THE REGULATION AND ROLE OF GLYCOPROTEIN C DURING HERPESVIRUS PATHOGENESIS

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor in Philosophy in VMS-Pathobiology
in the Graduate College of the
University of Illinois Urbana-Champaign, 2022

Urbana, Illinois

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ABSTRACT

Within the *Herpesviridae*, the *Alphaherpesvirinae* is a subfamily that infects a broad number of mammals, birds, and other vertebrates, causing diseases that affect humans and companion and agricultural animals. Marek's disease (MD) is a lymphoproliferative disease of chickens with high mortality rates caused by Marek's disease herpesvirus (MDV). Infections with virulent MDV strains incur severe economic losses due to high mortality rates, and prevention/vaccination against MD. MDV infects immune cells that circulate to the skin where they transfer the virus to feather follicle epithelial (FFE) cells. It is at this site where fully productive MDV replication occurs, and the virus is shed from the chickens into the environment that can then infect naïve chickens through inhalation of infectious dust and dander. Current vaccination against MD only reduces clinical signs and oncogenesis but does not prevent virus replication, shedding, and the subsequent establishment of latency. This presents an obstacle for controlling the disease, especially on poultry farms where even vaccinated chickens can continue to shed virulent virus. Previous research in our laboratory showed that the alphaherpesvirus conserved glycoprotein C (gC) is essential for horizontal transmission of MDV in chickens. We hypothesize that gC is a potential target to be used in the development of recombinant vaccines that can provide more efficient and effective protection against clinical signs, replication, and transmission of the virus. The research described in this thesis provides more insight on the regulation and the mechanistic roles gC proteins play during horizontal transmission of alphaherpesviruses. First, the regulation of MDV gC by the MDV-specific RLORF4 gene was studied and was conclusively shown that RLORF4 is not involved in regulation of gC expression. Secondly, the requirement of gC for homologous avian herpesviruses during

horizontal transmission was addressed showing gC is also required for an MD vaccine strain that is homologous to MDV. Thirdly, the conserved function of avian gC proteins was studied using classical gene exchange experiments where both complete and deficient compensation during horizontal transmission in chickens allowed us to elucidate potential conserved functions of gC proteins. In summary, these studies advance our knowledge regarding herpesvirus pathogenesis that may improve vaccine development against human and agricultural diseases.

ACKNOWLEDGMENTS

I am extremely grateful to my mentor and advisor, Dr. Keith Jarosinski, for his guidance, support, and encouragement during my PhD thesis and research. He was there to teach me techniques that will make me a better scientist in the future. I appreciate his mentorship and support whenever I ran into frustrations and hurdles in my research or while writing. He was also very supportive when I was struggling with my fear of public speaking.

I would also like to give thanks to my committee members Drs. Gee Lau, Rachel Whitaker, and Ying Fang, for their guidance and moral support during my studies. I also appreciate the support of the Department of Pathobiology for giving me the opportunity to share my research with not only the department, but the College of Veterinary Medicine as well. I am very grateful to the animal care staff at both the Veterinary Medicine Animal Facility (VMAF) and the Edward R. Madigan Laboratory (ERML) for their services and support during animal experiments.

It has been a pleasure to work with members of the Jarosinski Lab, and I appreciate their help and support throughout my PhD studies. Dr. Yvette Tien and Andrea Krieter, who have been my lab mates since the beginning, have always been a reliable support system, great company, and helped me collect samples whenever we had a deadline. Dr. Nagendra Prabhu Ponnuraj, and Huai Xu for helping with animal and research experiments, and Dr. Haji Akbar for not only helping with necropsies and sample collections, but also contributing to the statistical analysis of our data.

Specifically, I would like to give thanks to my late undergraduate advisor, Dr. Carlos Ruiz-Martínez, who was the first one to push me into research. He was always there in my highs

and lows, especially when it came time to shift from my premed courses into courses that would be beneficial for a career in research. He also told me a phrase that I have used as encouragement all these years: “You can fall 7 times, but you can get up an 8th time”.

Finally, I would like to dedicate this thesis to my loving family: my mom (Wendaliz Rodriguez-Torres), my dad (Ricardo Vega-Castillo), and my sister (Paola Vega-Rodriguez) for their love, support, and understanding throughout my upbringing and my PhD studies. My deepest gratefulness to them for supporting all my decisions and for celebrating with me my every achievement. I would like to also dedicate this thesis to my fiancé, Masaki Sugimoto, who has been there since the start. He was always there when I needed someone to talk to, and was my rock, especially when I was feeling home sick and/or frustrated with my research.

TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW ………………………………………………………………1

CHAPTER 2: MAREK'S DISEASE HERPESVIRUS (MDV) RLORF4 IS NOT REQUIRED FOR EXPRESSION OF GLYCOPROTEIN C AND HORIZONTAL TRANSMISSION..........13

CHAPTER 3: THE REQUIREMENT OF GLYCOPROTEIN C (gC) FOR HORIZONTAL TRANSMISSION IS A CONSERVED FUNCTION OF gC FOR AVIAN HERPESVIRUSES……………………………………………..…………………………...30

CHAPTER 4: THE REQUIREMENT OF GLYCORPOTEIN C FOR HORIZONTAL TRANSMISSION IS FUNCTIONALLY CONSERVED WITHIN THE ALPHAHERPESVIRUS GENUS (*MARDIVIRUS*), BUT NOT THE HOST (*GALLID*).………56

CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS ………………... 77

REFERENCES …………………………………………………………………………….... 79

CHAPTER 1: LITERATURE REVIEW

HERPESVIRUSES

Herpesviruses are a family of DNA viruses that are associated with infections within specific host species, based on viral-to-host adaptation in their natural setting (1). Within the *Herpesviridae*, there are three subfamilies divide based on their host range, genome structure, and the cells in which they establish latency (2). The *Alphaherpesvirinae* is a subfamily of herpesviruses that infect mammals and birds, among other vertebrates and invertebrates. This family has a wide host range and is known for their ability to establish infection and latency in the peripheral nervous system. Human viruses within this family include herpes simplex viruses (HSV) 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV). On the other hand, the *Betaherpesvirinae* have a more restrictive host range, replicate at a much slower rate than alphaherpesviruses, and they establish latency in leukocytes. Some of the viruses in this subfamily are human cytomegalovirus (HCMV) and human herpesvirus 6A and 6B (HHV-6A and HHV-6B). Lastly, the *Gammaherpesvirinae* also have a restricted host range and can establish latent infection in lymphocytes, primarily in B cells. The two most studied human viruses in this subfamily are Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV).

ALPHAHERPESVIRUSES

Among the different *Herpesviridae* subfamilies, this thesis will focus on the *Alphaherpesvirinae*. Within this subfamily are the *Simplexvirus, Varicellovirus, Scutavirus, Mardivirus,* and *Iltovirus* genera. This subfamily is known to cause diseases in humans, including *human alphaherpesvirus* 1, 2, and 3 (HHV-1, HHV-2, and HHV-3), better known as HSV-1, HSV-2, and VZV, respectively; and agriculturally relevant species like *Equid alphaherpesvirus*-1 (EHV-

1), *Bovid alphaherpesvirus*-1 (BoHV-1), and avian herpesviruses *Gallid alphaherpesvirus* (GaHV) -2 (also referred to as Marek's disease herpesvirus or MDV), and GaHV-1 or infectious laryngotracheitis virus (ILTV).

GLYCOPROTEINS EXPRESSED BY ALPHAHERPESVIRUSES

Many of the genes encoded by herpesviruses are conserved among the *Herpesviridae*, while others are conserved among the subfamilies, and still others are unique for the respective virus. For example, glycoproteins (g)B, gL, gH, gM, and gN are conserved among all members of the *Herpesviridae*, while gD and gC are unique to the *Alphaherpesvirinae*. These glycoproteins are incorporated into the viral envelope and play important roles during cell free virus infection (Figure 1). Most of our knowledge on alphaherpesvirus glycoprotein roles and functions have been extensively studied based on HSV-1 that has led to the discovery of conserved and divergent functions among other alphaherpesviruses such as VZV, EBV, BoHV, pseudorabies virus (PRV), MDV, and ILTV. The functions of these glycoproteins are summarized in Table 1.

Herpesvirus envelope gB is one of the most studied glycoproteins among the *Alphaherpesvirinae*. HSV-1 gB has been shown to form the virus entry complex with the heterodimer gH/gL and gD to mediate cell fusion and entry of the viral capsid into the cell (3-15). gD is an essential glycoprotein for most of the *Alphaherpesvirinae*; however, VZV does not encode a gD homolog and MDV gD is dispensable for virus replication *in vitro* and in replication in chickens (16, 17). MDV gD expression appears to be silenced *in vitro* and it has been suggested its expression is restricted to the feather follicle epithelium (FFE) of infected chickens (18, 19), and is not required for horizontal transmission (20).

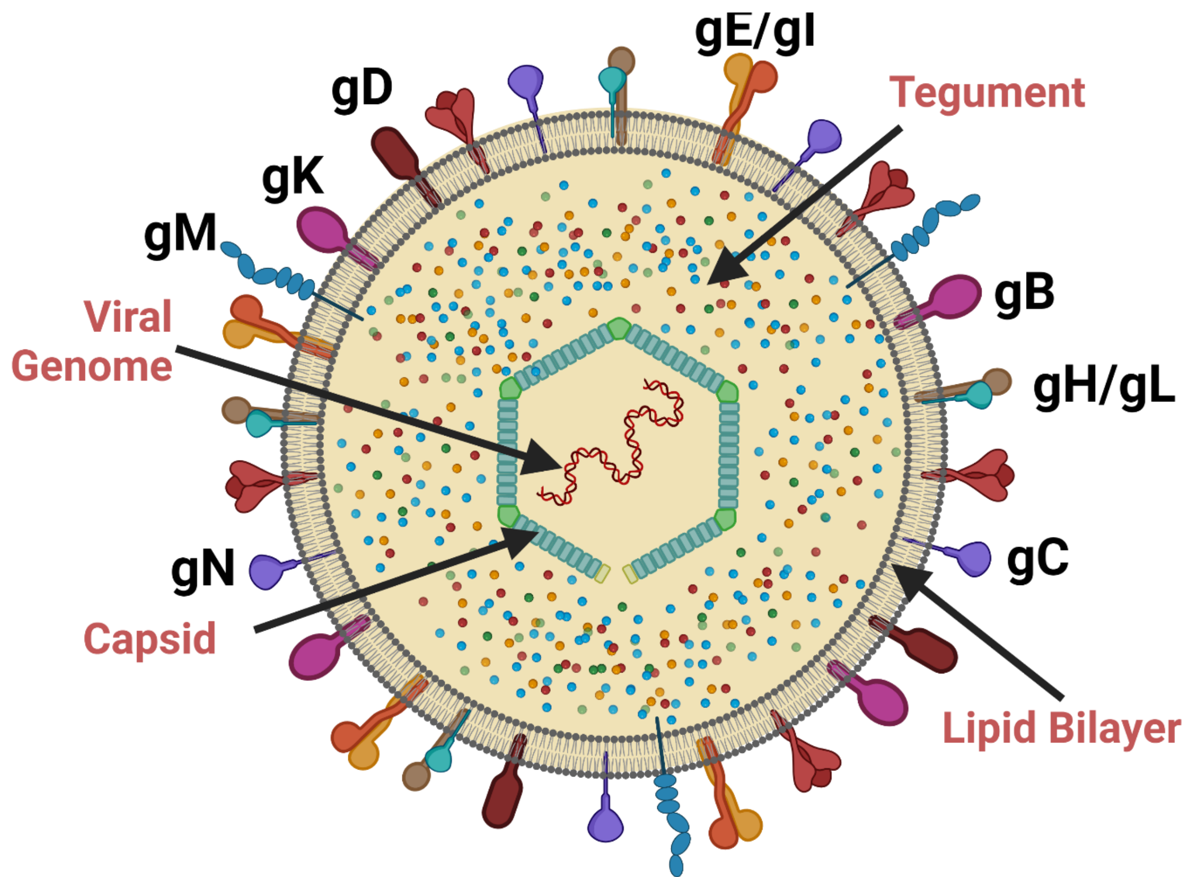


Figure 1. The alphaherpes virion. Schematic representation of the virion depicting its major components: the viral genome, capsid, tegument, and envelope (lipid bilayer membrane). Embedded in the envelope are the viral glycoproteins: gB, gC, gE/I, gH/L, gK, gN, and gM. Image was created with BioRender.com.

