected transcripts. The initial set was GFP-3'SV40 C1 (an unrelated viral protein), -3'RUNX1, 3'RBL2, and 3'BRCA2 as affected reporters. The unaffected reporters included GFP-3'hGH, -3'STAT1, -3'STAT3, and 3'GAPDH. A representative image is in Figure 3-1. However, this representative was one of the times that the reporters were neatly affected. In order to quantify the impact on GFP, we utilized flow cytometry to measure GFP levels. This was when the inconsistency across the unaffected reporters were observed (Figure 3-2). The transfections were done in 12-well and 24-well, and could alter the level of inhibition by ORF10 for some reporters. In other cases, the ratio of transfection (9:1 or 6:1 between ORF10 to reporter) could affect whether a reporter was considered affected or not. Then in another case, replicates did not confirm each other. The set presented in Figure 3-2 is one of the many sets of flow cytometry done in an attempt to resolve the issue with the reporter, with no definite

solution. The GFP reporter library had also been transferred from a vector backbone with no intron to one that had an intron. While this led to an overall higher expression of GFP protein, it did not provide a solution to the reporter inconsistency.

A crystallographic study on the ORF10-Rae1 determined that ORF10 had the ability to bind RNA and possesses its own RNA-binding domain[18]. While this does not completely disprove the need for a trans-acting element such as RNA-binding protein for the complex to perform its function, our lab's preliminary mass spectrometry data could benefit from refinement. The isolated ORF10-Rae1 complex used for our mass spectrometry study was isolated by lysing the whole cell then performing the sequential immunoprecipitation. ORF10 is localized around the nuclear envelope[17]. Therefore, a refinement on the mass spectrometry study would be to isolate the ORF10-Rae1 complex from the nuclear envelope. It is possible that the use of whole cell lysis allowed for artificial interactions with proteins that the ORF10-Rae1 complex would not typically encounter.

Table 3. RNA-binding proteins potentially interacting with ORF10-Rae1 as detected by mass spectrometry.

HNRNPUL1	Heterogeneous nuclear ribonucleoprotein U-like protein 1
ROA2	Heterogeneous nuclear ribonucleoproteins A2/B1
HNRPU	Heterogeneous nuclear ribonucleoprotein U
CPSF6	Cleavage and polyadenylation specificity factor subunit 6
SF3B2	Splicing factor 3B subunit 2
IF4B	Eukaryotic translation initiation factor 4B
HNRPK	Heterogeneous nuclear ribonucleoprotein K
HNRPM	Heterogeneous nuclear ribonucleoprotein M
FIP1	Pre-mRNA 3'-end-processing factor FIP1
HNRH1	Heterogeneous nuclear ribonucleoprotein H
HNRH2	Heterogeneous nuclear ribonucleoprotein H2
NONO	Non-POU domain-containing octamer-binding protein
HNRPF	Heterogeneous nuclear ribonucleoprotein F
SRRM2	Serine/arginine repetitive matrix protein 2
FLNA	Filamin-A

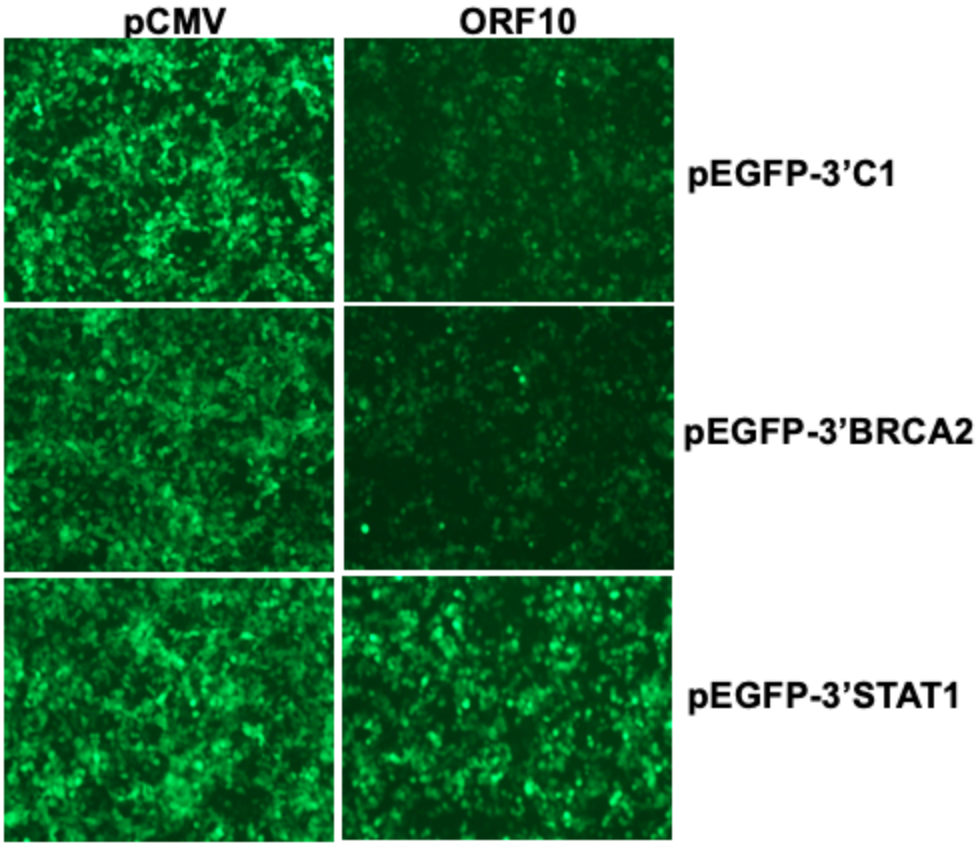


Figure 3-1. Representative image of GFP reporters that are affected and unaffected by ORF10 expression.

The GFP reporters were co-transfected with ORF10-FLAG into 293T cells. With fluorescent microscopy (above) as well as western blot and flow cytometry, it is possible to assess ORF10 effect on the reporters.

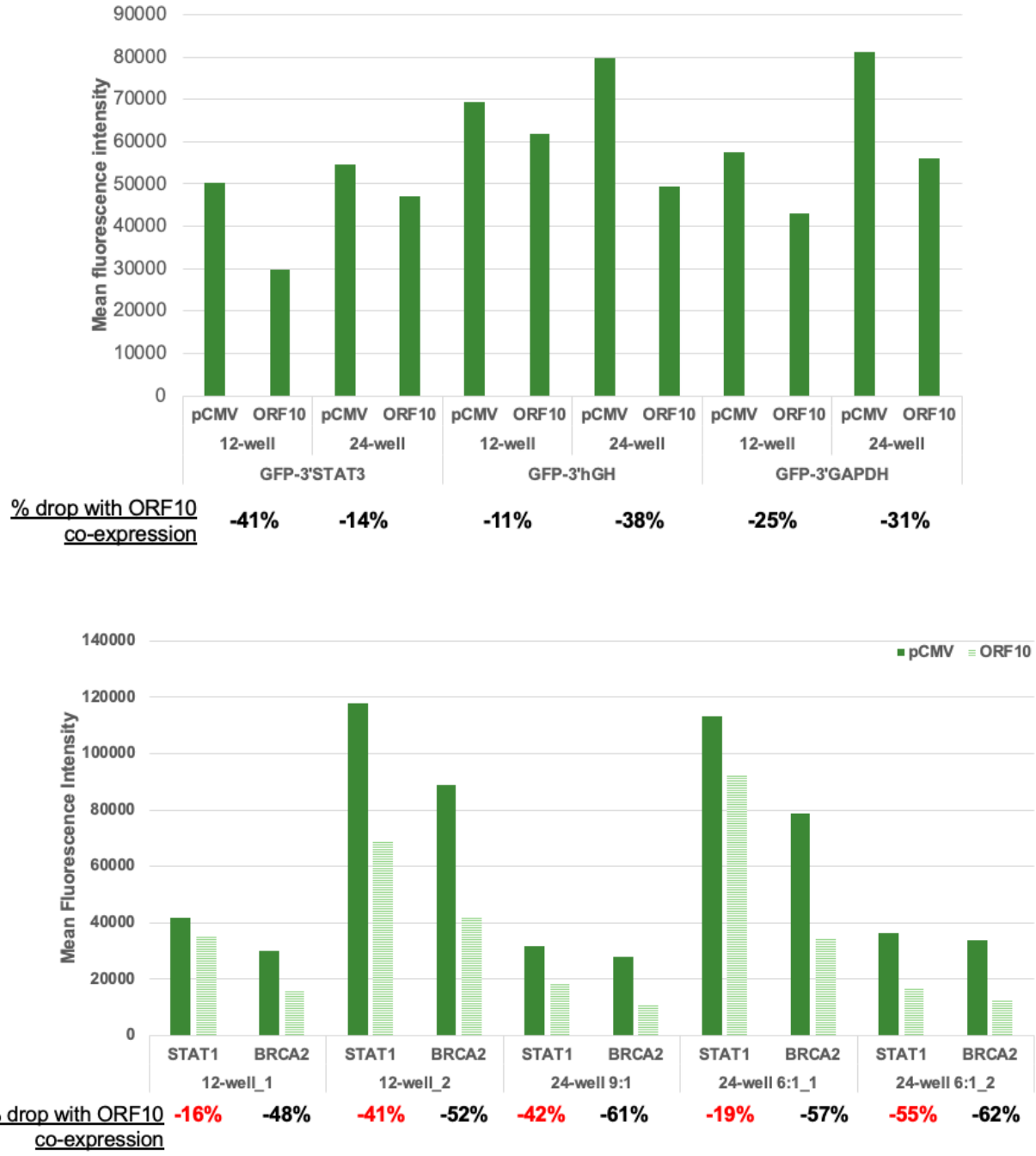


Figure 3-2. Inconsistency in ORF10-unaffected reporters

Flow cytometry indicated that well-size for transfection assay, ORF10:GFP reporter ratio, and replicates resulted in different sensitivity level of ORF10-unaffected reporter to ORF10 expression.

CHAPTER 4

KSHV ORF10 AND THE KINETICS OF VIRAL GENE EXPRESSION

INTRODUCTION

Due to the narrow host range of KSHV, studying the virus in vitro becomes quite a challenge. For in vitro purposes, one of the solutions is through the generation of the iSLK cell line[32]. Derived from a renal cancer cell line, the cells are latently infected with recombinant KSHV.219 that constitutively expresses GFP under the control of EF1a promoter to indicate the presence of latent infection. For reactivation, ORF50 that encodes the RTA (reactivation and transcription activator) is reactivated by doxycycline through a tet-on inducible system. RTA is a sequence-specific, trans-regulator that is a transcriptional activator. RTA binds to RTA-responsive elements (RRE) to initiate cap-independent expression of KSHV genome. Additionally, non-specific chemical treatments can be used to enhance the reactivation of KSHV, such as sodium butyrate (NaB), which is a histone deacetylase (HDAC) inhibitor (Figure 4-1). In previous work by the lab[17], it was found through RNA-sequencing that the absence of ORF10 in iSLK leads to an attenuation in the expression of late genes. However, this finding is based on reactivation of iSLK cells lines using both doxycycline and sodium butyrate. This chapter describes the preliminary findings that without ORF10 in the KSHV.219 genome cassette, there was an accelerated expression of viral genes, when the iSLK cell lines were reactivated using only doxycycline.