Additionally, gB of HSV-1, BoHV-1, and PRV has been shown to target extracellular vesicle activities and modify the architecture of early and late endosomes (8, 9, 21, 22). gE and gI have been reported to be essential for cell-to-cell spread of HSV-1, HSV-2, VZV and MDV (23-25); however, their importance in VZV replication may be dependent on the cell type used for propagation (26, 27). The gE/gI complex is thought to function in virus egress and cell-to-cell spread when it interacts with tegument proteins (28-30). Both glycoproteins are dispensable for replication of most other alphaherpesviruses (31-35). Furthermore, HSV gE/gI forms an immunoglobulin G (IgG) Fc receptor (FcγR) that binds the Fc domain of anti-HSV-1 IgG antibodies inhibiting Fc-mediated functions *in vitro* and *in vivo*. (36, 37). Similarly, the

requirement of gM and its complex partner, gN (or UL49.5), are not required for *in vitro* replication of HSV-1, BoHV-1, EHV-1, or PRV (38-43). Deletion of either gM or gN (UL49.5) in MDV results in virus defective in cell-to-cell spread (44).

Some alphaherpesviruses encode gG, (45-52) and gJ (53-55). gG is known to be a viral chemokine binding protein (vCKBP) that binds a broad range of chemokines with high affinity to inhibit their activity or to enhance chemokine-mediated migration (56-59). Interestingly, VZV and MDV do not encode gG (53-55). It is believed that VZV gC may play a similar role as gG in that it can function as a vCKBP that binds chemokines and promotes chemokine-dependent leukocyte migration (60). Just like gG, gJ is not encoded by both VZV and MDV (53-55). For some herpesviruses, gJ is believed to inhibit host apoptotic processes (61, 62). Additionally, gK of various alphaherpesviruses can interact with UL20 to function in virion envelopment, fusion, and neuronal entry. They have been shown to be essential for infectious virus production and subsequent spread (63-82).

Table 1. Glycoproteins conserved among the *Alphaherpesvirinae*.

	Gene	Type of Membrane Protein	Functions						Post-translational Modifications		
									Glycosylation		Phosphorylation
			HSV	PRV	EHV	BoHV	VZV	MDV	N-linked	O-linked	
gB	**UL27/ORF31**	**SPT**[α]	**Present** Required for attachment and fusion with the host cell. (3-9, 83, 84)						**Y**	**N**	**Y**
gC	**UL44/ORF14**	**SPT**[α]	**Present** Immune evasion by binding to complement component C3. Increases the efficiency of HSV-1 infection. (85-107)				**Present** Enhances chemokine-mediated migration. (60)	**Present** Required for horizontal spread. (20, 108, 109)	**Y**	**N**	**Y**
gD	**US6**	**SPT**[α]	**Present** Binds to the herpesvirus entry mediator (HVEM), nectin-1, and nectin-2. (10-17, 92, 110-115)				**Absent** (16)	**Present** gD is not required for MDV horizontal spread. (17-20, 116)	**Y**	**N**	**N**
gE	**US8/ORF68**	**SPT**[α]	**Present** Dimerizes to form a stable complex with gI. HSV gE/gI forms an IgG FcγR to inhibit Fc-mediated functions *in vitro* and *in vivo*. (23, 25-32, 35-37, 117)						**Y**	**N**	**Y**
gG	**US4**	**SPT**[α]	**Present** vCKBP that inhibits or to enhances chemokine-mediated migration. (45-52, 56-59)				**Absent** (53, 55)	**Absent** (54)	**Y**	**N**	**N**
gH	**UL22/ORF37**	**SPT**[α]	**Present** Required for fusion with the host cell. Dimerizes to form a stable complex with gL. (12, 118-121)						**Y**	**Y**	**N**

Table 1 (cont.). Glycoproteins conserved among the *Alphaherpesvirinae*.

gI	**US7/ORF67**	**SPT**[α]	**Present** Dimerizes to form a stable complex with gI. HSV gE/gI forms an IgG FcγR to inhibit Fc-mediated functions *in vitro* and *in vivo*. (23, 25-32, 35-37, 117)			**Y**	**N**	**N**
gJ	**US5**	**SPT**[α]	**Present** Promotes cell-to-cell spread and syncytia formation. Inhibits host cell apoptosis. (61, 62)	**Absent** (53, 55)	**Absent** (54)	**Y**	**N**	**N**
gK	**UL53/ORF5**	**MPT**[β]	**Present** Interacts with gB and regulates gB fusion properties with pUL20. (63-82, 122)			**Y**	**N**	**N**
gL	**UL1/ORF60**	**PM**[γ]	**Present** Required for fusion with the host cell. Dimerizes to form a stable complex with gH. (12, 118-121, 123, 124)			**Y**	**Y**	**N**
gM	**UL10/ORF50**	**MPT**[β]	**Present** Important for virion assembly and egress. Required for cell-to-cell spread for MDV. Directs gN to the host TGN (38-44, 125)			**Y**	**N**	**N**
gN	**UL49.5/ ORF9A**	**SPT**[α]	**Present** Necessary for maturation of gM and modulation of gM's membrane fusion activity. Important for the virion's morphogenesis. (38-44, 125-128)			**N**	**Y**	**N**

[α]**Single-Pass membrane protein (SPT) –** transmembrane protein that spans the membrane once and is characterized by having either its C-terminus (Type I) or its N-terminus (Type II) in the cytosol.

[β]**Multiple-Pass membrane protein (MPT) –** transmembrane protein that spans the membrane more than once.

[γ]**Peripheral membrane protein (PM) –** transmembrane protein that is associated with a membrane to which it will temporarily bind. They can attach to integral membrane proteins and/or penetrate the lipid bilayer.

MAREK'S DISEASE VIRUS

MDV is one of the avian herpesviruses categorized into the *Alphaherpesvirinae* subfamily and is the causative agent of Marek's disease (MD) initially characterized by the Hungarian veterinary pathologist Dr. József Marek (129). Although it can infect other avian species, the main host of MDV are members of the *Gallus* genus (i.e., chickens) (130). MD is a fatal disease in susceptible chickens that greatly affects the poultry industry (131). Some of the clinical signs in susceptible chickens affected by MD are lethargy, paralysis, and proliferation of lymphomas in visceral organs (130, 132, 133). Disease severity is based on many factors including genetics and vaccination status of the host, environmental parameters, and the virulence potential of the MDV strain.

The natural route of MDV infection is through the inhalation of infectious dust and dander previously shed on the poultry farm (Figure 2). During the initial infection, the virus enters the respiratory tract and infects primarily pulmonary macrophages and B cells. These innate immune cells are then responsible for transmitting the virus to lymphoid organs to initiate replication (134). Infected B cells will result in semi-productive viral replication, which leads to suppression of the humoral and cellular mediated immunity (135). Infected B cells interact and transmit the virus to T cells and establish latency in these cells that are able to reactivate and undergo transformation to form lymphomas (136). MDV infects FFE cells in the skin where fully-productive replication is completed, forming infectious cell-free virus particles that are shed into the environment that is required for dissemination (133).

Vaccination against MD has significantly reduced economic losses in the poultry industry. Current live-modified cell-associated vaccines, composed of attenuated strains of MDV and non-pathogenic HVT and GaHV-3, provide protection against the clinical signs of MD (*i.e.,* tumor

formation, paralysis, lethargy, etc.), but do not provide sterilizing immunity against the pathogen. It is accepted that failure to induce sterilizing immunity introduces selective pressure for the virus to evolve and evade the vaccine-induced immunity, causing the emergence of highly virulent strains (135, 137). Neonatal chicks are highly susceptible to MD, for this reason, cell-associated vaccines are administered when chicks have hatched or *in ovo*.

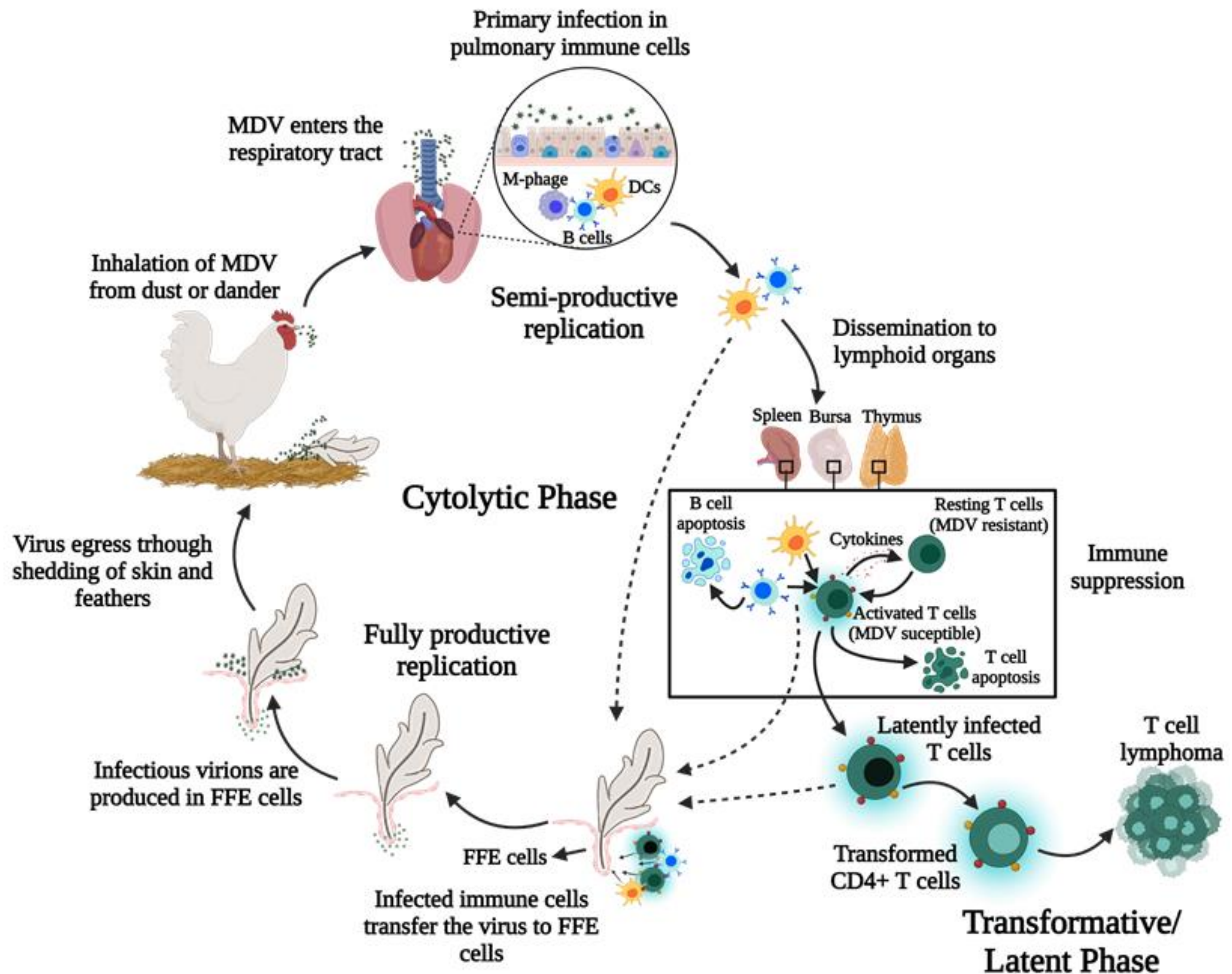


Figure 2. MDV Life Cycle. Schematic representation of the MDV life cycle. Image was created with BioRender.com.

CONSERVATION OF GLYCOPROTEIN C (gC) AMONG ALPHAHERPESVIRUSES

Classification of MDV as an alphaherpesvirus is mainly based on the similarities in genomic structure and conserved genes to HSV-1 (138). In addition, naming of genes encoded within MDV, GaHV-3, and HVT are based on the HSV system, where high conservation has been seen for both HSV and VZV (138, 139). Since then, sequencing of their genomes has led to a better understanding of their structure (140-144) (Figure 3).