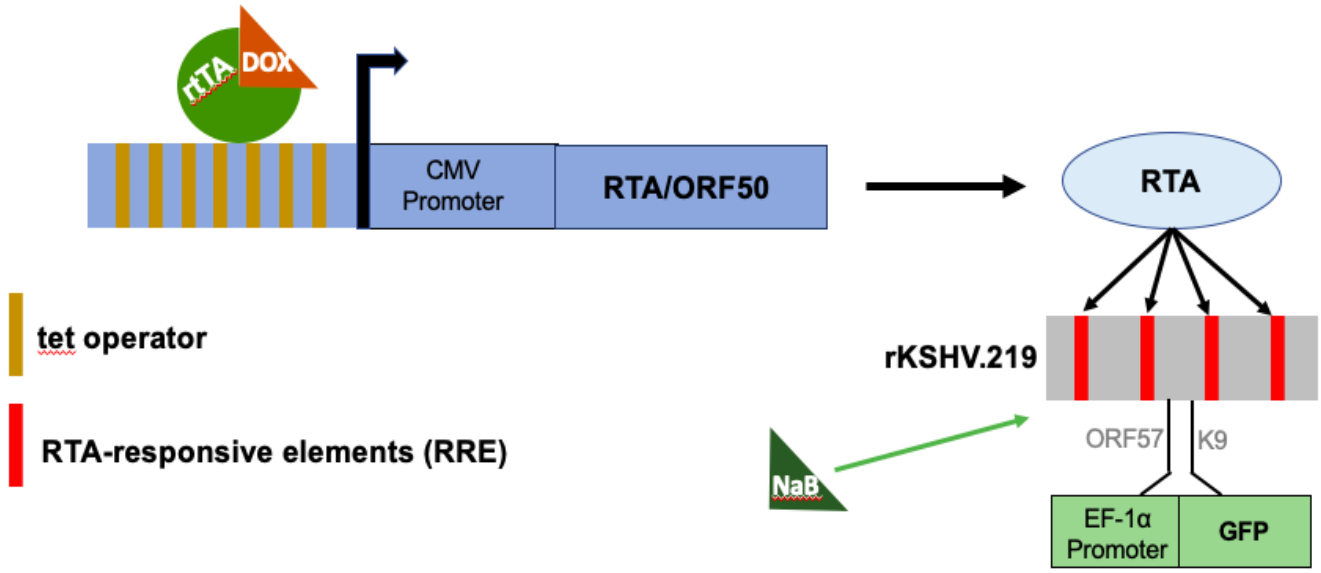


Figure 4-1. Schematic of the iSLK reactivation cell line.

Doxycycline initiates the tet-on controlled expression of RTA (encoded by ORF50), which is a sequence-specific, trans regulator that is a transcriptional activator. It binds to the RTA-responsive elements (RRE) within the KSHV genome to initiate cap-independent expression of KSHV genome. The recombinant KSHV.219 virus, which constitutively expresses GFP, is under the EF1a promoter and is an indicator if the iSLK cell line is still latently infected by KSHV. To enhance viral expression, sodium butyrate (NaB) can be used, which is a histone deacetylase (HDAC) inhibitor that loosens the genome and increases access to the KSHV genome by the replication machinery.

THE STUDY

The use of NaB is ideal to increase virion production by the cell line but not ideal in studying host-viral interaction due to its non-specific mechanism of action as an HDAC inhibitor. The end goal of the study was to send iSLK cells in different reactivation stages for mass spectrometry to look at differential expression of proteins between iSLK with the complete KSHV genome (iSLK WT) and iSLK with a genome lacking the ORF10 gene (iSLK 10S).

We determined the kinetics of KSHV gene expression with doxycycline only. The surrogate to look at the level of virion production is a common KSHV glycoprotein, K8.1. While doxycycline alone was sufficient to induce reactivation of the virus, we noticed different kinetics of K8.1 expression (Figure 4-2). By day 1, K8.1 expression was already detected in the iSLK 10S group while no K8.1 was detected in the iSLK WT group. By day 2 post reactivation, iSLK WT and 10S were equivalent. By day 3 post reactivation, the K8.1 level dropped in the iSLK 10S group but the level was maintained in iSLK WT.

We then decided to look at the expression of immediate-early genes, K8a and ORF45 (Figure 4-3). K8a was detected by day 1 post reactivation in both sets, but the expression is stronger in the iSLK 10S group. At day 2 post reactivation, the expression level evened out between the two groups, like it did in K8.1 expression. At day 3 post reactivation, unlike K8.1 for which 10S loses most of the expressed K8.1 while WT retained K8.1 expression, K8a expression attenuated to a similar level for both WT and 10S. For ORF45, the expression pattern was similar to K8.1 at day 1 post reactivation with faster

reactivation in the iSLK 10S group. At day 2 post reactivation, the 10S set had stronger expression of ORF45 compared to the WT set. By day 3 post reactivation, the level of ORF45 evened out between WT and 10S but the level is lower than the peaked level for 10S set at day 2 post reactivation.

Previously, the RNA-sequencing data determined that the absence of ORF10 led to the attenuation of late-gene levels. However, this was done in the presence of sodium butyrate. Based on the western blots, it seemed that to sustain K8.1 expression, ORF10 is required. The reason for this is unclear. Rather than a direct control over K8.1, it is possible that an additional factor (such a trans-acting factor) is needed for sustaining K8.1 level but the presence of this factor requires ORF10.

The division of early and late genes in gammaherpesviruses is based on the requirement for de novo protein synthesis in order to have viral gene expression during lytic replication. For example, viral DNA processing factors are expressed immediately upon the start of lytic replication, while structural proteins are expressed after de novo protein synthesis. ORF10 has a late-gene core that activates its expression after DNA replication takes place in the gammaherpesviral lytic cycle. However, ORF10 has a hybrid promoter and is expressed in minute quantities even before DNA replication takes place, i.e. when immediate early genes are being expressed. It is possible that this small quantity plays a role in controlling immediate-early gene expression. Thus, in the absence of ORF10, early expression of the immediate-early gene happens.

The next goal for this study is to send samples to mass spectrometry, comparing iSLK reactivation expression patterns to MHV-68 infection spectrometry. For iSLK reactivation, while the virus is KSHV, it lacks the entry process involved in infection, which would be accounted for in MHV-68.

We performed a first-round of mass spectrometry for the iSLK reactivation set, comparing unactivated or non-reactivated (UA) and 1 to 3 days post reactivation cells for iSLK WT and 10S. For UA cells, the comparison shows the impact of carrying latent infection of KSHV to the host and our mass spectrometry data showed different patterns in protein expression and GO pathway enrichments. For the reactivation cell lines, a group of genes downregulated by re-entry into lytic replication of KSHV WT were rescued in expression level the reactivation of KSHV 10S virus indicating the ORF10 role in downregulating the level of these proteins. Since ORF10 is expressed late during the lytic replication cycle and has been shown previously to affect the expression of viral late genes, we saw a consistency in that the most pronounced rescue level was during day 3 post reactivation.

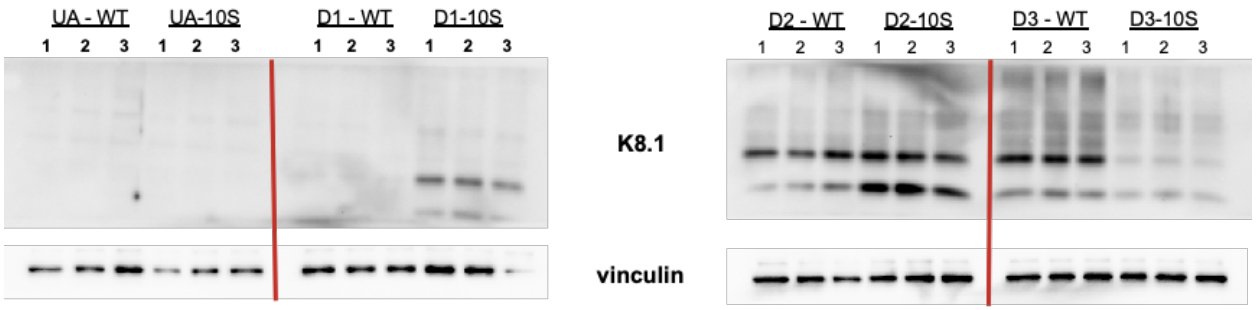


Figure 4-2. Kinetics of iSLK expression through reactivation by doxycycline only

In triplicates, iSLK WT and 10S were reactivated then collected at 1, 2, and 3 days post reactivation. A triplicate of non-reactivated (UA) cells were collected as control. The marker for reactivation is K8.1, a common glycoprotein of KSHV.

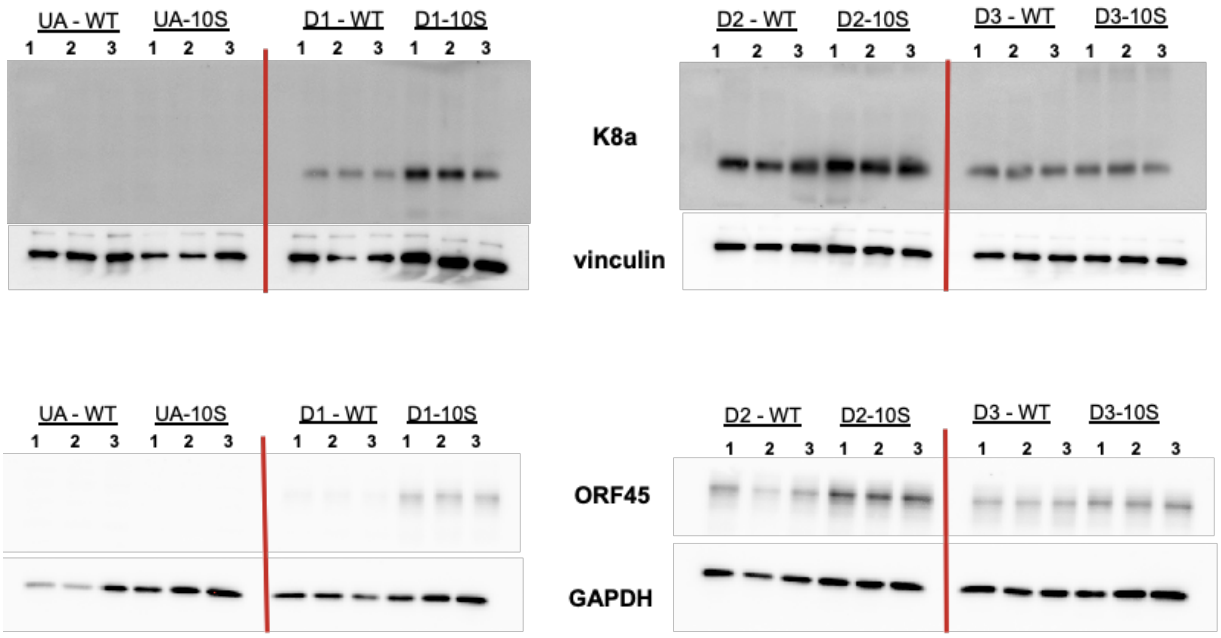


Figure 4-3. The levels of early KSHV gene expressions in iSLK reactivation by doxycycline only

In triplicates, iSLK WT and 10S were reactivated then collected at 1, 2, and 3 days post reactivation. A triplicate of non-reactivated (UA) cells were collected as control. K8a and ORF45 are two immediate-early genes assessed for expression level.

CHAPTER 5

MHV-68 ORF10 AND INTERFERON SIGNALING

ABSTRACT

Through a viral-genome wide, overexpression screen of individual MHV-68 open reading frames, it was determined that ORF10 was a candidate antagonist of type I interferon (IFN-I) response. In an immunocompetent host, the mutant MHV-68 virus lacking ORF10 (MHV-68 10S) was attenuated in the lungs. However, infection of an immunocompromised host did not lead to a rescue in the viral replication of the MHV-68 10S virus in the lungs. This suggested that while ORF10 may play a role in interferon signaling manipulation, MHV-68 did not depend on ORF10 for evading the host immune response.

INTRODUCTION

Type I interferon (IFN-I) signaling is the first line of host defense against viral infection. Thus, the success of a virus in infecting a host is dependent upon its ability to evade IFN-I response. A genome-wide MHV-68 screen was performed to determine candidate MHV-68 genes that could antagonize IFN-I signaling. Each open reading frame (ORF) was co-transfected with an interferon stimulated response element (ISRE) reporter that controls the expression of firefly luciferase. The transfected 293T cells were treated with IFN-a and eight genes were found to suppress the ISRE reporter by at least 50% compared to the control vector co-transfection (Figure 5-1). Two of the genes identified, M2 and M8, are unique to MHV-68 with no conservation with human gammaherpesviruses. Two of the genes, ORF50 and ORF64, are essential for viral replication. However, four of the ORFs are not essential for viral replication and are conserved among MHV-68 and the human gammaherpesviruses: ORF10, ORF11, OF36, and ORF54[33].

In addition to ORF10 overexpression, we aimed to investigate the role of ORF10 and IFN-I signaling antagonism through an infection model. Thus, we engineered the MHV-68 10S mutant, in which a stop codon was placed after the 41st amino acid of ORF10. We also created MHV-68 10R, a revertant mutant that restores the ORF10 gene into the 10S mutant.