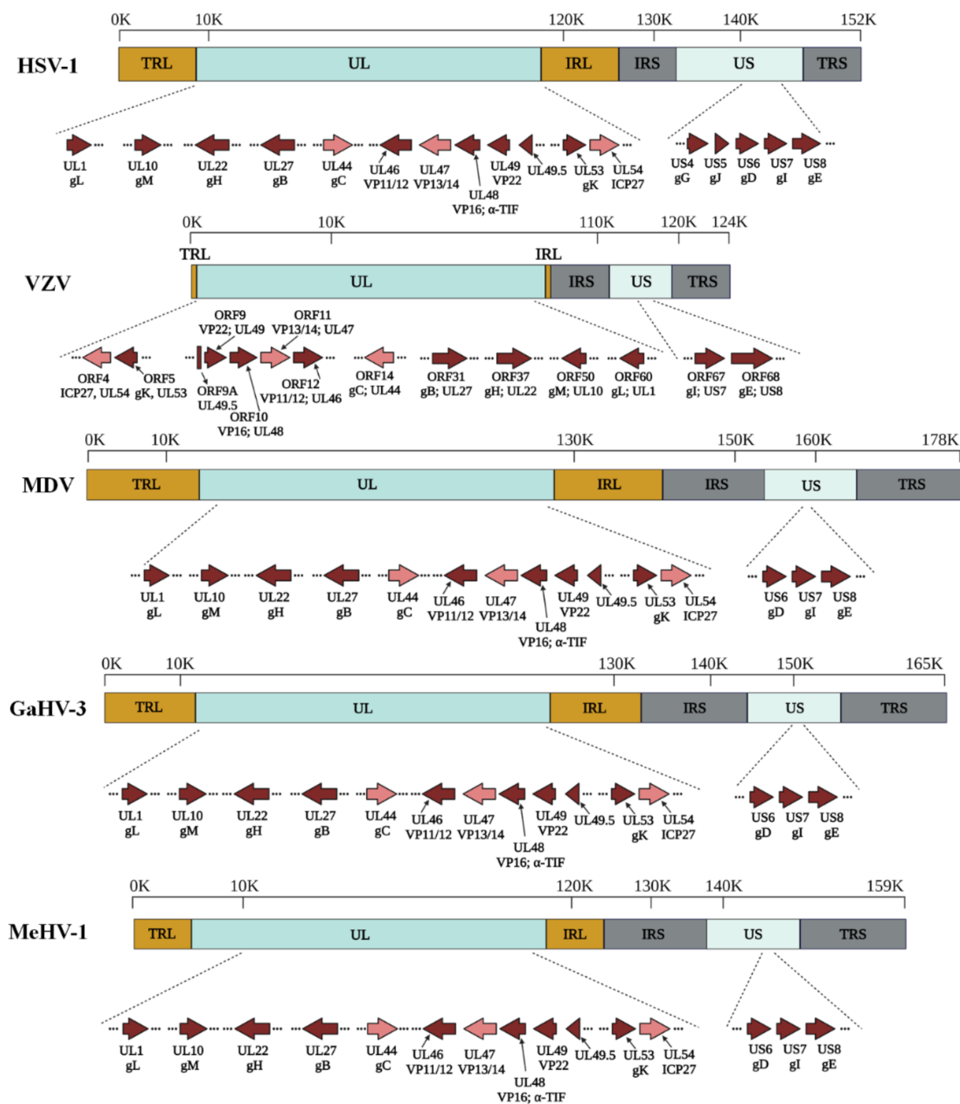


Figure 3. Human and avian alphaherpesvirus genomic structures. Schematic representation of the genomic differences among the different alphaherpesviruses. Sizes of genomic regions are not to scale. The UL and US regions are expanded to show differences in nomenclature and gene orientation. Image was created with BioRender.com.

The MDV genome is a linear double-stranded DNA composed of terminal and internal repeats denoted as terminal repeat long (TRL), terminal repeat short (TRS), internal repeat long (IRL) and internal repeat short (IRS) regions (Figure 4). These repeat sequences flank two unique sequences, a unique long (UL) and a unique short (US) sequence (145). In these sequences we can observe conserved genes present in all viruses in the family (146). However, as mentioned above, these genes have conserved and divergent functions dependent on the viruses. It is believed that the functions of these genes have evolved within their respective host in a host- or disease-specific manner. To gain understanding of the genes involved in host specific and pathogenesis functions, comparison of the virus' *in vitro* and *in vivo* replication characteristics needs to be understood. For example, a majority of the infectious HSV produced *in vitro* is cell-associated, however, HSV does produce infectious cell-free virus. In contrast, VZV is highly cell-associated *in vitro* and infectious virus does not egress as cell-free virus, but is instead within, or attached to, the cell (147). Considering some of the previously mentioned viruses, with respect to their cell-associated nature, members of the *Mardivirus* genus are similar to those in the *Varicellovirus* genus, while members of the *Iltovirus* genus are similar to viruses in the *Simplex* genus.

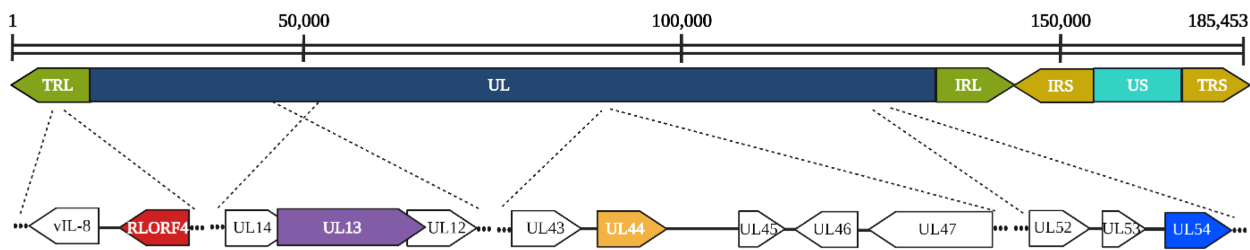


Figure 4. MDV genomic structure. Schematic representation of the MDV genome showing the TRL and IRL, TRS, IRS, UL, and US regions.

The gC envelope glycoprotein is conserved in the *Alphaherpesvirinae*. Research on gC has been focused on its functions and roles in viral pathogenesis based on its immunogenicity and high expression levels for the last few decades (148-151). It has long been known that

expression of MDV gC *in vitro* is not essential, and can actually be inhibitory for viral replication, as serial passaging in tissue culture results in decreased expression of gC and increased plaque sizes (108, 152, 153). Interestingly, the sequence of the open reading frame (ORF) encoding gC in attenuated strains with severely decreased or deficient gC expression show no apparent changes in gene or promoter sequences that would explain this phenomenon (154). Deletion mutants, in which the complete ORF of gC or mutation of the gC start codon have shown that MDV lacking gC have significantly increased cell-to-cell spread, while MDV "over-expressing" gC using high expressing promoters decreases cell-to-cell spread (151). The direct role gC plays in modifying or inhibiting cell-to-cell spread in tissue culture is not currently known.

gC homologues have been shown to be involved in binding of the virus to host cells. For HSV-1, HSV-2, PRV and BoHV-1, the initial interaction with cells involves gC binding to cell surface glycosaminoglycans like heparan (85-95). However, the presence of gC is not essential for a successful viral infection, where gB/gH/gL play the required role for HSV fusion and entry (96-98). In addition to interaction with cell surface molecules, the gC homologues for EHV-1 has been linked to post-entry events, such as virus egress or release of infectious virions (99). Furthermore, gC of other herpesviruses have been shown to usurp the immune system for their own benefit inhibiting the alternative complement pathway. The gC proteins of HSV-1, PRV, BoHV-1, and EHV-1 bind to complement component C3 and inhibit complement-mediated virus neutralization and lysis of infected cells (100-105). Additionally, studies in C3 reconstituted and knockout mice during HSV-1 infection confirmed gC's importance as a complement activation inhibitor (106). Huemer et al. (104), observed gC proteins' inhibition of C3 among several

animal alphaherpesviruses in a species-specific manner. This species-selective interaction could likely be due to the evolution of herpesviruses within their hosts.

Through the use of our natural virus-host system, where chickens are experimentally infected and housed with naïve birds to test natural infection, it was determined that MDV gC, and another gene (UL13) were essential for horizontal transmission of the virus (20, 108). The precise mechanisms by which MDV gC facilitates transmission has not been elucidated to date. However, gC is not important for replication of the virus within the chicken during experimental infection, where gC-negative MDV is inoculated directly into the chicken, replicates, and causes disease like wild-type MDV. Therefore, the essential role gC provides MDV is during horizontal transmission of the virus.

The purpose of the research described in this thesis is to expand our understanding of the essential role MDV gC plays during horizontal transmission. In particular, the regulatory and mechanistic roles that avian gC proteins play in this process. By understanding these functions, we believe we could generate better vaccines and therapies to block MDV spread in the flock.

CHAPTER 2: MAREK'S DISEASE HERPESVIRUS (MDV) RLORF4 IS NOT REQUIRED FOR EXPRESSION OF GLYCOPROTEIN C AND HORIZONTAL TRANSMISSION[1]

ABSTRACT

Marek's disease virus (MDV) is a lymphotropic alphaherpesvirus that causes Marek's disease (MD) in chickens. RLORF4 is a MDV-specific gene that is systematically deleted during attenuation of MDV *in vitro*. Concomitantly, the expression of glycoprotein C (gC) is diminished during attenuation, suggesting these two changes may be linked. Original studies in which RLORF4 was deleted utilized an infectious clone that lacked gC expression due to a frame-shift mutation within the gC open reading frame (UL44). Here, we utilized an infectious clone in which gC expression was restored to test our hypothesis that RLORF4 is important for expression of MDV gC, and subsequently, horizontal transmission. Contrary to our hypothesis, gC expression was unaltered during both *in vitro* and *in vivo* replication of RLORF4-null MDV and was able to efficiently transmit from chicken to chicken, conclusively showing that RLORF4 does not regulate gC expression and is not required for horizontal transmission.

INTRODUCTION

Marek's disease (MD) in chickens is caused by *Gallid alphaherpesvirus* 2 (GaHV-2), better known as Marek's disease herpesvirus (MDV). The most prominent sign of MD is the development of solid lymphomas in the viscera and other organs (155). Natural infection begins

[1] This chapter has been previously published on *Virology* 534, pp.108-113.
Vega-Rodriguez, W., Ponnuraj, N. and Jarosinski, K.W., 2019. Marek's disease alphaherpesvirus (MDV) RLORF4 is not required for expression of glycoprotein C and horizontal transmission. As per the Journal's specification, the author retains the right to reuse this article as a chapter in this thesis without the need to provide signed documentation.

through the respiratory route by inhalation of infectious virus where the virus initially infects pulmonary B lymphocytes and/or is taken up by pulmonary macrophages or dendritic cells (156) and transported to lymphoid organs. Following the initiation of primary cytolytic infection in the host, activated T lymphocytes recruited to the sites of infection become the primary cell type infected and latency can be established in these cells, where some can also undergo oncogenic transformation resulting in lymphoma formation. This event is ultimately a dead end for the virus. Important for dissemination in the population, migrating infected cells transport virus to feather follicle (FF) epithelial (FFE) cells in the skin, where infectious virus is shed into the environment and the virus life cycle can repeat in new hosts.

Previously referred to as the "A-antigen," MDV glycoprotein C (gC) is normally expressed at high levels during *in vitro* and *in vivo* propagation, but its expression is greatly reduced after 30-50 serial passages in tissue culture cells (108, 148-153). This decreased expression coincides with increased plaque sizes and attenuated characteristics. Wilson et al. (154) examined the relationship between gC expression and attenuation and found that although gC levels were related to decreased levels of gC mRNA produced by attenuated MDV, there were no alterations in the UL44 (gC) open reading frame or promoter region suggesting a MDV-encoded regulatory protein may regulate its expression.

The RLORF4 gene is unique to MDV and its role in attenuation was originally identified as a gene deleted following serial passage in cell culture cells (157-160). Formerly identified as a hypothetical reading frame within the repeat long (RL) regions of the MDV genome, the mRNA of RLORF4 was identified during both lytic and latent infection (157) as a single transcript encoding a 142 amino acid protein in wild-type strains. Further studies confirmed its expression at the protein level during lytic replication (161). Common among genes expressed within the

RL regions of MDV, multiple mRNA splice variants were identified between RLORF4, viral interleukin 8 (vIL-8), and the MDV oncoprotein, Meq (162); however, the functional importance of these transcripts is currently not known. Following the generation of an infectious clone of the RB-1B strain as a bacterial artificial chromosome (BAC), further experiments confirmed that deletion of RLORF4 within this BAC clone resulted in highly attenuated virus *in vitro* and *in vivo* (163). In this report, both wild-type and ΔRLORF4 viruses were unable to transmit to naïve contact chickens. This BAC clone was subsequently found to contain numerous frame-shift mutations (164), of which mutations within UL44 (gC) and UL13 protein kinase (CHPK) abrogated MDV spread from chicken to chicken (20, 108).

Since RLORF4 is consistently deleted during serial passage and expression of MDV gC is significantly reduced, we postulated that RLORF4 is important for gC expression and subsequently, horizontal transmission of MDV. However, this hypothesis could not be tested in the original studies due to a frame-shift mutation in UL44 (gC). Here, we tested this hypothesis using recombinant (r)MDV that expresses gC and is transmission competent. Contrary to our hypothesis, deletion of RLORF4 within a fully restored rMDV did not affect gC expression *in vitro* and *in vivo*, and RLORF4 played no role during horizontal transmission of MDV. These results directly address an important question in gC regulation *in vitro* and *in vivo* and conclusively show that RLORF4 is not involved in gC expression as originally hypothesized. In addition, the level of attenuation was not as dramatic as previously seen; indicating the severe attenuation previously reported was most likely due to compounding mutations within the rMDV used in that study.

MATERIALS AND METHODS

Cell culture and cells

Chicken kidney cells (CKCs) were prepared from 2-4 weeks-old specific-pathogen-free (SPF) chickens obtained from the University of Illinois at Urbana-Champaign (UIUC) Poultry Farm following standard methods (165). Briefly, primary CKCs were seeded in growth medium consisting of Medium 199 (Cellgro, Corning, NY, USA) supplemented with 10% tryptose-phosphate broth (TPB), 0.63% $NaHCO_3$ solution, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 4% fetal bovine serum (FBS). Confluent CKCs were maintained in F10.199 medium consisting of a 1:1 mixture of Ham's F10 (Cellgro) and Medium 199 supplemented with 7.5% TPB, 0.63% $NaHCO_3$, 0.2% FBS, and antibiotics. CKCs were maintained at 38°C in a humidified atmosphere of 5% CO_2.

The chicken DF-1-Cre fibroblast cell line (166) was cultivated in a 1:1 mixture of Leibovitz L-15 and McCoy 5A (LM) media (Gibco, Gaithersburg, MD, USA) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and maintained in 50 µg/ml Zeocin (Invitrogen, Carlsbad, CA). DF-1-Cre cells were maintained at 38°C in a humidified atmosphere of 5% CO_2.

Generation of ΔRLOF4 and ΔgC rMDVs

Coding sequences of RLORF4 and UL44 (gC) were deleted from a previously described fully virulent BAC clone (167) using two-step Red-mediated mutagenesis (168). Briefly, the I-*Sce*I-*aphAI* cassette from pEP-KanSII (169) was amplified by PCR with Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix using primers shown in Table 2 and used for mutagenesis in GS1783 *Escherichia coli* cells. Restriction fragment length polymorphism

(RFLP) analysis, analytical PCR, and DNA sequencing confirmed all clones were correct. Primers used for sequencing have been previously published (109, 157, 162).

Table 2. Primers used for generation of recombinant Marek's disease virus (rMDV).

Primer Name	Direction[a]	Sequence (5' → 3')[b]
ΔRLORF4	Forward	GTATATAGCGCAAGCGCGCAGGGCTGGTTCGGGTAAGGCGTTCACGCTAGTTTATGCCCCATCGTAGGGATAACAGGGTAATCGATTT
	Reverse	GATGCATTTTGTTTATTGAAAATTTCCATTCGATGGGGCATAAACTAGCGTGAACGCCTTACCCGCCAGTGTTACAACCAATTAACC
ΔgC	Forward	CATCCCGAAGAGACACCAAACGTAACCCTCTACATATCTTCCCTCTAATCTCATTGTTATGTAGTTTAGGGATAACAGGGTAATCGATTT
	Reverse	GAGTTATAAAAAATATGTTTAATAAATCACAACTACATAACAATGAGATTAGAGGGAAGATATGTAGCCAGTGTTACAACCAATTAACC

[a]Directionality of the primer.
[b]Bold nucleotides indicate stop codons for each respective gene and underlined sequences are priming sites within the mutagenesis template plasmid pEP-KanS.

rMDVs were reconstituted by transfecting DF-1-Cre cells, which efficiently remove the mini-F BAC sequences from the viral genome, with purified BAC DNA plus Lipofectamine 2000 (Invitrogen) using the manufacturers' instructions. Transfected DF-1-Cre cells were mixed with fresh primary CKCs until plaques formed, then further propagated in CKCs until virus stocks could be stored. All rMDVs were used at ≤ 5 passages for *in vitro* and *in vivo* studies.

Measurement of plaque areas

Plaque areas were measured as previously described (163). Briefly, CKCs were seeded on 6-well dishes and infected with 100 plaque-forming units (PFU) per well. After 5 days, cells were washed once with phosphate buffered saline (PBS), fixed and permeabilized with PFA buffer (2% paraformaldehyde, 0.1% Triton X-100) for 15 min, and washed twice with PBS.

Immunofluorescence assays (IFAs) were performed as previously described (163) using anti-MDV chicken sera and goat anti-chicken IgY-Alexa Fluor® 568 secondary antibody (Molecular Probes, Eugene, OR). Digital images of 50 to 75 individual plaques were collected using an EVOS™ FL Cell Imaging System (Thermo Fisher Scientific) and compiled using Adobe® Photoshop® CC 2015 version 7 SP1. Plaque areas were measured using ImageJ (170) version 1.51j8 software, and means were determined for each plaque population. Significant differences in mean plaque areas were determined using Student's *t* tests assuming equal variances in Microsoft® Excel 2016.

Ethics statement

All animal work was conducted at UIUC according to national regulations. The animal care facilities and programs of UIUC meet the requirements of the law (89 –544, 91–579, 94 –276) and NIH regulations on laboratory animals and are in compliance with the Animal Welfare Act, PL 279. The College of Veterinary Medicine at UIUC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures were in compliance with approval of UIUC's Institutional Animal Care and Use Committee.

***In vivo* experiment**

Commercial Pure Columbian × New Hampshire chickens were obtained from the UIUC Poultry Farm (Urbana, IL) and were from MD-vaccinated parents; therefore, considered to be maternal antibody positive. All experimental procedures were conducted in compliance with approved Institutional Animal Care and Use Committee protocols. Water and food were provided *ad libitum*. Five-day old chicks were experimentally infected by intra-abdominal inoculation of 2,000 PFU for each rMDV and housed in separate rooms (n = 8/group). For each

group, another 11 chickens were left uninfected to act as contacts to determine whether rMDVs were able to naturally infect naïve chickens by horizontal transmission. Chickens were evaluated for symptoms of MD daily, euthanized when birds showed clinical signs of MD (*e.g.*, lethargy, depression, paralysis, torticollis, etc.), and examined for gross MD lesions by a veterinarian blinded to the groups. Chickens positive for MD included birds succumbing to disease prior to the experimental termination date and birds positive for MD-related lesions at termination of the experiment. Fisher's exact tests were used to determine statistical differences between groups of chickens for MD incidence at a significance level of $p<0.05$.

DNA extraction from blood cells and qPCR assays

Whole blood was collected as previously described (20), and DNA was extracted using the E.Z. 96 blood DNA kit from Omega Bio-tek, Inc. (Norcross, GA). To quantify MDV genomic copies in blood, 2× Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific) was performed as previously described (171). Standard curves were generated for MDV ICP4 and chicken GAPDH using previously described templates (163) starting with approximately 500 pg of DNA. Total copy numbers were determined as previously described (172) using the C_T value for that sample. The coefficient of regression was always >0.99 for standard curves. Thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All qPCR assays were performed using an Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) and the results were analyzed using QuantStudio™ Design & Analysis Software v1.4.2 supplied by the manufacturer. Significant differences in MDV genomic copies at each time point were determined using Student's *t* tests assuming equal variances at a significance level of $p<0.05$ using Microsoft® Excel 2016.

Monitoring rMDV in feather follicles (FFs)

To track the time at which each rMDV reached the FFs, two flight feathers were plucked from the right and left wings (4 total) of inoculated birds weekly beginning at 7 dpi, and UL47eGFP expression was examined using a Leica M205 FCA fluorescent stereomicroscope with a Leica DFC7000T digital color microscope camera (Leica Microsystems, Inc., Buffalo Grove, IL). Feather plucking for all birds was discontinued after 48 dpi, since only a few experimentally infected birds remained.

Fluorescent microscopy of FFs

Whole feathers were plucked from chickens infected with different rMDVs and the FFs were fixed using PFA buffer, washed twice with PBS, and then blocked in 10% neonatal calf serum. Fixed FFs were stained with primary anti-gC monoclonal A6 (kindly provided by Jean-Francois Vautherot, INRA, Nouzilly, France) antibody (151) and anti-mouse Ig Alexa Fluor 568 (Molecular Probes, Eugene, OR) was used as secondary antibody. The Leica M205 FCA fluorescent stereomicroscope with a Leica DFC7000T digital color microscope camera (Leica Microsystems, Inc., Buffalo Grove, IL) was used to analyze stained FFEs. All images were compiled using Adobe® Photoshop® CC, 2017.0.1 release.

Western blot analysis

Western blot analyses were performed as previously described (151). To detect relative level of MDV infection, MAb H19 (149) was used at 1:10,000 dilution to detect MDV pp38 protein. To detect gC, MAb A6 was used at a 1:500 dilution. For protein loading control, anti-β-actin (ACTNO5; Abcam, Cambridge, MA) MAb was used at its recommended dilution. Secondary anti-mouse IgG peroxidase conjugate was purchased from GE Healthcare (Piscataway, NJ). The SuperSignal West Pico Chemiluminescent Substrate kit from Thermo

Fischer Scientific (Rockford, IL) was used to detect antigens using the manufacturer's instructions.

RESULTS

Generation of rΔRLORF4 and rΔgC rMDV

In order to test the ability of RLORF4 to regulate gC expression, the complete RLORF4 open reading from was deleted from a previously described fluorescent rMDV (vUL47eGFP) that is fully transmissible among chickens (167) using two step Red-mediated recombination (169). Since there are two copies of RLORF4 located in the RL regions, two rounds of integration and resolution were required. First, RLORF4 was removed within the internal RL (IRL) and then subsequently removed from the terminal RL (TRL) to generate vΔRLORF4 (data not shown). Figure 5A shows a schematic representation of parental clone rUL47eGFP and the RLORF4-null (rΔRLORF4) clone. As a control for gC expression and downstream studies, rΔgC was generated in which the complete UL44 gene encoding gC was removed using two-step Red-mediated recombination (Figure 5B). RFLP analysis of rUL47eGFP, rΔRLORF4, and rΔgC confirmed the integrity of the BAC clones as the predicted banding patterns were observed (Figure 5C). PCR and DNA sequencing was used to confirm that each clone was correct at the nucleotide level (data not shown) using previously described primers (157, 162).

Deletion of RLORF4 results in increased MDV replication in tissue culture

Following reconstitution of rUL47eGFP and rΔRLORF4 resulting in vUL47eGFP and vΔRLORF4, respectively, we first tested *in vitro* replication properties using plaque size assays. We hypothesized that deletion of RLORF4 would result in increased plaque sizes, similar to former studies using rMDV that lacked expression of gC and UL13 protein kinase (163). Consistent with the former work, RLORF4-null rMDV generated increased plaque sizes

compared to the vUL47eGFP (Figure 6). Also consistent with former studies (20, 108, 109), vΔgC generated significantly larger plaques than vUL47eGFP, but there was no difference between vΔRLORF4- and vΔgC-generated plaque sizes. These results show that even in the restored RB-1B rMDV, deletion of RLORF4 results in increased plaque sizes (replication) that is indicative of attenuation *in vitro* (158, 159, 163).