RESULTS AND DISCUSSION

The in vitro growth curve generated through a time-course infection of NIH 3T3 cells indicated that after 72 hours, the 10S virus grew at a similar rate as the 10R and wild type MHV-68 viruses (Figure 5-2). A more complete picture of the immune system response towards viral infection is provided by an animal model. Since we aimed to study host-viral interaction of 10S virus within mice, we first infected 10R and 10S viruses into immunocompetent BALB/c mice to determine the level of productive infection and latency establishment for the 10S virus. We found that there was an attenuation by both lytic replication (Figure 5-3) and latency establishment (Figure 5-4) by the 10S virus. The reason for the attenuation was unclear.

Previously, an interferon-stimulated response element (ISRE) screen of individual MHV-68 open reading frames determined ORF10 to be a candidate for antagonizing type I interferon (IFN-1) signaling. Thus, we infected IFNAR1-/- mice with the 10R and 10S virus to determine if interferon signaling was the reason for the attenuation. However, despite the absence of interferon signaling, the attenuation from the 10S virus remained (Figure

5-5). Only the absolute titer of each group increased by 100-fold compared to the infection of immunocompetent mice but the gap between 10R and 10S remained.

With the non-rescue of viral replication of the 10S virus in the lungs of IFNAR1-/- mice, the results so far indicated that while ORF10 may play a role in interferon signaling manipulation, the MHV-68 virus itself does not depend on ORF10 for in vivo replication in the presence of IFN-I signaling. In a sense, this result was not surprising as the ISRE screen indicated that other ORFs may have the ability to compensate for the loss of ORF10 when it came to combating or evading host interferon response.

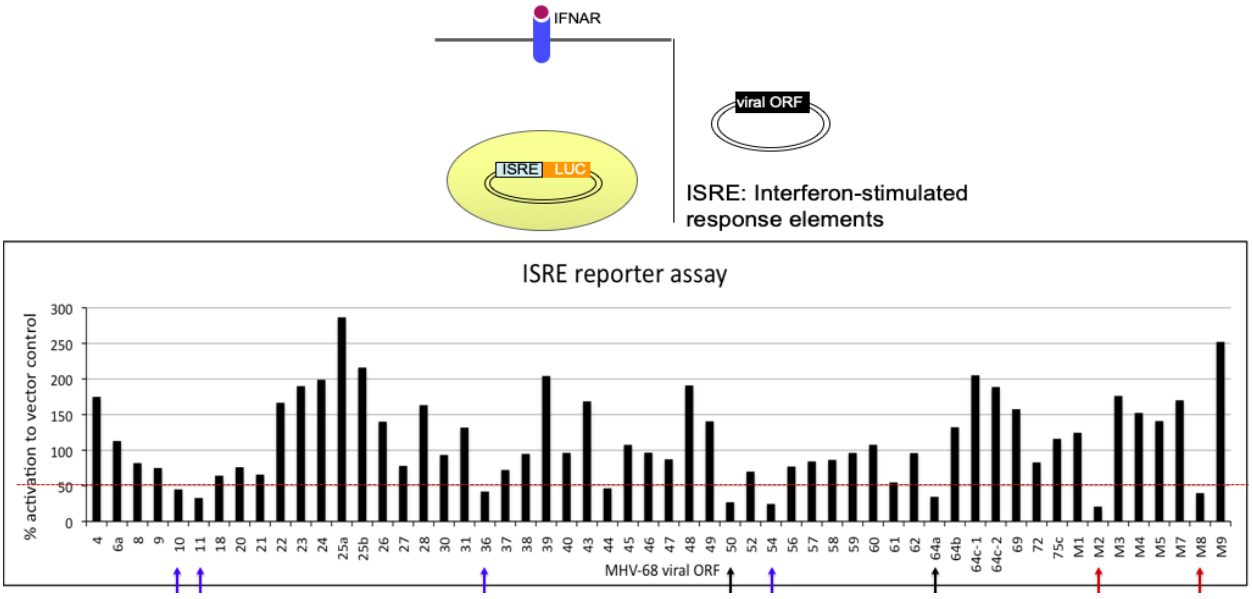


Figure 5-1. ISRE reporter screen to determine candidate MHV-68 genes involved in antagonizing interferon signaling.

Red arrows indicate ORFs present only in MHV-68. Black arrows indicate ORFs conserved between MHV-68 and KSHV but are essential to viral survival. Blue arrows indicate conserved ORFs that are non-essential to viral survival. ORF10 falls under the conserved and non-essential ORFs.

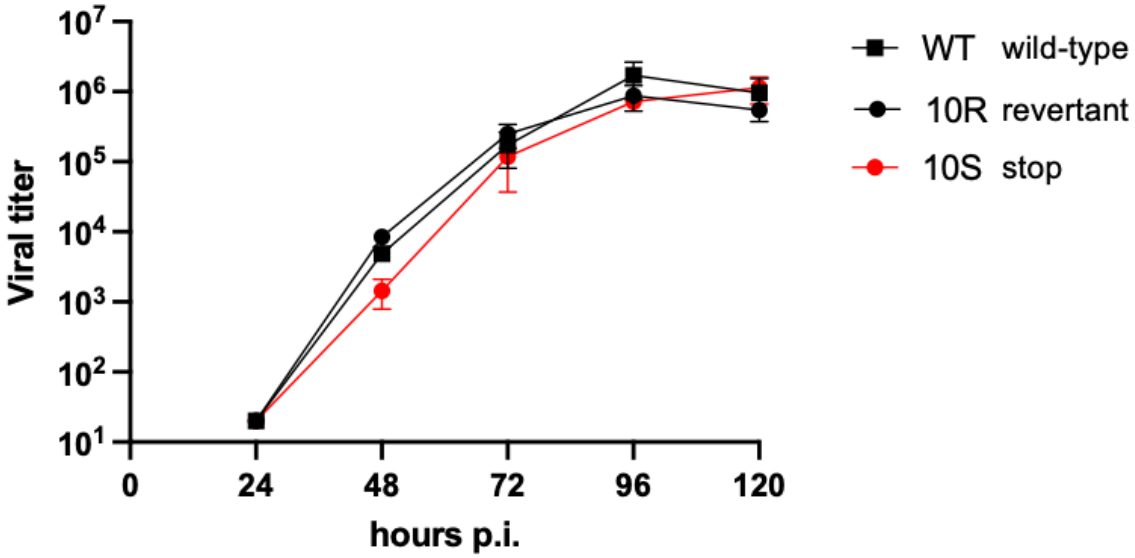


Figure 5-2. In cell culture, MHV-68 ORF10S virus had a similar replication kinetics as MHV68 ATCC WT and 10R.

At 50% confluence, NIH 3T3 cells were infected with ATCC WT, 10R, or 10S at the MOI of 0.05. At every 24-hour interval, the infections were collected from 24 hours to 120 hours post infection, freeze-thawed three times, then titered to produce the kinetic

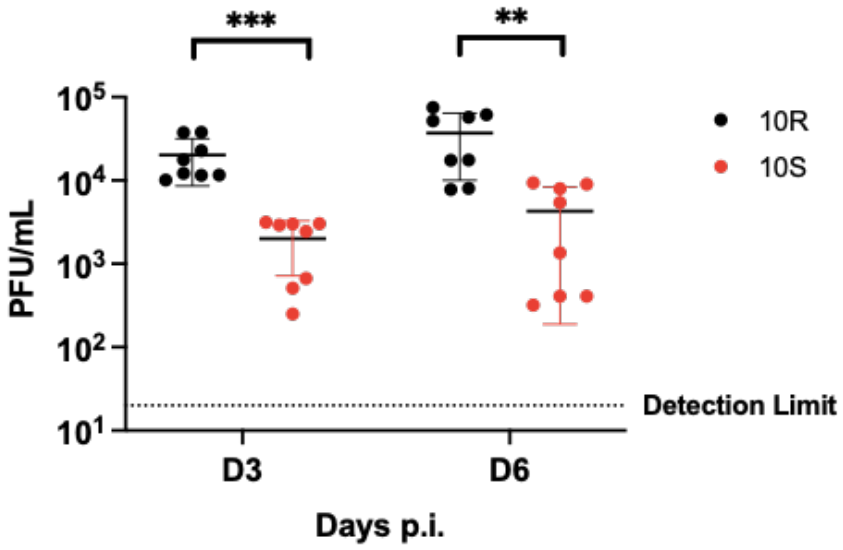


Figure 5-3. MHV-68 ORF10S virus had an attenuated productive infection level in immunocompetent mice.

BALB/c mice were infected intranasally with 10,000 pfu of MHV-68 10R or 10S virus then the mice were sacrificed at 3 and 6 days post infection. The lungs were collected and mechanically homogenized then used for plaque assays to determine the productive infection of the viruses in vivo.

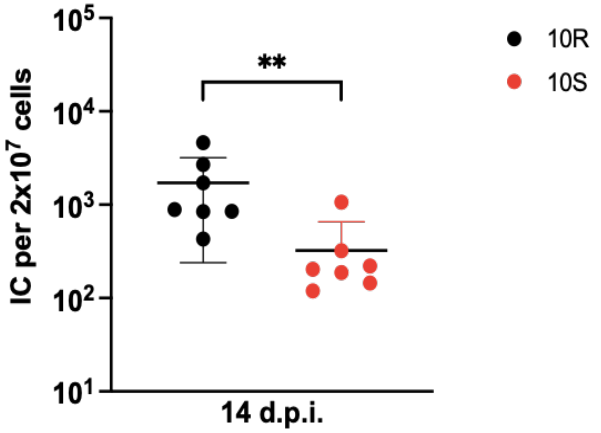


Figure 5-4. MHV-68 ORF10S virus had an attenuated latency establishment in immunocompetent mice.

BALB/c mice were infected intranasally with 5000 pfu of MHV-68 10R or 10S virus then the mice were sacrificed at 14 days post infection. The spleens were collected and processed to obtain single-cell splenocytes then used for infectious center assays to determine the latency load of the viruses in vivo.

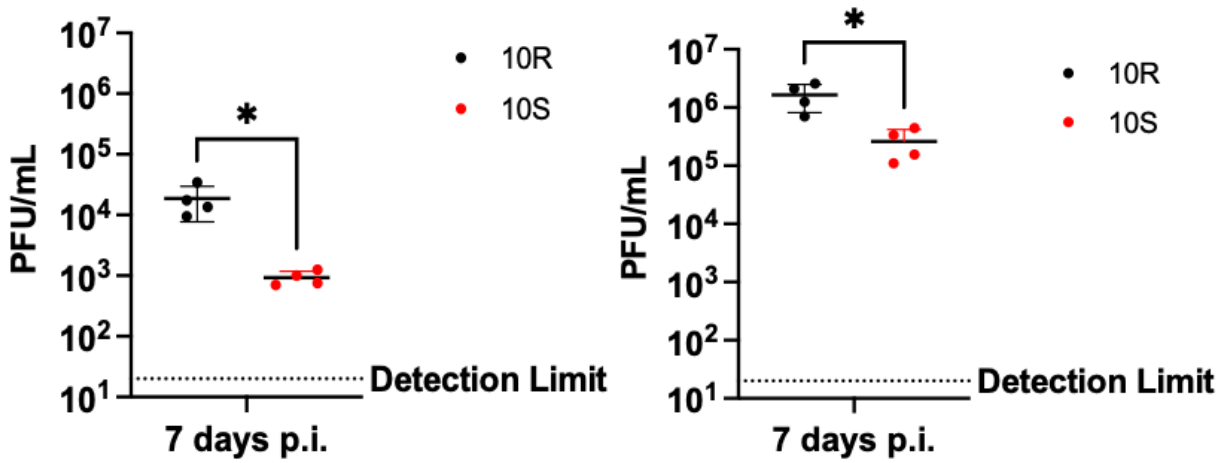


Figure 5-5. Intranasal infection of IFNAR1-/- did not lead to a rescue in 10S virion production.

IFNAR1-/- mice were infected intranasally with 10,000 pfu of MHV-68 10R or 10S virus then the mice were sacrificed at 7 days post infection. The lungs were harvested to determine the level of productive virions at the primary site of infection via plaque assays. The experiment was repeated twice and each panel shows the lung titers from a separate IFNAR1-/- in vivo experiment.