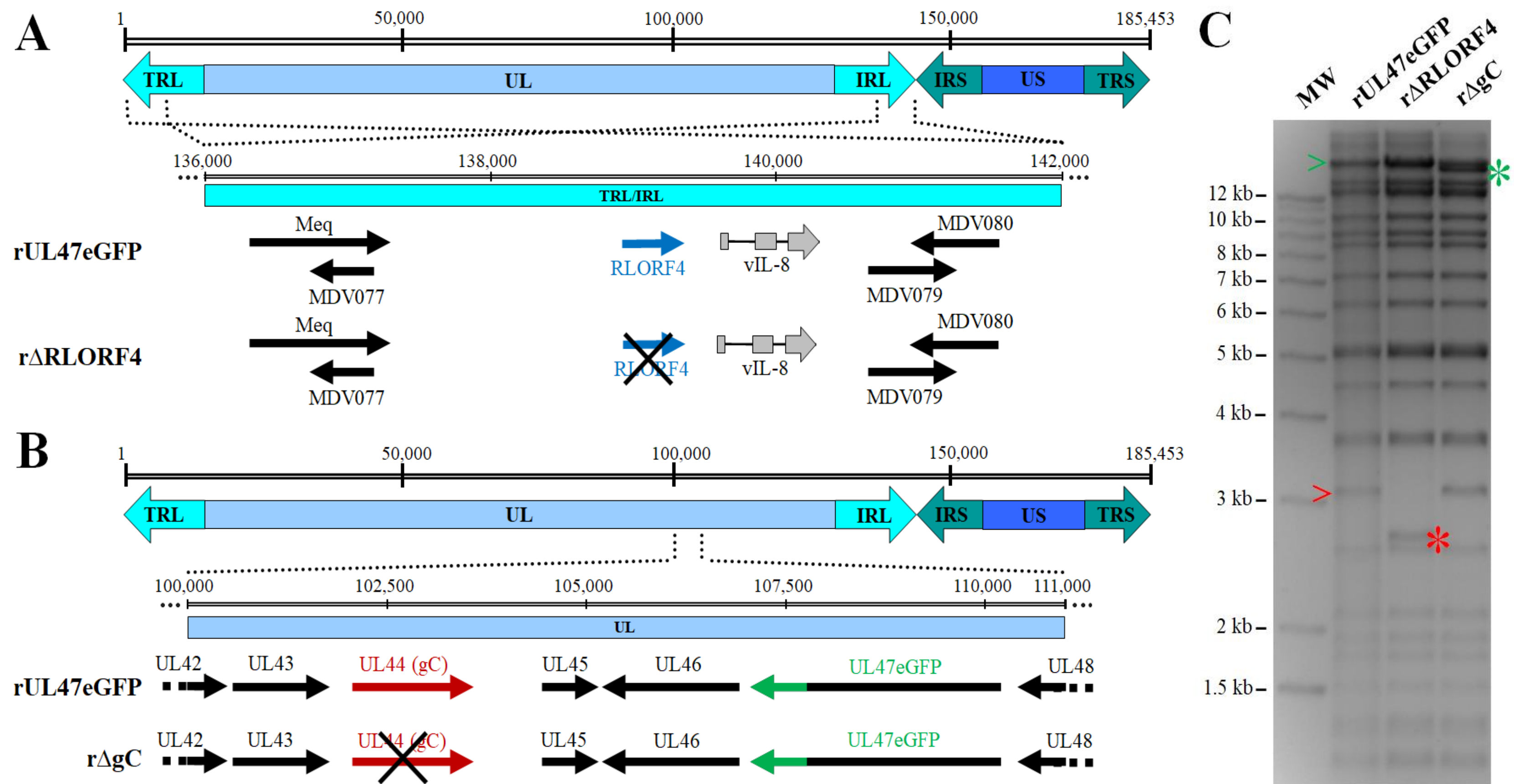


Figure 5. Generation of rMDVs. (A) Schematic representation of the MDV genome depicting the locations of the terminal repeat long (TRL) and short (TRS), internal repeat short (IRS), and unique long (UL) and short (US) regions. The TRL/IRL region is expanded to show the relevant genes within this region and deletion of RLORF4 in the rΔRLORF4 rMDV. (B) Schematic representation of the MDV genome with the UL region spanning nucleotides 100,000 to 111,000 expanded to show relevant genes within this region, including eGFP tagged UL47 (UL47eGFP) and removal of gC (vΔgC). (C) BAC DNA obtained for rUL47eGFP, rΔRLORF4, and rΔgC were digested with BamHI and examined using RFLP analysis. Integrates are excluded for simplicity. Removal of both copies of RLORF4 results in shifting of the BamHI-L fragments of rUL47eGFP (>) from 3,098 bp to 2,672 bp (*) in vΔRLORF4. Removal of the UL44 ORF (gC) results in a shift of the BamHI-A fragment from 18,748 in rUL47eGFP (>) down to 17,245 bp in rΔgC (*). The molecular weight marker used was the 1 kb Plus DNA Ladder from Invitrogen, Inc. (Carlsbad, CA). No extraneous alterations are evident.

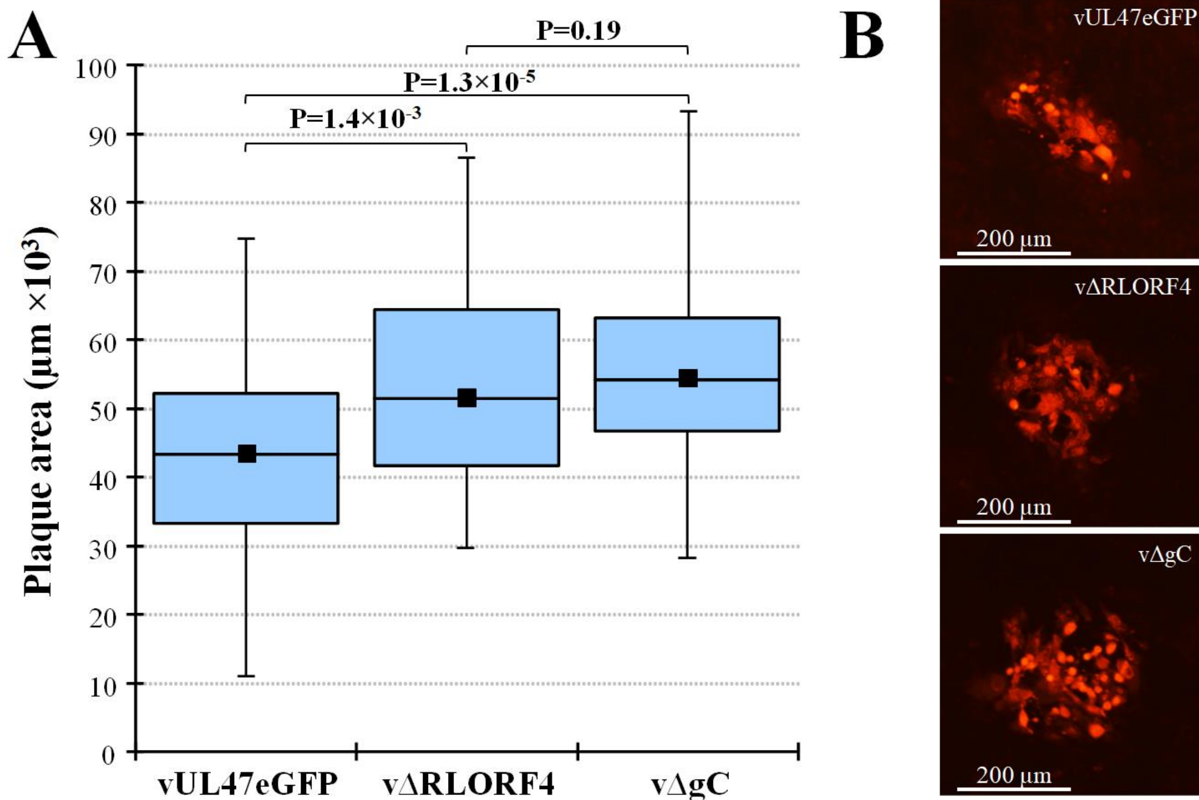


Figure 6. Replication in tissue culture cells. (A) Plaque areas were measured for viruses reconstituted from rUL47eGFP (vUL47eGFP), rΔRLORF4 (vΔRLORF4), and rΔgC (vΔgC). Both vΔRLORF4 and vΔgC generated significantly larger plaque areas than the parental vUL47eGFP using Student's *t* tests. (B) Representative plaques induced by vUL47eGFP, vΔRLORF4, and vΔgC are shown below each group.

RLORF4 is not required for horizontal transmission

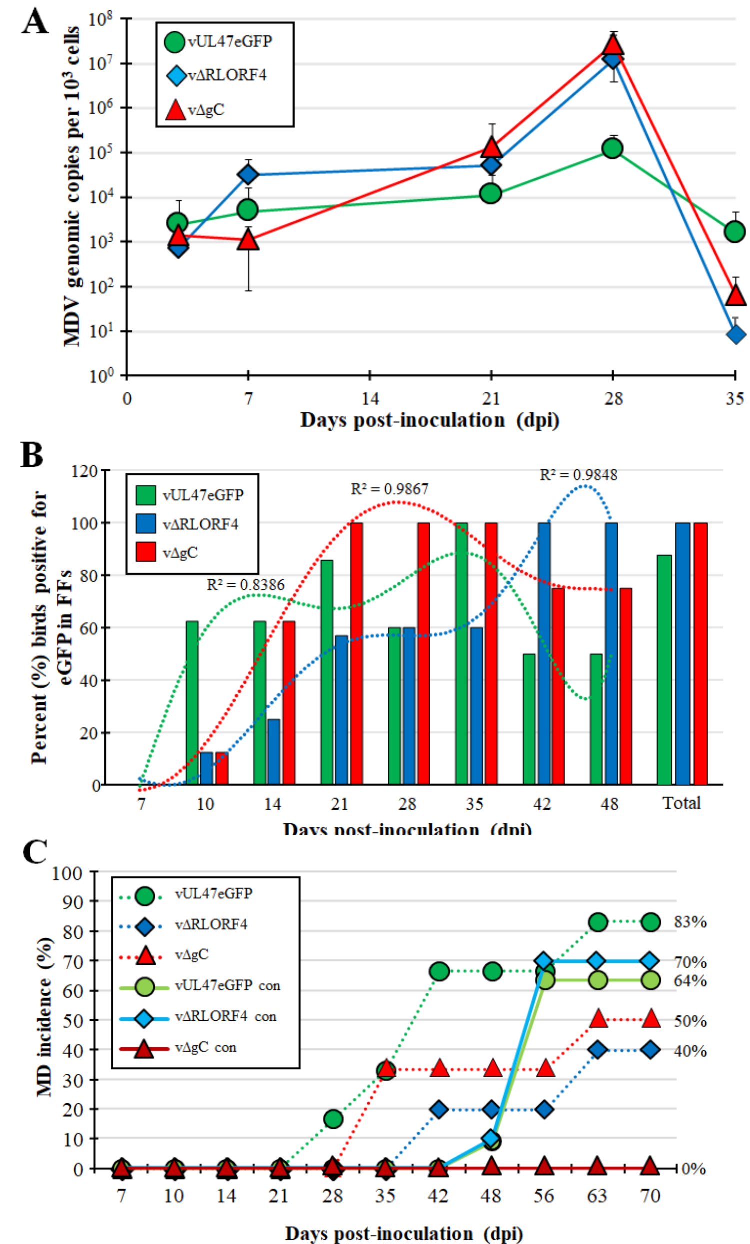
Although RLORF4 has been shown to be important for pathogenesis of transmission-deficient MDV (163), its importance during *in vivo* attenuation and horizontal transmission has not been evaluated in transmission-competent MDV. To test *in vivo* replication, chickens were inoculated with 2,000 PFU of vUL47eGFP, vΔRLORF4, or vΔgC and naïve contact chickens were housed with experimentally infected birds to evaluate horizontal transmission through natural infection. A third group vΔgC groups was included as a negative control for horizontal transmission and gC expression.