CHAPTER 6

MHV-68 ORF10 AND VIRAL DISSEMINATION

ABSTRACT

Typically, MHV-68 wild type virus gains the ability to replicate in peripheral organs, such as the spleen and liver with the removal of interferon response from the host. However, the lack of rescue in the lung viral titer for MHV-68 10S in an immunocompromised host was accompanied by a surprising phenotype. In the spleen and liver of the IFNAR1-/- mice, the 10S virus remained deficient in viral replication. This deficiency was overcome when the 10S virus was introduced more directly into these peripheral organs. Further in vitro investigation determined that the basis for this in vivo phenotype was the role the ORF10 held in the MHV-68's ability to perform cell-to-cell spread in order to perform viral dissemination within the peripheral organ or tissue.

INTRODUCTION

Upon MHV-68 infection, the virus moves from one organ to another in a process called viral trafficking. Typically, MHV-68 is introduced into a laboratory mouse model via intranasal infection. MHV-68 then undergoes lytic replication in the lung epithelium[14]. The virus then spreads to the lung-draining lymph nodes through dendritic cell trafficking. In this compartment, other leukocytes are infected including B-cells[34,35]. In the lymph nodes, the virus infects the B-cells and establishes latency. From the lymph nodes, the latently infected B-cells can enter the circulation then reach peripheral organs, such as the spleen and the liver. In the case of the spleen, MHV-68 spreads from the macrophage to the B-cells within the splenic marginal zone. The marginal B-cells then enter the follicular zone to infect dendritic cells, which then infect the follicular B-cells[36]. Within the spleen, the latently infected follicular B-cells can undergo germinal center reaction, proliferate, and

differentiate in order to become memory B-cells that establish the lifelong latent reservoir of virus within the host. These infected cells can also enter reactivation to replenish the latent reservoir and source new viruses for viral transmission to a new host[37,38].

For an efficient viral trafficking, within a particular organ or tissue, the virus must have the ability to spread within an organ before moving on infecting another (peripheral) organ or loading virus into the circulation. This involves what we here define as viral dissemination, which is the ability of the virus to spread from an infected cell to an uninfected cell within a tissue or organ. There is a myriad of methods by which viruses, including the gammaherpesvirus family, perform viral dissemination from one cell to another within an organ or tissue type[39,40]. Broadly speaking, these mechanisms are divided into two types: cell free and cell-to-cell spread. In cell free spread, the virus exits the cell of origin (for example, through lysis of the original host) then enters the circulation and is free to infect a new cell host. This method is energetically costly, requires high levels of viral amplification, and is prone to immune surveillance detection. In cell-to-cell spread, the virus takes advantage of normal cell-to-cell contact features, such as the presence of tight junctions, receptors, and the fluidity of plasma membrane, in order to move viruses from one cell to a neighboring cell.

RESULTS

In vivo assessment of ORF10 and viral dissemination

For the wild type MHV-68 virus, the removal of interferon signaling within the host leads to the ability for the virus to undergo lytic replication in peripheral organs such as the spleen and the liver[41]. Expecting that this would be the case for the 10S virus, instead we

were presented with a surprising phenotype. In the intranasally infected IFNAR1-/- mice, it was found that despite the removal of interferon response, there was still a complete absence of lytic replication in the spleen and the liver (Figure 6-1). Of note, the viral copy number between 10R and 10S for the spleen and liver are similar when compared between the viruses and the organs. This indicates that while the viruses were able to enter the organs, the lytic replication for the 10S virus failed to materialize (Figure 6-2).

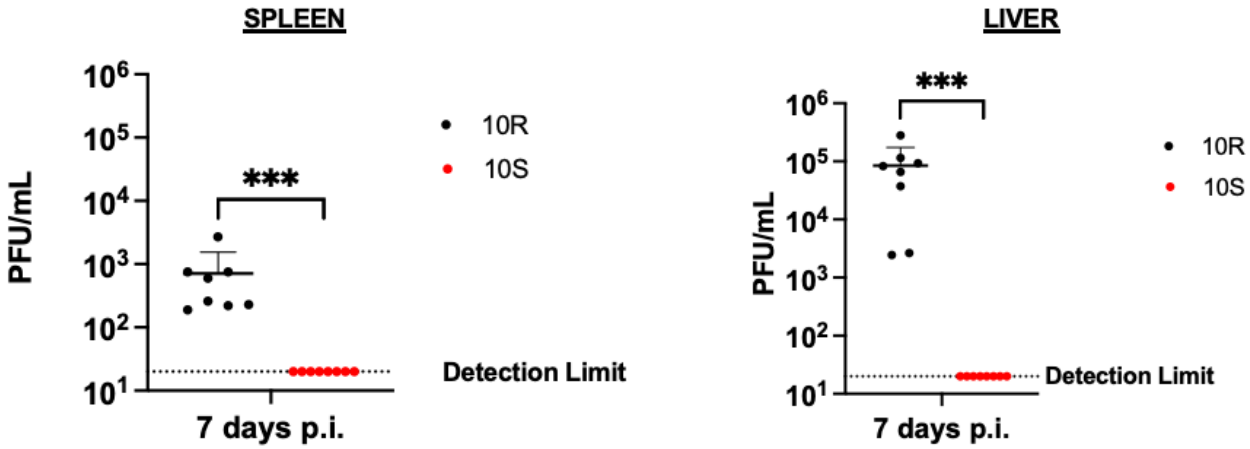


Figure 6-1. The removal of host interferon response did not lead to lytic replication by MHV-68 10S in the peripheral organs.

IFNAR1-/- mice were infected intranasally with 10,000 pfu of MHV-68 10R or 10S virus then the mice were sacrificed at 7 days post infection. The spleens and livers were harvested to determine the level of productive virions at the peripheral sites of infection via plaque assays.

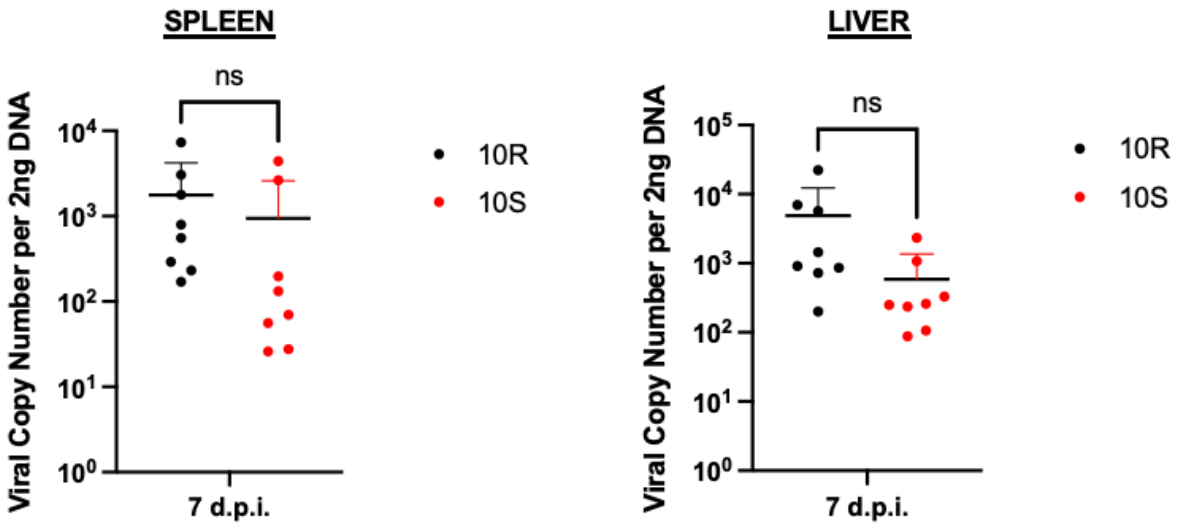


Figure 6-2. The viral copy number of MHV-68 10R and 10S were equivalent in host without interferon response.

IFNAR1-/- mice were infected intranasally with 10,000 pfu of MHV-68 10R or 10S virus then the mice were sacrificed at 7 days post infection. The spleens and livers were homogenized and an aliquot of the samples were taken and processed for genomic DNA extraction. The DNA was then used to perform droplet PCR against MHV-68 ORF6 to determine the viral copy number of each sample.

The reason for the lack of lytic replication by the 10S virus even in the absence of host interferon response could be due to two potential reasons. One possible reason is that ORF10 is required for the site-dependent viral dissemination of MHV-68. Thus, the absence of ORF10 hampered the ability of the virus to replicate in a specific peripheral organ/tissue after intranasal infection. Another possible reason is that ORF10 is required for the route-dependent viral dissemination of MHV-68, and its absence involves a particular defect in the viral trafficking process from the lung epithelium all the way to the spleen. To validate the site-dependent hypothesis, we performed intraperitoneal (IP) injection. Through this method, the virus enters the circulation directly through the portal vein, which is a more direct introduction into the spleen and liver. This injection method bypasses the need to travel from the lung epithelium to the spleen via the lymph nodes. Indeed, IP injection led to an ability of the 10S virus to replicate in the spleen and liver (Figure 6-3), indicating that ORF10 is not involved in site-dependent dissemination.

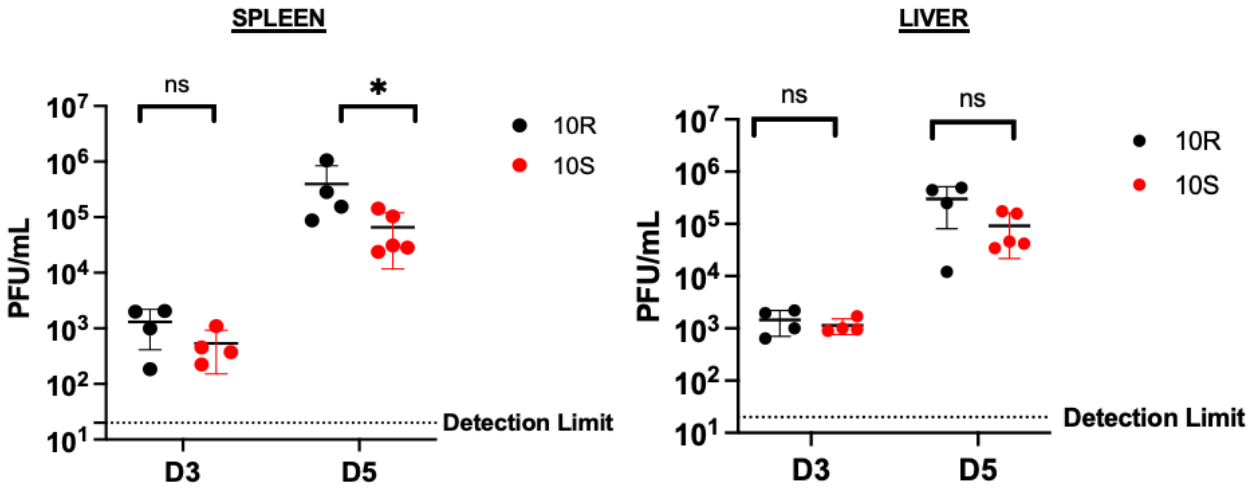


Figure 6-3. Intraperitoneal injection of MHV-68 10S into hosts without interferon response led to lytic replication in peripheral organs.

IFNAR1-/- mice were infected intraperitoneally with 5000 pfu of MHV-68 10R or 10S virus then the mice were sacrificed at 3 and 5 days post infection. The spleens and livers were homogenized then used for plaque assays to determine the viral titer.

In vitro assessment of ORF10 and viral dissemination

In both in vivo and in vitro studies, we observed that there was a starting dose dependency on the efficiency of viral replication. In the in vivo studies, intranasal infection led to a low starting dose for infecting peripheral organs while intraperitoneal infection gave a high starting dose for peripheral organ infection. In the IN infection, there was an all-or-nothing difference in 10R vs. 10S titer (Figure 6-1), while in the latter, there was virtually no difference (Figure 6-3). Correspondingly, we observed that high MOI infection (MOI 5), led to a small difference in 10R vs. 10S titer in NIH 3T3 cells (Figure 6-4A). However, with lower MOIs, after permitting the cells to replicate and spread over 4 days (using vero cells that can withstand overconfluent growth), we observed a difference in 10R and 10S titers (Figure 6-4B). At MOI 0.0125 and 0.025, the difference began to appear. With 10-fold reduction in the starting dose (0.00125 and 0.0025), which depended mainly on cell-to-cell spread in order to infect the entire well, we observed a stark difference between 10R and 10S titer. Therefore, the question became what was the reason for the 10S viral mutant's sensitivity towards the starting infection dose?

To assess the requirement of ORF10 in route-dependent dissemination, we determined the blood viral load by collecting the plasma and peripheral blood mononuclear cells (PMBCs) to determine the presence of the virus in the circulation. Both 10R and 10S could enter the circulation but 10S has a 10-fold lower titer (Figure 6-5). This indicated that the 10S virus has the ability to enter the circulation during viral trafficking despite the lower ability. Therefore, the lack of ORF10 leading to a defect in lytic replication at peripheral organs in an immunosuppressed host had to do with events at the organs.

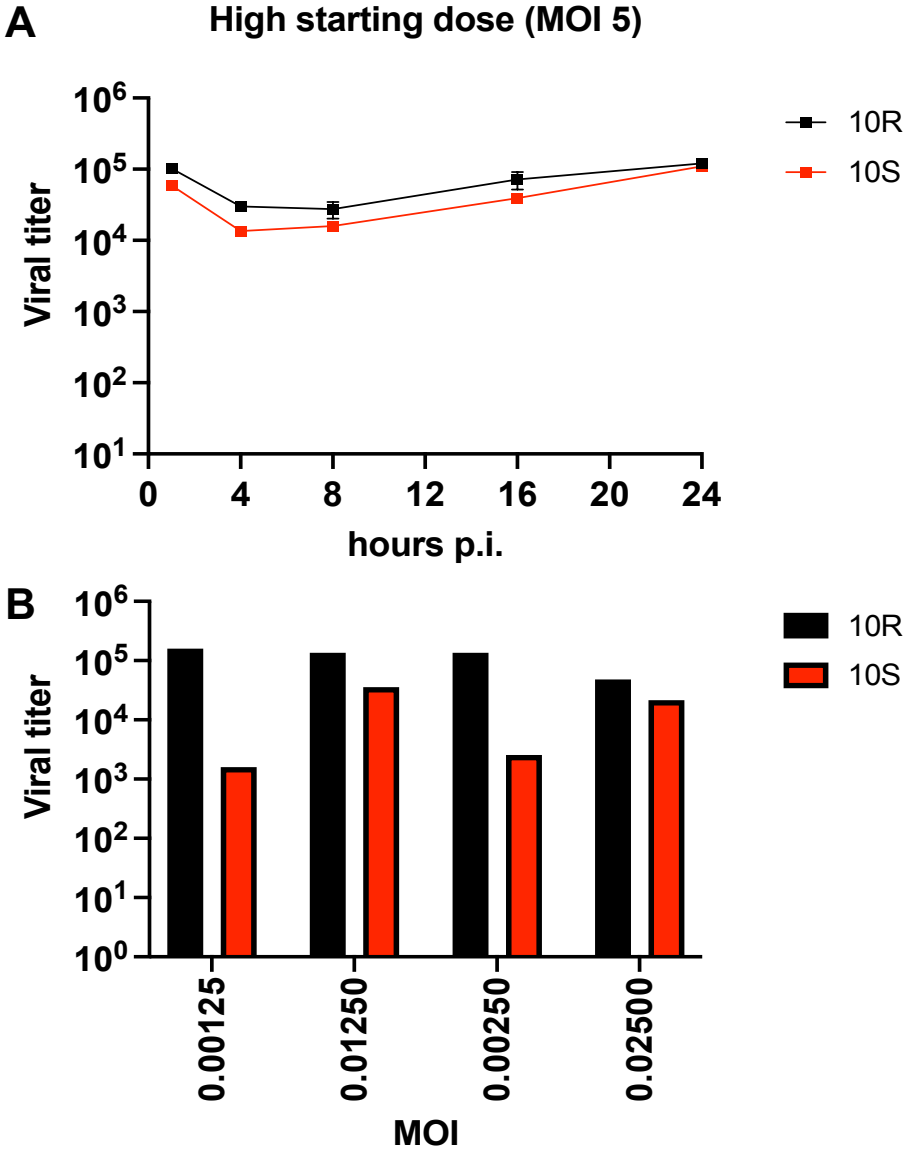


Figure 6-4. High vs. low starting dose influenced the ability to observe titer difference between MHV-68 10R and 10S.

(A) NIH 3T3 cells were infected at MOI of 5 to create single-step growth curve. **(B)** Vero cells were infected at 10-fold difference low MOIs and harvested at four days post infection.

One phenotype of the 10S virus was observed during plaque assays: it produced smaller plaques compared to the 10R and WT viruses. This is a typical indication of a defect in cell-to-cell spread by a virus[42–44]. Therefore, we investigated the sensitivity of the 10S virus towards the presence of neutralizing antibody. In this experiment, we utilized serum from mice that were intraperitoneally injected with MHV-68 ATCC WT virus or PBS. The virus-injected mice generated serum with neutralizing antibodies against MHV-68 (NAb serum) while the PBS-injected mice generated mock serum with no antibodies against MHV-68. The application of NAb serum into the growth medium of a cell line forced on-going viral spread in cell culture to be mostly through only cell-to-cell spread, while mock serum allows for both cell free and cell-to-cell spread infection. Our output in measuring viral sensitivity towards NAb serum's effect were measuring the cluster size through K8.1 glycoprotein surface staining (Figure 6-6) and measuring the viral titer of cell-associated viruses (Figure 6-7). Both measurements indicated that the 10S virus was significantly more sensitive to the presence of NAb serum in the cell culture media, suggesting a role of ORF10 in cell-to-cell spread. 10R infection maintained in mock or NAb serum had comparable levels of productive infection while compared to maintenance in mock serum, 10S productive infection dropped by nearly 10-fold in the presence of neutralizing antibodies.

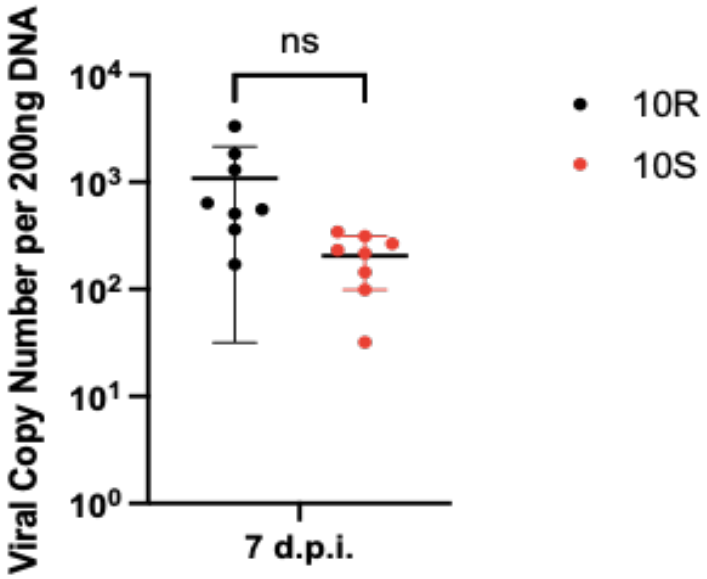
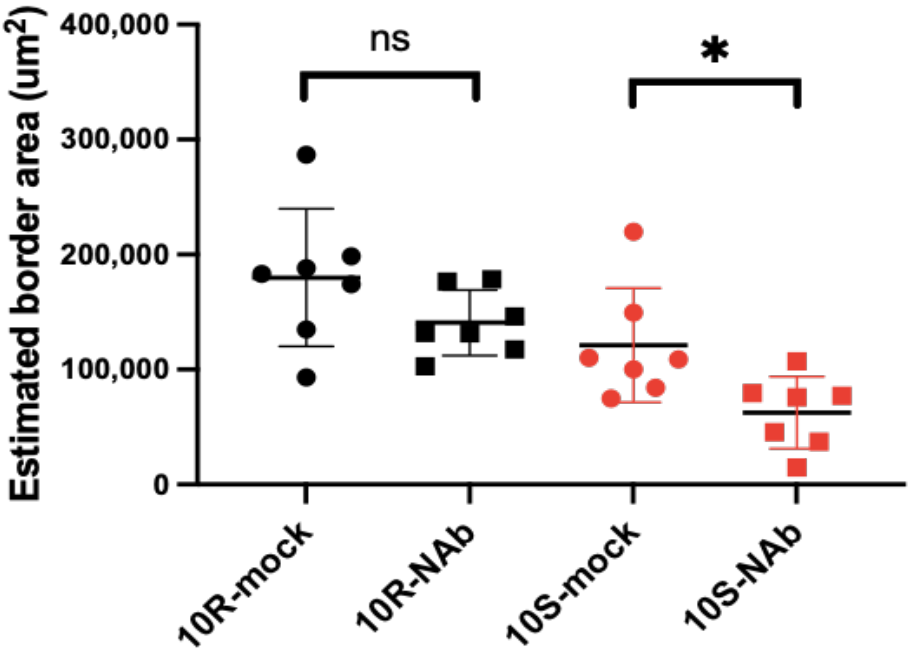


Figure 6-5. Blood viral load comparison in intranasally infected IFNAR-/- mice.

IFNAR1-/- mice were infected intranasally with 10,000 pfu of MHV-68 10R or 10S virus then the mice were sacrificed at 7 days post infection. The mice were bled through cardiac puncture to obtain whole blood kept in EDTA tubes to prevent blood coagulation. The plasma and PBMCs were isolated from whole blood through centrifugation then processed for genomic DNA extraction for droplet PCR use to determine the blood viral load.


Estimated border area (um2)
400,000
300,000
200,000
100,000
0
ns
*
10R-mock
10R-NAb
10S-mock
10S-NAb

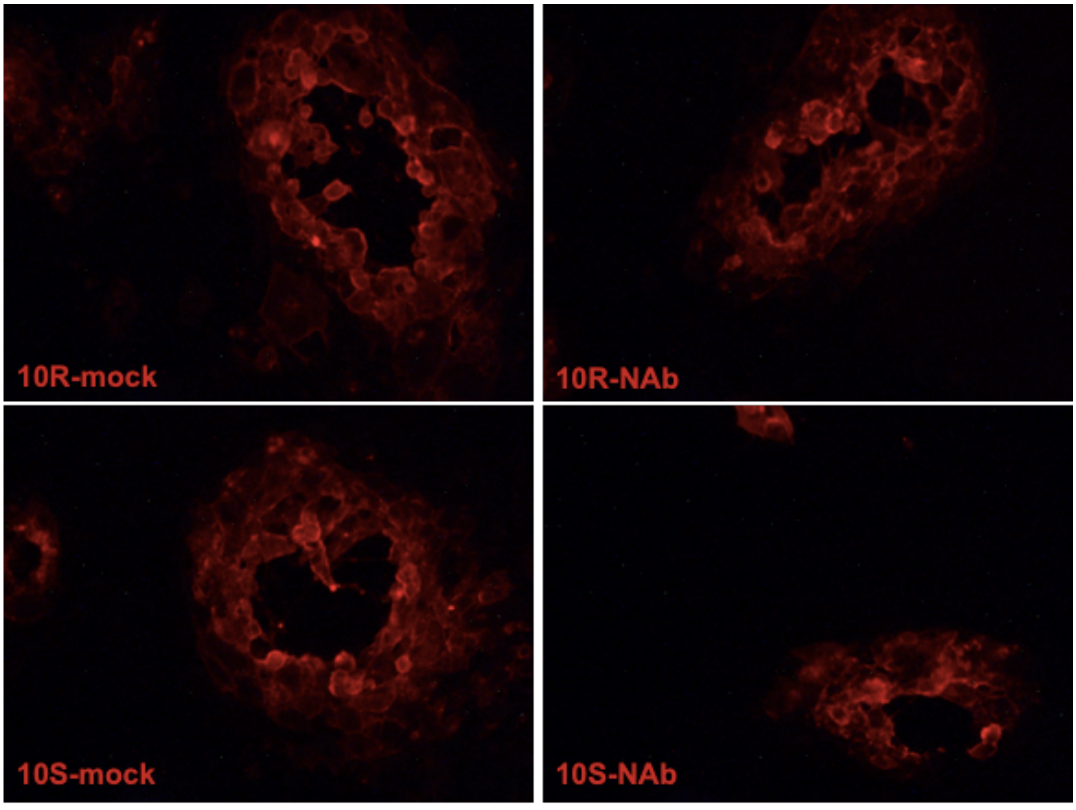

10R-mock
10R-NAb
10S-mock
10S-NAb

Figure 6-6. The presence of neutralizing antibodies in the cell culture media led to smaller clusters produced by the 10S virus compared to other conditions.