MDV genomic copies were measured in blood from experimentally infected chickens over 35 days pi and showed no significant difference between all groups at each time point (Figure 7A). In order to determine whether there was a difference in each virus reaching the FFs required for transmission, FFs were plucked from each infected bird and expression of vUL47eGFP in FFs was observed using a fluorescent stereoscope. Figure 7B shows that each virus readily reached the FFs with 100% of vΔRLORF4 and vΔgC infected chickens having positive FFs by 48 days, while 88% of vUL47eGFP birds were positive. There was no significant difference between each group, though vUL47eGFP tended to reach the FFs faster than both vΔRLORF4 and vΔgC.

MD incidence was determined for each group in both experimentally infected and contact chickens. MD was induced in 83, 40, and 50% of chickens experimentally infected with vUL47eGFP, vΔRLORF4, and vΔgC, respectively (Figure 7C); however, these were not significant using Fisher's exact test. These results show that there were no significant differences between each group in virulence when birds were experimentally infected.

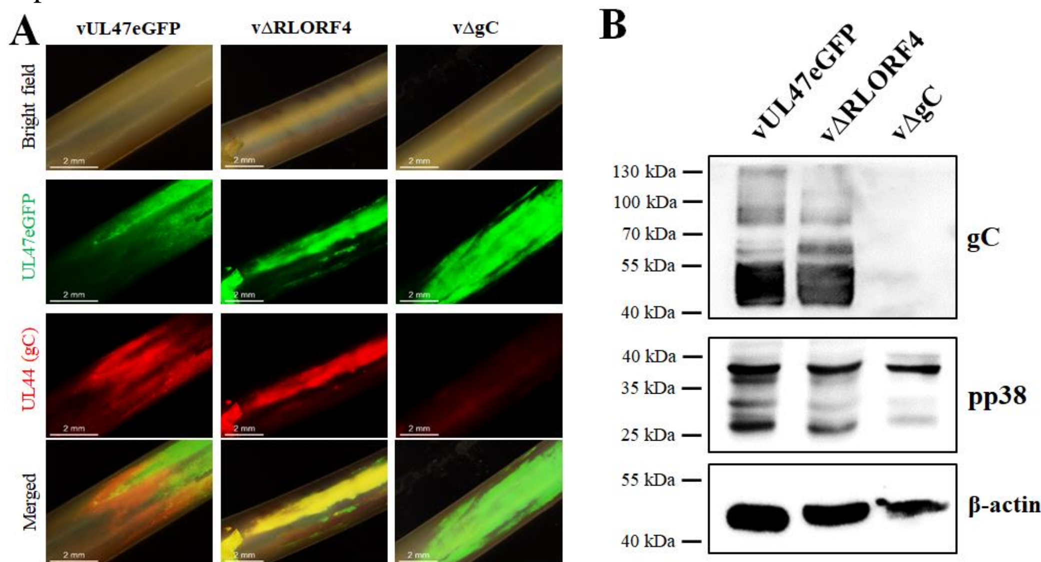
No contact chickens housed with vΔgC (vΔgC con) developed MD over the course of 70 days and these birds were determined to be uninfected based on a lack of viral DNA in the blood using qPCR assays and anti-MDV antibodies in their serum using IFAs at termination of the experiment (data not shown). This is consistent with the requirement of MDV gC for horizontal transmission in chickens (20, 108, 109). In contrast, vUL47eGFP and vΔRLORF4 were able to efficiently infect contact chickens with 64 and 70%, respectively, developing MD. These result show that RLORF4 is not required for horizontal transmission or natural infection of MDV. In all, these results show that deletion of RLORF4 in a fully virulent, transmission-restored rMDV is not required for horizontal transmission of MDV.

Figure 7. Replication, MD incidence, and transmission of rMDVs in chickens. Pure Columbian × New Hampshire chickens were experimentally infected with vUL47eGFP, vΔRLORF4, or vΔgC as described in the Materials and Methods (n = 8/group) for 70 days. (A) Replication was monitored in experimentally infected chickens by quantification of genomic MDV genomes in the blood over the first 5 weeks of infection. Shown is the mean MDV genomic copies per 10^3 blood cells ± standard error of means. No significant differences were observed between all groups and time points using Student's *t* tests. (B) Quantitative analysis of the percent of birds with rMDV in the FFs (UL47eGFP) over the course of the experiment, including the total number of birds over the course of the experiment. Using One-Way ANOVA, there was no significant difference in the total number of chickens positive for rMDV in the FFs (*f*-ratio value = 1.20103; *p*-value = 0.327724). Trend lines for each group were added using Microsoft Excel using the polynomial setting at an order of 6 and R^2 values for each trend line are shown. (C) Total MD incidence was determined by identification of gross lesions in euthanized chickens in both experimentally- and naturally- (contact) infected chickens. Although MD incidence induced by vΔRLORF4 (40%) and vΔgC (50%) in experimentally chickens was much lower than vUL47eGFP (83%), these differences were determined to be not significant using Fisher's exact test at $p < 0.05$ (vUL47eGFP vs. vΔRLORF4, $P = 0.2424$; vUL47eGFP vs. vΔgC, $P = 0.5455$). In contact chickens naturally infected, 64 and 70% of chickens housed with vUL47eGFP (vUL47eGFP con) or vΔRLORF4 (vΔRLORF4 con) developed MD, while 0% of contact chickens housed with vΔgC (vΔgC con) developed MD. There was no significant difference between vUL47eGFP con and vΔRLORF4 con groups, while the vΔgC con group was significantly different between vUL47eGFP ($P = 0.0039$) and vΔRLORF4 ($P = 0.0031$).



RLORF4 is not required for gC expression in FFE cells

Our data thus far show that RLORF4 is not important for replication and chicken-to-chicken transmission of MDV. We originally hypothesized that RLORF4 was important for gC expression and subsequently host-to-host transmission; however, our data suggests this is not the case. To address our original question directly, we used IFA and western blotting to evaluate gC expression in FFE cells of infected chickens. Feathers plucked from infected chickens were fixed and stained using anti-MDV gC antibody. Figure 8A shows both vUL47eGFP- and vΔRLORF4-infected FFs abundantly express gC, while vΔgC-infected FFs were negative for gC protein. Figure 8B shows western blotting for gC in FFE cells scraped from FFs, consistent with IFA staining of FFs. Thus, RLORF4 does not affect gC expression in FFs which is consistent with it being dispensable for horizontal transmission in chickens.



[2]**Figure 8. Expression of gC in FFs of infected chickens.** (A) Feathers were plucked from vUL47eGFP-, vΔRLORF4-, and vΔgC-infected birds at 28 dpi, fixed, then stained using anti-MDV gC antibody. FFs obtained from vUL47eGFP- and vΔRLORF4-infected chickens were positive for gC protein, while FFs from vΔgC-infected birds were negative. (B) Western blot analysis for gC in FFEs. Whole cell protein lysates were collected from FFE cells scraped from FFs infected with vUL47eGFP, vΔRLORF4, or vΔgC, electrophoresed through a 15% SDS-PAGE gel, transferred to nitrocellulose membranes, and probed for MDV gC as described in the Materials and Methods. Anti-β-actin antibody was used as internal cellular control.

[2] Figure 8A was generated by Dr. Nagendra Prabhu Ponnuraj.

DISCUSSION

In this report, we tested the importance of RLORF4 for expression of gC and horizontal transmission of MDV. Based on former data from multiple laboratories, we hypothesized that the decrease in expression of MDV gC and inability of attenuated MDV to transmit from chicken-to-chicken following passage *in vitro* was directly due to deletion of RLORF4. However, the most direct study of RLORF4 in attenuation used a rMDV that lacked gC expression and was unable to transmit (163). To address the role RLORF4 has on gC expression and, consequently, chicken-to-chicken transmission, gC and transmission competent rMDV was used in this report.

Deletion of both copies of RLORF4 resulted in increased plaque sizes *in vitro*, which was consistent with attenuated nature of MDV (163) and rMDV lacking gC (vΔgC) generated similar plaque sizes as vΔRLORF4. In former studies, it was presumed the increased plaque sizes seen with attenuated MDV was because these viruses lacked RLORF4, and subsequently, gC expression was ablated, resulting in larger plaques sizes. However, our results show that the increased plaque sizes induced by viruses lacking RLORF4 or gC are independent of each other, thus RLORF4 enhances replication in tissue culture cells through a mechanism independent from gC.

In chickens experimentally infected with rMDV, no significant differences were observed between each group for replication or MD incidence (Figure 7). This is consistent with former studies on MDV gC playing no role in replication and MD incidence in experimentally infected chickens (20, 108, 109). However, our previous studies showed that RLORF4 was important for replication and disease induction in experimentally infected chickens (163). Although disease induction was reduced from 83% for vUL47eGFP- down to 40% for vΔRLORF4-infected chickens (Figure 7C), this was not a significant decrease and therefore suggest no attenuation.

There are at least three potential reasons why the level of MD incidence was not significant. First, it could be due to the low number of experimentally infected birds for each group (n = 5), since our primary goal was to examine transmission and used more birds for this group (n = 11). Secondly, the lack of attenuation could be due to using different chicken lines in the former and current studies, as P2a chickens (173) were used previously, while Pure Columbian × New Hampshire chickens were used in the current study. The PC × NH line has not been characterized for MD resistance/susceptibility to date. Thirdly, the rMDV used previously had numerous mutations (164), including frameshifts within the UL13 protein kinase, UL44 (gC), and US6 (gD) genes that generated truncated proteins, and lacked the US2 gene; while the rMDV used in the current study was fully repaired. Most likely, these additional mutations contributed to the severely attenuated nature of RLORF4-null virus in the former studies, while the current report suggests only minor attenuation based on the combined *in vitro* and *in vivo* data. In all, these three reasons could explain the insignificant result in MD induction (Figure 7).

Consistent with abundant gC expression in FFs (Figure 8), vΔRLORF4 was able to efficiently transmit from chicken-to-chicken as wild type virus (vUL47eGFP) conclusively showing that RLORF4 is not involved in gC expression nor interindividual transmission of MDV. Currently, it is not known why expression of gC is severely affected following serial passage tissue culture cells, but the data presented here conclusively show that RLORF4 is not involved in this process. Recently, the role of the *Herpesviridae* conserved ICP27 regulatory protein, encoded by MDV UL54 (ICP27) has been shown to be essential for expression of MDV gC in cell culture (171). Future work in our laboratory focuses on the role ICP27 plays in attenuation and gC expression.

CHAPTER 3: THE REQUIREMENT OF GLYCOPROTEIN C (gC) FOR HORIZONTAL TRANSMISSION IS A CONSERVED FUNCTION OF gC FOR AVIAN HERPESVIRUSES[3]

ABSTRACT

We have formerly shown that glycoprotein C (gC) of *Gallid alphaherpesvirus* 2, better known as Marek's disease (MD) alphaherpesvirus (MDV), is required for horizontal transmission in chickens. Since gC is conserved within the *Alphaherpesvirinae* subfamily, we hypothesized gC was important for horizontal transmission of other alphaherpesviruses. To test this hypothesis, we first generated a fluorescent protein tagged clone of *Gallid alphaherpesvirus* 3 MD vaccine strain 301B/1 to track virus replication in cell culture and chickens using fluorescent microscopy. Following validation of this system, we removed the open reading frame of 301B/1 gC from the genome and determined whether it was required for horizontal transmission using experimental and natural infection studies. Horizontal transmission of MD vaccine 301B/1 was abrogated by removal of 301B/1 gC. Rescuent virus in which 301B/1 gC was inserted back into the genome efficiently spread among chickens. To further study the conserved function of gC, we replaced 301B/1 gC with MDV gC and this virus also efficiently spread in chickens. These data suggest the essential function of alphaherpesvirus gC proteins is

[3] This chapter has been previously published on *Scientific Reports 11*(1), pp.1-12.
Vega-Rodriguez, W., Xu, H., Ponnuraj, N., Akbar, H., Kim, T. and Jarosinski, K.W., 2021. The requirement of glycoprotein C (gC) for horizontal transmission is a conserved function of gC for avian herpesviruses. As per the Journal's specification, the author retains the right to reuse this article as a chapter in this thesis without the need to provide signed documentation. http://creativecommons.org/licenses/by/4.0/. The changes made to this article are as follow: interindividual spread was changed to horizontal transmission in every occurrence, and Figure 4 was divided into two separate figures.

conserved and can be exploited during the generation of future vaccines against MD that affects the poultry industry worldwide.