Vero cells were seeded at over 90% confluence in 24-well plates then infected with 300 pfu of 10R or 10S virus. After a 1-hour adsorption period to facilitate viral entry, the viral inocula were removed and the cells were washed with PBS three times. Then cell culture media with mock or NAb serum were added at a 1:250 dilution to the appropriate wells. Three days post infection, the cells were harvested by removal of the growth media then three PBS washes. The cells were then fixed and surface stained for K8.1 glycoprotein to perform immunofluorescence assay to measure cluster size via automated estimation by the NIS Element Viewer.

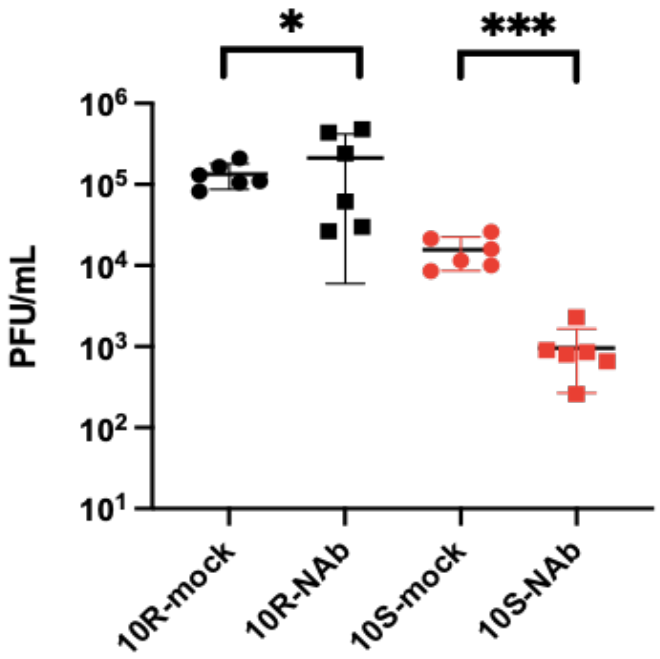


Figure 6-7. The presence of neutralizing antibodies in the cell culture media led to a significantly lower cell-associated viral titer produced by the 10S virus compared to other conditions.

Vero cells were seeded at over 90% confluence in 24-well plates then infected with 300 pfu of 10R or 10S virus. After a 1-hour adsorption period to facilitate viral entry, the viral inocula were removed and the cells were washed with PBS three times. Then cell culture media with mock or NAb serum at a 1:250 dilution were added to the appropriate wells. Three days post infection, the cells were harvested by removal of the growth media then three PBS washes. Fresh cell culture media were then added then the cells were subjected to three freeze-thaws to release cell-associated viruses into the media to allow for viral titering.

The next query was the mechanism by which ORF10 contributed to the cell-to-cell spread process. Previously, our lab determined that both the KSHV and MHV-68 ORF10 interacted with the cellular export factor, Rae1, to selectively inhibit host transcript export[17]. We wondered if the mechanism by which ORF10 played a role in cell-to-cell spread could be related to the Rae1 interaction. We first generated lentiviral constructs in order to transiently express MHV-68 or KSHV ORF10. A conserved segment at the C-terminus of ORF10 has also been determined to be essential for Rae1 interaction[18]. Thus, we also generated C-terminal deletion mutants of both MHV-68 and KSHV ORF10 (Ctd mutants) and confirmed that without the C-termini, both MHV-68 and KSHV ORF10 lost the interaction with Rae1 (Figure 6-8A). Then we infected the ORF10-expressing vero cells with MVH-68 10R or 10S viruses, in the presence of mock or Nab serum.

Comparing the infection maintained in the presence of NAb serum, we observed a mild rescue at best, in the MHV-68 ORF10 complementation. However, a clear-cut rescue by KSHV ORF10 complementation was observed (Figure 6-8B). The MHV-68 ORF10 WT did not have a dramatic rescue on 10S-NAb cell-to-cell infection; it was a partial rescue at best when comparing the WT complementation to the CTD complementation. The titer of 10R was reduced to reach the level of 10S-NAb as if MHV-68 ORF10 expression had a suppressive effect towards infection. For M10-CTD complementation, there was still a difference between 10R and 10S but no more sensitivity towards serum type. However, the KSHV ORF10-CTD complementation had the staircase pattern similar to the results from the untransduced vero NAb experiment. On the other hand, the KSHV ORF10 WT complementation raised the 10S-mock and NAb up to the level of the 10R replication

level, indicating a much more dramatic rescue effect by KSHV ORF10 on 10S virus infection.

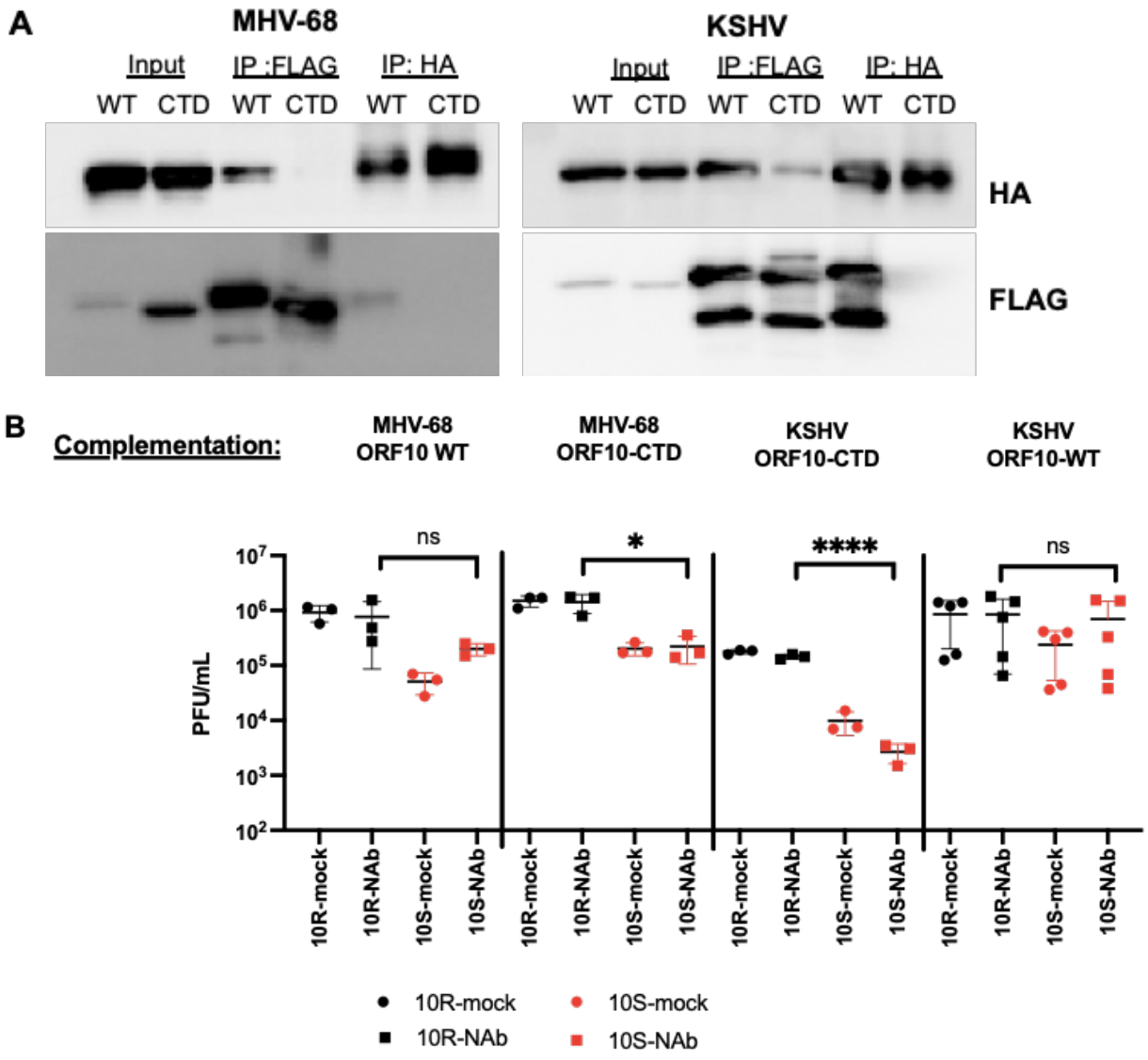


Figure 6-8. Lentiviral complementation with gammaherpesvirus ORF10 WT or C-terminal deletion (CTD).

(**A**) Co-immunoprecipitation between ORF10-FLAG and Rae1-HA showing the loss of ORF10-Rae1 interaction upon the deletion of the C-terminal deletion (**B**) Two sets of complementation studies. Vero cells were transduced with lentiviral construct in order to transiently express ORF10 WT or CTD of MHV-68 or KSHV. The transduced cells were infected with 300 pfu of 10R or 10S virus then maintained in DMEM FBS with 1:250

dilution of mock or NAb serum. The infected cells were then washed with PBS three times before media replacement and freeze-thaw to prepare for titering.

DISCUSSION

Despite the lack of ORF10 importance on interferon signaling antagonism, the same set of in vivo experiments indicated the lack of lytic replication in peripheral organs even after the removal of interferon signaling. With an intact IFN-I response, lytic replication would not take place in peripheral organs, such as the spleen and the liver. This inhibition is linked to IFN-I response[41]. Therefore, the surprising finding in this study was that in the absence of IFN-I response in the host, the 10S virus remained incapable of replicating in the spleen and liver replication (Figure 6-1) in contrast to the revertant mutant in our experiment or known findings in literature. There were two possibilities for this. One possibility involved the extensive trafficking and dissemination the virus would have to perform from the lung epithelium to the dendritic cells and B-cells of lymph nodes then the B-cells of the spleen then to the spreading within the organ[7,45]. It was possible that in the absence of 10S, this dissemination process was interrupted (route-specific requirement for ORF10). Another involved the inability to infect specific organs, such as the spleen and liver (site-specific requirement for ORF10). Through intraperitoneal injection of the IFNAR1-/- mice, which introduced the virus more directly to the spleen and liver, we eliminated the ORF10 requirement for organ- or site-specific infection. By skipping the requirement for viral trafficking, we showed that 10S is capable of performing lytic replication in the peripheral organs (Figure 6-3).

Subsequently, the question became what was the part of viral dissemination required ORF10. An initial hypothesis was a specific cell type required ORF10 to be infected. However, in the spleen, infection from the marginal to the follicular zone required the

sequential infection of macrophage then B-cells in the marginal zone, followed by dendritic then B-cells in the follicular zone. The 10S virus was still capable of doing so as shown in the intraperitoneal data in which 10S direct entry into the spleen led to a robust lytic replication (Figure 6-3). 10S virus was also capable, albeit attenuated, in entering the circulation that it should be able to seed peripheral organ infection (Figure 6-5). After in vivo studies, we decided to follow-up the observed phenotype by also considering an in vitro phenotype we had observed in the 10S virus amplification. In plaque assays involving the 10S virus, we noticed that 10S consistently formed significantly smaller plaques compared to ATCC WT or 10R viruses. This indicates inefficiency in cell-to-cell spread[43,44].

Many viruses, including gammaherpesviruses, have the ability to perform cell-free spread and cell-to-cell spread. Cell-free spread is possible but requires high levels of viral genome expressions as well as the packaging components[46]. The high requirement needed for cell-free spread leads to cell-to-cell spread being the other major route for viral dissemination since cell-to-cell contact can facilitate efficient viral assembly. Cell-to-cell spread often occurs through the tight points of cell-to-cell contact to ensure the efficiency of viral dissemination[39] as well as aiding in immune evasion during dissemination[47]. At the tight contact points, some viruses have evolved to manipulate existing cellular structures, such as disseminations that take advantage of cellular tight junction networks[46]. Other viruses carry the ability to drive the restructuring of cell membranes, such as the formation of viral synapses[48]. We further investigated this hypothesis by performing neutralizing antibody (NAb) experiments. The presence of NAb in the growth

medium forced the viral dissemination within the cell culture to rely largely on cell-to-cell spread. Indeed, we observed that 10S infection was more significantly impacted by the presence of NAb compared to 10R. Additionally, the infected cluster area size was significantly reduced by the presence of NAb for 10S but not for 10R.

Next, our question was the mechanism by which ORF10 controlled cell-to-cell spread. Our lab previously determined that ORF10 interacts with Rae1, a cellular export factor, leading to the interaction in selectively inhibiting host transcript export[17]. Further studies determined that the interaction of ORF10 with Rae1 occurs through the C-terminal of the ORF10[18]. The amino acid sequence motif of the C-terminal is the so-called EEPM motif that is generally conserved across viral proteins that interact with Rae1, such as M protein of VSV[19,20] and SARS-COV2 ORF6[21] among others. When we deleted the C-terminal of gammaherpesvirus ORF10, we confirmed the loss of interaction with Rae1 for both MHV-68 and KSHV ORF10. Through lentiviral complementation expressing wild type or C-terminal deleted ORF10, we were able to implicate Rae1 into ORF10's role in cell-to-cell spread. The KSHV ORF10 complementation set had clearer data in demonstrating the ability of returning ORF10 expression to rescue 10S virus infection in the presence of neutralizing antibodies. The cross-species rescue of a murine viral infection by a human viral gene expression highlighted the conservation of the ORF10 cell-to-cell spread function in vivo.

CHAPTER 7

SUMMARY AND PERSPECTIVE

SUMMARY

CHAPTER 1 describes general background on the herpesviral family as well as what is known so far on the gammaherpesvirus ORF10.

CHAPTER 3 elaborates on the reason for the discontinuation of a previous project as a doctoral dissertation project. The project involves further investigation on the molecular mechanism by which the KSHV ORF10-Rae1 complex performs its function in selective host transcript export inhibition. The specific significance of this project for gammaherpesviral pathogenesis is still unclear, yet involves the manipulation of host transcript expression level by a KSHV gene through its interaction with a common viral target, Rae1, which is a cellular export factor. Despite the discontinuation of this project, the project in Chapter 6 still involves the investigation of Rae1's involvement in the ORF10 role in controlling cell-to-cell spread and viral dissemination within the host, which may elucidate a reason for the Rae1 hijacking by gammaherpesviruses.

CHAPTER 4 documents preliminary findings related to KSHV infection of a host through the reactivation cell line, the iSLK cell line, and the possibility that the absence of ORF10 leads to an accelerated expression of other viral genes. While still in its preliminary stage, this project could further elucidate the host protein expression manipulation by KSHV infection. An interesting phenotype also showed that in the absence of KSHV ORF10, there is a potential alteration in the expression kinetics of KSHV genes, which could impact the tightly controlled expression sequence and timing of KSHV genes in order to

amplify the virus efficiently and to maintain the perfect balance between its lytic and latent viral cycle.

CHAPTER 5 introduces the genome-wide screen that links MHV-68 ORF10 with the evasion of type I interferon signaling, a first-line of defense against viral infection by the mammalian host. In our investigation, it was found that antagonizing type I interferon signaling might not necessarily be the primary role of ORF10 in vivo.

However, CHAPTER 6 follows on a surprising phenotype from Chapter 5 that becomes the center point of this dissertation that implicates MHV-68 ORF10 in the role of viral dissemination and cell-to-cell spread through in vivo experiments. We then followed-up on these phenotypes with in vitro experiments to confirm the role of MHV-68 ORF10 in cell-to-cell spread. Additionally, we demonstrated that KSHV ORF10 was capable of rescuing the MHV-68 ORF10S viral infection in cell-to-cell spread, highlighting the conserved nature and importance of ORF10's role in cell-to-cell spread and viral dissemination.

PERSPECTIVE

The major findings described in this dissertation involved a novel role of gammaherpesvirus ORF10 in viral dissemination and cell-to-cell spread. A previous in vitro screen shows that ORF10 has a potential role in antagonizing host interferon response[33] but this did not seem to be the role of ORF10 in vivo. In the absence of IFN-I signaling, MHV-68 has the ability to undergo lytic replication in the spleen and liver[41]. However, we discovered a surprising phenotype for MHV-68 10S that still lacked the ability to perform lytic replication even in the absence of IFN-I signaling. Correspondingly, productive infection in the liver, another secondary organ, was not detected for MHV-68 10S. An in vitro phenotype that was noticeable for MHV-68 10S infection was the smaller plaque size compared to MHV-68 WT and 10R, which indicates inefficient viral spread of the 10S mutant virus. Through neutralizing antibody experiments and lentiviral complementation, we confirmed that MHV-68 without ORF10 was significantly more affected by the presence of neutralizing antibodies in terms of producing infectious virions. Complementarily, lentiviral expression of KSHV ORF10 was able to rescue the infectious virion level of MHV-68 10S. The mild rescue by MHV-68 ORF10 complementation may indicate an additional function that ORF10 has for the MHV-68 virus that KSHV never possessed, no longer performed, or performed to a much lesser extent. Nonetheless, the apparent rescue by KSHV ORF10 still capable of interacting with Rae1 implicated ORF10 in the gammaherpesvirus viral dissemination process within peripheral organs through cell-to-cell spread.

Tentative role of ORF10 in cell-to-cell spread

Cell-to-cell transmission of viruses offers several advantages compared to cell-free transmission[39,46]. For example, cell-to-cell transmission is more resistant to antiviral immunity that acts on virions, such as complement and neutralizing antibodies, than cell-free transmission. Another major advantage is that cell-to-cell transmission relies less on the efficiency of virion production and release because virions are directly delivered to target cells that are in contact with producing cells. Therefore, it is not surprising that viruses encode for multiple proteins to facilitate cell-to-cell spread to enable efficient viral dissemination within a host.

Several viral proteins in the gammaherpesviral family are known to target different parts of the cytoskeleton due to the dynamic nature of cytoskeletal components for reshaping cell structure. It is known that the MHV-68 gp48 manipulates actin structures of the host for optimal viral dissemination[50]. The ORF52 of rhesus monkey rhadinovirus and KSHV ORF52 have a conserved leucine zipper motif that leads to a distinct bundling of microtubules for viral genome transport[51]. In the case of our findings, Rae1 is known to be non-essential for the viability of the cells, which makes it an ideal target for viral manipulation since it allows for the virus to maintain a fine-tuned balance of manipulating the host without causing premature damage[49]. However, to perform RNA export, the host cell itself must rearrange the cell membrane structure[26]. Thus, Rae1 becomes an attractive target for viruses to target in order to gain access to this dynamic process in the host to facilitate viral dissemination within host tissue or organs.

Typically, viral dissemination and cell-to-cell spread involve structural proteins such as glycoproteins[52,53], viral tegument[54,55], and the viral envelope[56,57]. For gammaherpesviruses, glycoproteins such as gp150 and the co-synergy of ORF27-ORF58, play essential roles in viral dissemination[43,50,58]. Small non-coding RNAs have also been implicated in the cell-to-cell spread process[59,60]. ORF10 is not known to be a viral structural protein. It is localized around the nuclear envelope and manipulates host transcript export selectively[17]. The impact of this action for the survival of the virus is unclear. However, with our recent findings on ORF10's new role in cell-to-cell spread, it is possible that ORF10 hijacked the Rae1 pathway not for manipulating the protein expression of the host, but for controlling the host cell structure to facilitate cell-to-cell spread via a capability that Rae1 could hold as a cellular export factor.

It is possible that ORF10 is direct in controlling the cell-to-cell spread. However, it could also be indirect, in that the absence of ORF10 leads to the downregulation level of late viral transcripts[17]. One possible effect of manipulating late viral gene level and expression is an alteration in the virion structure and surface glycoprotein structure. This is the case for another gammaherpesvirus protein encoded by ORF37. When ORF37 is mutated in a certain way, it leads to the production of viruses with different virion-surface profiles, which leads to significantly lower sensitivity to neutralizing antibodies[61,62]. This could also be the case for ORF10, especially given ORF10's tentative role in affecting the expression of late genes, which include those that encode for structural proteins found on the virion surface. We performed a preliminary neutralization assay and found that 10S and 10R in

vitro had similar sensitivity to the NAb serum. However, whether this is true in vivo remains to be investigated and is one of the immediate follow-up studies to this project.

The 10S virus lacks the ability to enter lytic replication even in permissive conditions, i.e. lack of interferon response, such as the vero cell line and IFNAR1-/- mice. However, it can enter circulation well as seen in blood titer and even in organs lacking productive infection, the viral gene level is similar to that of 10R. It is possible that ORF10 also plays a role in nuclear egress. Thus, it can replicate the viral genome well but has difficulty in assembling and disassembling or vesicle formation for efficient exit of new virions. ORF10 is localized at the nuclear envelope. The known components of the nuclear egress complex (NEC) for gammaherpesvirus are encoded by ORF67 and ORF69, which initiate vesicle budding[63]. It could be that ORF10 is a part of this complex to engage Rae1-Nup98 for the manipulation of the host plasma membrane. Alternatively, ORF10-Rae1 could be a complex that performs this vesicle budding function redundantly in a distinct mechanism from that of ORF67 and ORF69.

Significance and future direction

During the persistent infection of herpesvirus, neutralizing antibodies would prevent the infection of cell-free virions but not cell-to-cell spread. Therefore, despite the initial finding that the absence of ORF10 had a minor impact on latency establishment, our subsequent findings on ORF10 and its role in cell-to-cell spread showed that ORF10 could be relevant in disease spread and transmission due to ORF10's role on cell-to-cell spread. It is plausible that the virus could reactivate from latently infected B cells then amplify itself in

the mucosal epithelium. Without efficient cell-to-cell spread, the ability of the virus to produce sufficient virion level could be severely compromised, which in turn could attenuate viral transmission.

Engineering C-terminal deletion mutant viruses would allow for the controls to determine if Rae1 interaction is responsible for any phenotype observed. Mass spectrometry studies could be used to determine the differential expression impact of gammaherpesvirus infection with and without ORF10, as well as infection profile with and without Rae1 interaction within different systems (iSLK reactivation for KSHV infection and in vitro infection for MHV-68). Additionally, utilizing peptides to physically interrupt ORF10-Rae1 interaction could yield a cleaner overall effect since the interruption of a cellular export factor's function could lead to other effects unrelated to the true effects of the ORF10-Rae1 complex.

Follow-up studies would involve the investigation of whether ORF10-Rae1 direct cell-to-cell spread directly or indirectly. ORF10-Rae1 could hold a similar function to ORF27-ORF58 synergy that reorganizes cytoskeletal structure through actin manipulation. On the other hand, ORF10-Rae1 could be the complex that regulates the ORF27-ORF58 synergy. To investigate the potential cell structure manipulation role of ORF10, the immunofluorescence assay can be used in order to determine the change in cytoskeletal structure under the expression of ORF10 as well as comparing cytoskeletal structure formation under the infection of MHV-68 10R vs. 10S virus. The localization of ORF10 near the altered cytoskeleton can also give more clues on how direct the ORF10

contribution is to restructuring the host cytoskeleton. A thorough investigation can then be done via cryogenic electron microscopy (cryoEM) studies in order to investigate the morphology of the cell host's cytoskeletal structure, which is how the ORF27-OF58 synergy is resolved[43,44]. ORF10-Rae1 could also control cell-to-cell spread by interacting or regulating other viral proteins whose function in cell-to-cell spread is yet to be elucidated. Therefore, mass spectrometry to determine if ORF10-Rae1 actually interacts with other viral proteins would be informative for directing the project's future direction.

BIBLIOGRAPHY

1. Barton, E., Mandal, P. & Speck, S. H. Pathogenesis and host control of gammaherpesviruses: lessons from the mouse. *Annu. Rev. Immunol.* **29**, 351–397 (2011).

2. Virgin, H. W., 4th *et al.* Complete sequence and genomic analysis of murine gammaherpesvirus 68. *J. Virol.* **71**, 5894–5904 (1997).