INTRODUCTION

Herpesviruses have co-evolved with their respective hosts for millions of years. In most cases, each herpesvirus and host have reached a relatively stable relationship with many hosts infected with multiple herpesviruses including humans that currently have nine associated herpesviruses (174). Although there is a tremendous amount of information on herpesvirus-host interactions in cell culture, little is known about their relationships pertaining to horizontal transmission and the viral and cellular genes that mediate this important aspect of the virus lifecycle. This is primarily due to the difficulty of studying the mechanistic nature of human herpesviruses in humans, as well as a lack of many natural animal models. For this, we turn to natural herpesvirus-host models to address questions on horizontal transmission and dissemination in populations.

For much of the 1990's, there was a tremendous amount of attention paid to conserved herpesvirus glycoprotein C (gC) homologues due to their high expression levels and immunogenicity where they were shown to perform multiple functions *in vitro.* Some of the functions identified include primary attachment of cell-free virus to heparin sulfate (HS)- and chondroitin-like glycosaminoglycans (GAGs) on the surface of cells (97, 175), and involvement in late steps of virus egress from cultured cells (97, 99). Although gC is not essential for most herpesviruses studied thus far, it significantly increases the efficiency of infection by providing an additional binding mechanism (107) and helps shield the virus from antibody neutralization (176). In addition to viral attachment and egress, gC homologues are thought to have immune evasion functions mediated by binding to and inhibiting the action of complement component C3

(101, 102); (100, 103-105) as well as a role in chemokine-mediated leukocyte migration (60). Similar to MDV, gC homologues for herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV) appear to play a minor role in tissue culture model systems but are critical for HSV-1 and VZV replication in human skin cells using the SCID-hu mouse model (177) suggesting gC homologues may perform conserved functions during natural infection of the host. However, studies on the role of gC homologues are limited due to a lack of natural animal host model systems.

We have formerly shown that gC of Marek's disease alphaherpesvirus (MDV) is dispensable for *in vitro* and *in vivo* replication but is required for horizontal transmission from chicken to chicken (20, 108, 109, 178). MDV causes Marek's disease in chickens, presenting with severe clinical symptoms including the development of solid lymphomas in the viscera and other organs; metabolic dysfunction; and neurological signs like paralysis and ataxia (179). It is a major economic problem in the poultry industry due to its global distribution and transmissibility (131). Natural infection of MDV begins through the respiratory route by inhalation of infectious virus where pulmonary B lymphocytes and macrophages or dendritic cells (156) are initially infected and transport the virus to lymphoid organs. Primary cytolytic infection ensues in activated T lymphocytes recruited to the sites of infection, which become the primary cell type infected, and latency is established in these cells. Depending on the line of chicken or MDV strain, oncogenic transformation of latently infected T cells results in lymphoma formation that is ultimately a dead-end for the virus. Important for dissemination in the population, migrating infected cells transport MDV to feather follicle (FF) epithelial (FFE) cells in the skin, where infectious cell-free virus is shed into the environment, and the virus life cycle can repeat in new hosts.

There are currently eight herpesviruses identified in avian species with all characterized within the subfamily *Alphaherpesvirinae* in the *Herpesviridae* family (174). Of the eight avian herpesviruses, six belong to the *Mardivirus* genus of which MDV or *Gallid alphaherpesvirus* 2 (GaHV-2) is the prototypic virus within this genus. MD is controlled through vaccination with attenuated MDV strains and homologous non-oncogenic avian herpesviruses, including *Gallid alphaherpesvirus* 3 (GaHV3) and turkey herpesvirus (HVT: *Meleagrid alphaherpesvirus*; MeHV1). However, the current vaccines are efficient at reducing tumor formation and disease, but not block horizontal transmission of virulent MDV resulting in increasing virulence over the decades (180). It is generally accepted that GaHV-3 and HVT have similar horizontal transmission pathogeneses as MDV.

Here, we hypothesized that the absolute requirement of gC for MDV horizontal transmission is conserved among other avian herpesviruses. To test this hypothesis, we used a recently generated infectious bacterial artificial chromosome (BAC) clone of the MD vaccine strain 301B/1 (181) in experimental and natural infections of chickens to determine whether 301B/1 gC is required for 301B/1 transmission. Our results conclusively showed that 301B/1 gC is required for horizontal transmission and that MDV gC could compensate for 301B1 gC in this process. These results suggest the importance of gC homologues in horizontal transmission may be a conserved function and draws importance to studying this glycoprotein during horizontal transmission of other herpesviruses.

MATERIALS AND METHODS

Cell culture and cells

All cells were maintained at 38°C in a humidified atmosphere of 5% CO_2. Chicken embryo cells (CEC) were prepared from 10-11-day-old specific-pathogen-free (SPF) embryos

obtained from the University of Illinois at Urbana-Champaign (UIUC) Poultry Farm following standard methods (165). Briefly, primary CEC cultures were seeded in growth medium consisting of Medium 199 (Cellgro, Corning, NY, USA) supplemented with 10% tryptose-phosphate broth (TPB), 0.63% $NaHCO_3$ solution, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 4% fetal bovine serum (FBS). Confluent CEC cultures were maintained in Medium 199 supplemented with 7.5% TPB, 0.63% $NaHCO_3$, 0.2% FBS, and antibiotics.

The chicken DF-1-Cre fibroblast cell line (166) was cultivated in a 1:1 mixture of Leibovitz L-15 and McCoy 5A (LM) media (Gibco, Gaithersburg, MD, USA) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and maintained in 50 µg/ml Zeocin (Invitrogen, Carlsbad, CA).

Generation of two-step Red recombination shuttle vectors.

To produce pEP-301BgC-in, 301B/1 UL44 was Gibson assembly cloned from r301B/1 BAC DNA into pcDNA3.1 using primers shown in Table 3. Briefly, 301B/1 UL44 was amplified by PCR using a set of primers encompassing the complete UL44 gene, gel purified, and cloned into the pcDNA3.1 TOPO vector (Life Technologies) using Gibson Assembly reaction mix (NEB) according to the manufacturer's instructions to generate pc301BgC. Next, a 3×Flag epitope was cloned into pc301BgC from a previously described r3×Flag54 BAC clone (171) using Gibson assembly to generate pc3×Flag301BgC. Next, the *aphAI*-I-*Sce*I cassette was amplified from pEP-KanS2 using primers shown in Table 3 and inserted into pc3×Flag301BgC using Gibson Assembly cloning to generate pEP-301BgC-in. All clones at each step were confirmed by PCR and DNA sequencing. For insertion of MDV gC (RB1B strain) into r301B/1, a previously described pEP-MDVgC-in shuttle vector was used (109).

Table 3. Primers used for cloning and generation of shuttle vectors using Gibson assembly cloning.

Construct[a]	Direction[b]	Sequence (5'→3')
pc301B gC	Vector For	ACATATTACTTTCGTCCGTCGGTAAGCCTATCCCTAACCCTCTCC
	Vector Rev	GACGCGTGCATGGGGAAAATTCCGAGCTCGGTACCAAGCTTAACTAG
	Insert For	AGCTTGGTACCGAGCTCGGAATTTTCCCCATGCACGCGTCACG
	Insert Rev	GGGTTAGGGATAGGCTTACCGACGGACGAAAGTAATATGTATTTTTTCCCGG
pc3×Flag301B gC	Vector For	ACAAGGATGACGACGATAAGATTAACCCCGATCTAGCTACACCC
	Vector Rev	CCGTCATGATCCTTGTAATCGCTAGCGCTTAGGACGCG
	Insert For	GCCGCGTCCTAAGCGCTAGCGATTACAAGGATCATGACGGAGATTACAAGG
	Insert Rev	GTAGCTAGATCGGGGTTAATCTTATCGTCGTCATCCTTGTAATCGATGT
pEP-301BgC-in	Vector For	GGCATAGAAATATCATCAGCCGAATATTACTCC
	Vector Rev	CGATTACCCTGTTATCCCTAGCTGATGATATTTCTATGCCGCTTGAG
	Insert 1 For	GGCATAGAAATATCATCAGCTAGGGATAACAGGGTAATCGATTTATTCAACAAAG
	Insert 1 Rev	CCTGCAAAGACCTGTAACCAGCCAGTGTTACAACCAATTAACCAAT
	Insert 2 For	TAATTGGTTGTAACACTGGCTGGTTACAGGTCTTTGCAGGACCC
	Insert 2 Rev	TCATCTTGGAGTAATATTCGGCTGATGATATT

[a]Construct generated with the set of primers.
[b]Directionality of the primer and product produced for Gibson assembly cloning.

Generation of r301B/1 clones

To create 301B/1 expressing fluorescent-tagged pUL47, the coding sequence of the monomeric red fluorescent protein (mRFP) gene was inserted in frame at the C-terminus of the 301B/1 UL47 open reading frame (ORF) by two-step Red-mediated mutagenesis (168) in an infectious bacterial artificial chromosome (BAC) clone of 301B/1. Briefly, the mRFP-I-*Sce*I-*aphAI* cassette was amplified from pEP-mRFP-in (169) using primers shown in Table 4 and used

for mutagenesis in GS1783 *Escherichia coli* cells. Multiple integrates and resolved clones were screened by RFLP analysis, analytic PCR, and DNA sequencing using primers shown in Table 5.

To create r3ΔgC, the coding sequence of 301B UL44 (gC) was deleted from r301B47R. Briefly, the I-*Sce*I-*aphAI* cassette from pEP-KanS2 was amplified by PCR with Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix using primers shown in Table 4 and used for mutagenesis in GS1783 *E. coli* cells. Following removal of UL44 in the r301B47R clone, 301B/1 gC with a 3×Flag epitope inserted at the N-terminus after the predicted signal sequence, or MDV gC were inserted into r3ΔgC using two-step Red recombination. Briefly, 3×Flag301B/1 gC or MDV gC were PCR amplified from pEP-301BgC-in or pEP-MDVgC-in, respectively, using primers shown in Table 4 and used for mutagenesis as described above. RFLP analysis, analytical PCR, and DNA sequencing confirmed all clones were correct. Primers used for MDV gC have been previously published (109, 157, 162), while primers for sequence 301B/1 gC are listed in Table 5.

r301B/1s were reconstituted by transfecting DF-1-Cre cells with purified BAC DNA plus Lipofectamine 2000 (Invitrogen) using the manufacturers' instructions as previously described (182). Transfected DF-1-Cre cells were mixed with fresh primary CEC cultures until plaques formed, then further propagated in CEC cultures until virus stocks could be stored. All viruses were used at ≤ 5 passages for *in vitro* and *in vivo* studies.

Table 4. Primers used for generation of recombinant *Gallid alphaherpesvirus* 3 301B/1.

Modification[a]	Direction[b]	Sequence (5'- 3')[c]
UL47mRFP	Forward	AGAAGATGCGAAGGAGGCGATCTTCAAAAAAACGGACCGG*ATGGCCTCCTCCGAGGACG*
	Reverse	TCACCACGATCTGCACGCCGCTCCGTGCGCTTTTTTTTTA*CAAGGCGCCGGTGGAGTG*
ΔgC	Forward	ATATACGCTCTCGGAGACGCGGCTCGCACGCCAGCTGAAATATTTTCCCCTAGTTTGCGGTGACATTGAT*TAGGGATAACAGGGTAATCGATTT*
	Reverse	TACAAGAGCTCGGGGCATATAATGAGCCAGATCAATGTCACCGCAAACTAGGGGAAAATATTTCAGCTGG*GCCAGTGTTACAACCAATTAACC*
ΔgC-R (3×Flag301BgC)	Forward	GGCTCGCACGCCAGCTGAAATATTTTCCCC*CCCATGCACGCGTCACG*
	Reverse	AATGAGCCAGATCAATGTCACCGCAAACTA*GACGGACGAAAGTAATATGTATTTTTTCCCG*
MDV gC	Forward	ATATACGCTCTCGGAGACGCGGCTCGCACG*TATCTTCCCTCATGCTCACG*
	Reverse	TACAAGAGCTCGGGGCATATAATGAGCCAG*CATAACAATGAGATTATAAT*

[a]Modification to the 301B/1 genome using two-step Red-mediated recombination. [b]Directionality of the primer. [c]Underlined sequence indicates start and stop codons for 301B/1 UL44 gene**.** *Italics indicate the template-binding region of the primers for PCR amplification with pEP-mRFP-in, pEP-KanS, pEP-301BgC-in, or pEP-MDVgC-in.* Red indicates unique upstream integration sequences.