3. Rochford, R., Lutzke, M. L., Alfinito, R. S., Clavo, A. & Cardin, R. D. Kinetics of murine gammaherpesvirus 68 gene expression following infection of murine cells in culture and in mice. *J. Virol.* **75**, 4955–4963 (2001).

4. Zhu, F. X., Cusano, T. & Yuan, Y. Identification of the immediate-early transcripts of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* **73**, 5556–5567 (1999).

5. Brown, J. C. & Newcomb, W. W. Herpesvirus capsid assembly: insights from structural analysis. *Curr. Opin. Virol.* **1**, 142–149 (2011).

6. Barton, E. S. *et al.* Herpesvirus latency confers symbiotic protection from bacterial infection. *Nature* **447**, 326–329 (2007).

7. Dittmer, D. P. & Damania, B. Kaposi sarcoma-associated herpesvirus: immunobiology, oncogenesis, and therapy. *J. Clin. Invest.* **126**, 3165–3175 (2016).

8. Ackermann, M. Pathogenesis of gammaherpesvirus infections. *Vet. Microbiol.* **113**, 211–222 (2006).

9. Weed, D. J. & Damania, B. Pathogenesis of Human Gammaherpesviruses: Recent

Advances. *Curr Clin Microbiol Rep* **6**, 166–174 (2019).

10. Cesarman, E. Gammaherpesviruses and lymphoproliferative disorders. *Annu. Rev. Pathol.* **9**, 349–372 (2014).

11. Wen, K. W. & Damania, B. Kaposi sarcoma-associated herpesvirus (KSHV): molecular biology and oncogenesis. *Cancer Lett.* **289**, 140–150 (2010).

12. Speck, S. H. & Virgin, H. W. Host and viral genetics of chronic infection: a mouse model of gamma-herpesvirus pathogenesis. *Curr. Opin. Microbiol.* **2**, 403–409 (1999).

13. Wang, Y., Tibbetts, S. A. & Krug, L. T. Conquering the Host: Determinants of Pathogenesis Learned from Murine Gammaherpesvirus 68. *Annu Rev Virol* **8**, 349–371 (2021).

14. Sunil-Chandra, N. P., Efstathiou, S., Arno, J. & Nash, A. A. Virological and pathological features of mice infected with murine gamma-herpesvirus 68. *J. Gen. Virol.* **73 (Pt 9)**, 2347–2356 (1992).

15. Flaño, E., Husain, S. M., Sample, J. T., Woodland, D. L. & Blackman, M. A. Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. *J. Immunol.* **165**, 1074–1081 (2000).

16. Coleman, C. B., Nealy, M. S. & Tibbetts, S. A. Immature and transitional B cells are latency reservoirs for a gammaherpesvirus. *J. Virol.* **84**, 13045–13052 (2010).

17. Gong, D. *et al.* A Herpesvirus Protein Selectively Inhibits Cellular mRNA Nuclear

Export. *Cell Host Microbe* **20**, 642–653 (2016).

18. Feng, H. *et al.* Molecular mechanism underlying selective inhibition of mRNA nuclear export by herpesvirus protein ORF10. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 26719–26727 (2020).

19. Faria, P. A. *et al.* VSV disrupts the Rae1/mrnp41 mRNA nuclear export pathway. *Mol. Cell* **17**, 93–102 (2005).

20. Quan, B., Seo, H.-S., Blobel, G. & Ren, Y. Vesiculoviral matrix (M) protein occupies nucleic acid binding site at nucleoporin pair (Rae1 • Nup98). *Proc. Natl. Acad. Sci. U. S. A.* **111**, 9127–9132 (2014).

21. Miorin, L. *et al.* SARS-CoV-2 Orf6 hijacks Nup98 to block STAT nuclear import and antagonize interferon signaling. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 28344–28354 (2020).

22. Fowler, V. L. *et al.* Genome Sequences of Nine Vesicular Stomatitis Virus Isolates from South America. *Genome Announc.* **4**, (2016).

23. Cao, C. *et al.* The architecture of the SARS-CoV-2 RNA genome inside virion. *Nat. Commun.* **12**, 3917 (2021).

24. Wu, T.-T. *et al.* Construction and characterization of an infectious murine gammaherpesivrus-68 bacterial artificial chromosome. *J. Biomed. Biotechnol.* **2011**, 926258 (2011).

25. Smith, G. A. & Enquist, L. W. A self-recombining bacterial artificial chromosome and

its application for analysis of herpesvirus pathogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4873–4878 (2000).

26. De Magistris, P. The Great Escape: mRNA Export through the Nuclear Pore Complex. *Int. J. Mol. Sci.* **22**, (2021).

27. Macveigh-Fierro, D., Rodriguez, W., Miles, J. & Muller, M. Stealing the Show: KSHV Hijacks Host RNA Regulatory Pathways to Promote Infection. *Viruses* **12**, (2020).

28. Enninga, J., Levy, D. E., Blobel, G. & Fontoura, B. M. A. Role of nucleoporin induction in releasing an mRNA nuclear export block. *Science* **295**, 1523–1525 (2002).

29. Fessler, S. P. & Young, C. S. Control of adenovirus early gene expression during the late phase of infection. *J. Virol.* **72**, 4049–4056 (1998).

30. Pardamean, C. I. & Wu, T.-T. Inhibition of Host Gene Expression by KSHV: Sabotaging mRNA Stability and Nuclear Export. *Front. Cell. Infect. Microbiol.* **11**, 270 (2021).

31. Matoulkova, E., Michalova, E., Vojtesek, B. & Hrstka, R. The role of the 3' untranslated region in post-transcriptional regulation of protein expression in mammalian cells. *RNA Biol.* **9**, 563–576 (2012).

32. Myoung, J. & Ganem, D. Generation of a doxycycline-inducible KSHV producer cell line of endothelial origin: maintenance of tight latency with efficient reactivation upon induction. *J. Virol. Methods* **174**, 12–21 (2011).

33. Leang, R. S. *et al.* The anti-interferon activity of conserved viral dUTPase ORF54 is

essential for an effective MHV-68 infection. *PLoS Pathog.* **7**, e1002292 (2011).

34. Frederico, B., Milho, R., May, J. S., Gillet, L. & Stevenson, P. G. Myeloid infection links epithelial and B cell tropisms of Murid Herpesvirus-4. *PLoS Pathog.* **8**, e1002935 (2012).

35. Gaspar, M. *et al.* Murid herpesvirus-4 exploits dendritic cells to infect B cells. *PLoS Pathog.* **7**, e1002346 (2011).

36. Frederico, B., Chao, B., May, J. S., Belz, G. T. & Stevenson, P. G. A murid gamma-herpesviruses exploits normal splenic immune communication routes for systemic spread. *Cell Host Microbe* **15**, 457–470 (2014).

37. Willer, D. O. & Speck, S. H. Long-term latent murine Gammaherpesvirus 68 infection is preferentially found within the surface immunoglobulin D-negative subset of splenic B cells in vivo. *J. Virol.* **77**, 8310–8321 (2003).

38. Weck, K. E., Barkon, M. L., Yoo, L. I., Speck, S. H. & Virgin HW, I. V. Mature B cells are required for acute splenic infection, but not for establishment of latency, by murine gammaherpesvirus 68. *J. Virol.* **70**, 6775–6780 (1996).

39. Sattentau, Q. Avoiding the void: cell-to-cell spread of human viruses. *Nat. Rev. Microbiol.* **6**, 815–826 (2008).

40. Cifuentes-Munoz, N., El Najjar, F. & Dutch, R. E. Viral cell-to-cell spread: Conventional and non-conventional ways. *Adv. Virus Res.* **108**, 85–125 (2020).

41. Barton, E. S., Lutzke, M. L., Rochford, R. & Virgin, H. W., 4th. Alpha/beta interferons

regulate murine gammaherpesvirus latent gene expression and reactivation from latency. *J. Virol.* **79**, 14149–14160 (2005).

42. Goh, K. C. M. *et al.* Molecular determinants of plaque size as an indicator of dengue virus attenuation. *Sci. Rep.* **6**, 1–11 (2016).

43. de Lima, B. D., May, J. S. & Stevenson, P. G. Murine gammaherpesvirus 68 lacking gp150 shows defective virion release but establishes normal latency in vivo. *J. Virol.* **78**, 5103–5112 (2004).

44. Gredmark-Russ, S. *et al.* A gammaherpesvirus ubiquitin-specific protease is involved in the establishment of murine gammaherpesvirus 68 infection. *J. Virol.* **83**, 10644–10652 (2009).

45. Thorley-Lawson, D. A. Epstein-Barr virus: exploiting the immune system. *Nat. Rev. Immunol.* **1**, 75–82 (2001).

46. Zhong, P., Agosto, L. M., Munro, J. B. & Mothes, W. Cell-to-cell transmission of viruses. *Curr. Opin. Virol.* **3**, 44–50 (2013).

47. Johnson, D. C. & Huber, M. T. Directed egress of animal viruses promotes cell-to-cell spread. *J. Virol.* **76**, 1–8 (2002).

48. Eduardo Garcia, V. P. Virological Synapse for Cell-Cell Spread of Viruses. *Cell-Cell Channels* 288 (2006) doi:10.1007/978-0-387-46957-7_22.

49. Ren, Y., Seo, H.-S., Blobel, G. & Hoelz, A. Structural and functional analysis of the interaction between the nucleoporin Nup98 and the mRNA export factor Rae1. *Proc.*

Natl. Acad. Sci. U. S. A. **107**, 10406–10411 (2010).

50. Gill, M. B., Edgar, R., May, J. S. & Stevenson, P. G. A gamma-herpesvirus glycoprotein complex manipulates actin to promote viral spread. *PLoS One* **3**, e1808 (2008).

51. Loftus, M. S., Verville, N. & Kedes, D. H. A Conserved Leucine Zipper Motif in Gammaherpesvirus ORF52 Is Critical for Distinct Microtubule Rearrangements. *J. Virol.* **91**, (2017).

52. Dingwell, K. S. & Johnson, D. C. The herpes simplex virus gE-gI complex facilitates cell-to-cell spread and binds to components of cell junctions. *J. Virol.* **72**, 8933–8942 (1998).

53. Dingwell, K. S. *et al.* Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. *J. Virol.* **68**, 834–845 (1994).

54. Chandran, B. & Hutt-Fletcher, L. Gammaherpesviruses entry and early events during infection. in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis* (eds. Arvin, A. et al.) (Cambridge University Press, 2011).

55. Nozawa, N. *et al.* Herpes simplex virus type 1 UL51 protein is involved in maturation and egress of virus particles. *J. Virol.* **79**, 6947–6956 (2005).

56. Jha, H. C. *et al.* Gammaherpesvirus Infection of Human Neuronal Cells. *MBio* **6**, e01844–15 (2015).

57. Tanner, J., Weis, J., Fearon, D., Whang, Y. & Kieff, E. Epstein-Barr virus gp350/220

binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. *Cell* **50**, 203–213 (1987).

58. May, J. S., Walker, J., Colaco, S. & Stevenson, P. G. The murine gammaherpesvirus 68 ORF27 gene product contributes to intercellular viral spread. *J. Virol.* **79**, 5059–5068 (2005).

59. Diebel, K. W. *et al.* Gammaherpesvirus small noncoding RNAs are bifunctional elements that regulate infection and contribute to virulence in vivo. *MBio* **6**, e01670–14 (2015).

60. Feldman, E. R. *et al.* Virus-encoded microRNAs facilitate gammaherpesvirus latency and pathogenesis in vivo. *MBio* **5**, e00981–14 (2014).

61. Kronstad, L. M. & Glaunsinger, B. A. Diverse virus-host interactions influence RNA-based regulation during γ-herpesvirus infection. *Curr. Opin. Microbiol.* **15**, 506–511 (2012).

62. Glaunsinger, B., Chavez, L. & Ganem, D. The exonuclease and host shutoff functions of the SOX protein of Kaposi's sarcoma-associated herpesvirus are genetically separable. *J. Virol.* **79**, 7396–7401 (2005).

63. Lv, Y. *et al.* Functional Identification and Characterization of the Nuclear Egress Complex of a Gammaherpesvirus. *J. Virol.* **93**, (2019).