Table 5. Primers used for sequencing.

Gene[a]	Direction[b]	Sequence (5'→3')
UL47cTerm	Forward	CCTTCTCGGCACGCTAGCCT
	Reverse	TTTTGGGACGCGAAGTGGCC
UL44 (gC)	Forward	GCTAAGTTGCGCAGGCAGAG
	Reverse	GGGCCGGATGTACCTATACG
	Forward	GACCCGCCTCGGTCGACG
	Reverse	ACATAGACGGAGCCCGGTGG
	Forward	GCCATCGACGAGGGGGT
	Reverse	GCCGGAATACTTGACGGGTTG

[a]Gene sequenced with the set of primers.
[b]Directionality of the primer.

Immunofluorescence assays (IFA)

CEC cultures were infected with different r301B/1 viruses on sterile glass coverslips at 100 plaque-forming units (PFU) per well. At 5 days post-infection (p.i.), cells were fixed with PFA buffer (2% paraformaldehyde, 0.1% Triton X-100) for 15 min and then washed twice with PBS. Fixed coverslips were blocked in 10% neonatal calf serum and stained with anti-GaHV-3 chicken sera and goat anti-chicken IgY-Alexa Fluor® 488 secondary antibody (Molecular Probes, Eugene, OR). To detect 3×Flag301BgC, mouse anti-Flag® M2 (Sigma-Aldrich) was used. Anti-gC monoclonal A6 (kindly provided by Jean-Francois Vautherot, INRA, Nouzilly, France) antibody (151) was used to detected MDV gC expression. Anti-mouse Ig Alexa Fluor 488 (Molecular Probes, Eugene, OR) was used as secondary antibody for both anti-Flag and -MDV gC monoclonal antibodies. The virus plaques were observed using an EVOS™ FL Cell Imaging System (Thermo Fisher Scientific) and compiled using Adobe® Photoshop® version 21.0.1.

Measurement of plaque areas

Plaque areas were measured in CEC cultures exactly as previously described (163) using anti-GaHV-3 chicken sera and goat anti-chicken IgY-Alexa Fluor® 488 secondary antibody (Molecular Probes, Eugene, OR). Digital images of 50 individual plaques were collected using an EVOS™ FL Cell Imaging System (Thermo Fisher Scientific) and compiled with Adobe® Photoshop® version 21.0.1. Plaque areas were measured using ImageJ (170) version 1.53d software. Box and Whisker plots were generated, and significant differences were determined using IBM® SPSS® Statistics Version 27 software.

Viral DNA replication in cell culture

To measure replication of viruses in cell culture qPCR assays were used to measure the relative level of replication as previously described (181). Briefly, CEC cultures were prepared in 6-well tissue culture plates and the next day inoculated with 100 PFU/well. Total DNA was collected from the inoculum and at 48, 72, 96, and 120 h following infection using the QIAamp® DNA Mini Kit (Qiagen, Germantown, MD). Quantification of 301B/1 genomic copies in CEC cultures was performed using primers and probe previously described (181) and were used in duplex PCR reactions with previously described primers and probes against chicken iNOS (172). All qPCR assays were performed in an Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) and the results were analyzed using the QuantStudio™ Design & Analysis Software v1.4.2 supplied by the manufacturer. The fold-increase over inoculum was used in triplicate for each virus and time point.

Western blot analysis

Western blot analyses were performed as previously described (151). Total protein was collected from infected CEC cultures or scraped from FFE previously described (178). In some experiments, infected CEC cultures media was collected to detected secreted proteins. To detect the relative level of 301B/1 infection, mouse anti-GaHV-3 Y5.9 (149) was used at 1:500 dilution to detect GaHV-3 specific gB. To detect mRFP tagged 301B/1 pUL47, rabbit anti-mRFP antibody (ab62341; Abcam®) was used with secondary sheep anti-rabbit IgG (H+L)-HRP conjugate (A16172; Life Technologies, Inc.). To detect MDV gC, monoclonal antibody A6 was used at a 1:500 dilution. To detect 3×Flag tagged 301B/1 gC, anti-Flag® M2 was used and for protein loading control, mouse anti-β-actin (ACTNO5; Abcam, Cambridge, MA) was used at their recommended dilutions. Anti-bovine serum albumin (BSA, Thermo Fisher Scientific) mAb

was used at its recommended dilution as a loading control for infected cell media. Secondary anti-mouse IgG peroxidase conjugate was purchased from GE Healthcare (Piscataway, NJ) and used for mouse monoclonal antibodies. The SuperSignal West Pico Chemiluminescent Substrate kit from Thermo Fischer Scientific (Rockford, IL) was used to detect antigens using the manufacturer's instructions.

Ethics statement

All animal work was conducted according to national regulations and ARRIVE guidelines. The animal care facilities and programs of UIUC meet the requirements of the law (89 –544, 91–579, 94 –276) and NIH regulations on laboratory animals and are compliant with the Animal Welfare Act, PL 279. UIUC and the College of Veterinary Medicine at UIUC are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures were conducted in compliance with approval of UIUC's Institutional Animal Care and Use Committee (IACUC).

Animal experiments

Pure Columbian (PC) chickens were obtained from the UIUC Poultry Farm (Urbana, IL) and were from MD-vaccinated parents; therefore, considered to be maternal antibody positive. To test replication of v301B47R, 7-day old chicks (n = 10) were experimentally infected by intra-abdominal inoculation of 4,000 PFU for v301B47R and housed with another ten chickens that were left uninfected to act as contacts to determine whether v301B47R can naturally infect naïve chickens by horizontal transmission. To test the ability of v301B47R, v3ΔgC, v3ΔgC-R, v3-MDVgC to replicate and horizontal transmission in chickens, 3-day old PC chicks (n = 8-10/group) were inoculated with 10,000 PFU with each respective virus and housed in separate rooms. To test natural infection through horizontal transmission, 6-8 age-match, naïve contact

chickens were housed experimentally infected chickens for eight weeks. Water and food were provided *ad libitum* for all animal experiments.

DNA extraction from blood cells and qPCR assays

To measure replication in chickens, whole blood was obtained by wing-vein puncture and DNA was extracted using the E.Z. 96 blood DNA kit from Omega Bio-tek, Inc. (Norcross, GA) as previously described (20). Quantification of 301B/1 genomic copies in CEC cultures or blood using qPCR was performed using primers and probe previously described (181) and were used in duplex PCR reactions with previously described primers and probes against chicken iNOS (172). All qPCR assays were performed in an Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) and the results were analyzed using the QuantStudio™ Design & Analysis Software v1.4.2 supplied by the manufacturer. The final viral loads were obtained after normalizing with chicken iNOS used as an internal control gene.

Monitoring v301B47R and its derivatives in feather follicles (FFs)

To track the time at which each r301B47R or its derivatives reached the FFs, two flight feathers were plucked from the right and left wings (4 total) of inoculated birds weekly and pUL47mRFP expression was examined using a Leica M205 FCA fluorescent stereomicroscope with a Leica DFC7000T digital color microscope camera (Leica Microsystems, Inc., Buffalo Grove, IL).

IFA of feather follicles (FFs)

Whole feathers were plucked from chickens infected with different r301B/1s and the FFs were fixed using PFA buffer, washed twice with PBS, and then blocked in 10% neonatal calf serum (Sigma-Aldrich). Fixed FFs were stained with primary mouse anti-Flag® M2 (Sigma-Aldrich) or anti-gC A6 (151) antibodies with anti-mouse Ig Alexa Fluor 488 (Molecular Probes,

Eugene, OR) used as secondary antibody. The Leica M205 FCA fluorescent stereomicroscope with a Leica DFC7000T digital color microscope camera (Leica Microsystems, Inc., Buffalo Grove, IL) was used to analyze stained FFEs. All images were compiled using Adobe® Photoshop® version 21.0.1.

RESULTS

Generation of recombinant (r)301B/1 expressing pUL47mRFP (r301B47R)

We and others have shown that fusing fluorescent proteins to the C-terminus of alphaherpesvirus pUL47 (VP13/14) allows the visualization of infected cells and does not affect replication in cell culture and *in vivo* for numerous herpesviruses (167, 183-186). Therefore, we inserted monomeric red fluorescent protein (mRFP) at the C-terminus of the pUL47 in a recently described BAC clone of 301B/1 (181) to generate r301B47R (Figure 9). RFLP analysis confirmed the integrity of the BAC clones as the predicted banding pattern was observed described in Figure 10. In addition, DNA sequencing was used to confirm that each clone was correct at the nucleotide level (data not shown) using primers specific for each gene (Table 3).

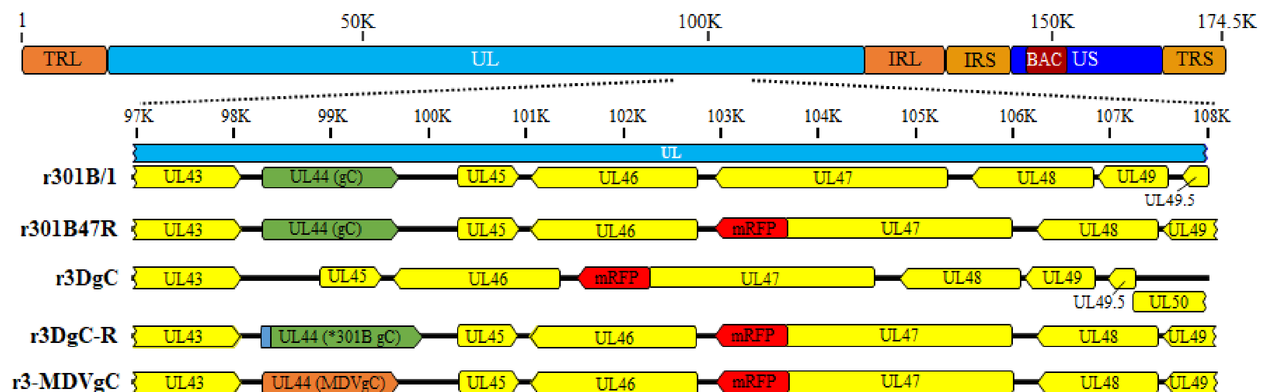


Figure 9. r301B/1 clones. (A) Schematic representation of the 301B infectious clone (181) genome depicting the locations of the terminal repeat long (TRL) and short (TRS), internal repeat long (IRL) and short (IRS), and unique long (UL) and short (US) regions. The location of the mini-F (BAC) sequence is also shown located within the US region. The region of the UL spanning UL43 to UL50 is expanded to show the relevant genes within this region including the mRFP tag on pUL47, deletion of UL44 (gC), and insertion of 3×Flag301BgC or MDV gC.

Replication of v301B47R in cell culture

Following reconstitution of r301B/1 and r301B47R resulting in v301B/1 and v301B47R, respectively, we first tested *in vitro* replication properties using plaque size assays. Consistent with fusing fluorescent proteins to pUL47 in other herpesviruses, there was no change in replication between parental v301B/1 and v301B47R during cell culture replication in chicken embryo cells (CEC) cultures (Figure 11A). Additionally, while reconstituting r301B47R in DF-1-Cre (data not shown) and propagating in CEC cultures, mRFP expression was abundantly expressed that could be visualized using fluorescent microscopy (Figure 11B). Expression of pUL47mRFP was almost exclusively found in the nucleus, consistent with MDV, though levels appeared to more abundant than observed for MDV (167, 182). Western blotting using anti-mRFP antibody showed mRFP expression was fused to the pUL47 since mRFP alone is ~26 kDa in size, while fused to pUL47 would create ~115 kDa protein (Figure 10C). These results show that fusing mRFP to the C-terminus of pUL47 of 301B/1 resulted in no change in viral replication in tissue culture and allowed the direct visualization of 301B/1 replication in cells. This data is consistent with former studies fusing fluorescent proteins to pUL47 homologues (167, 184, 185, 187).

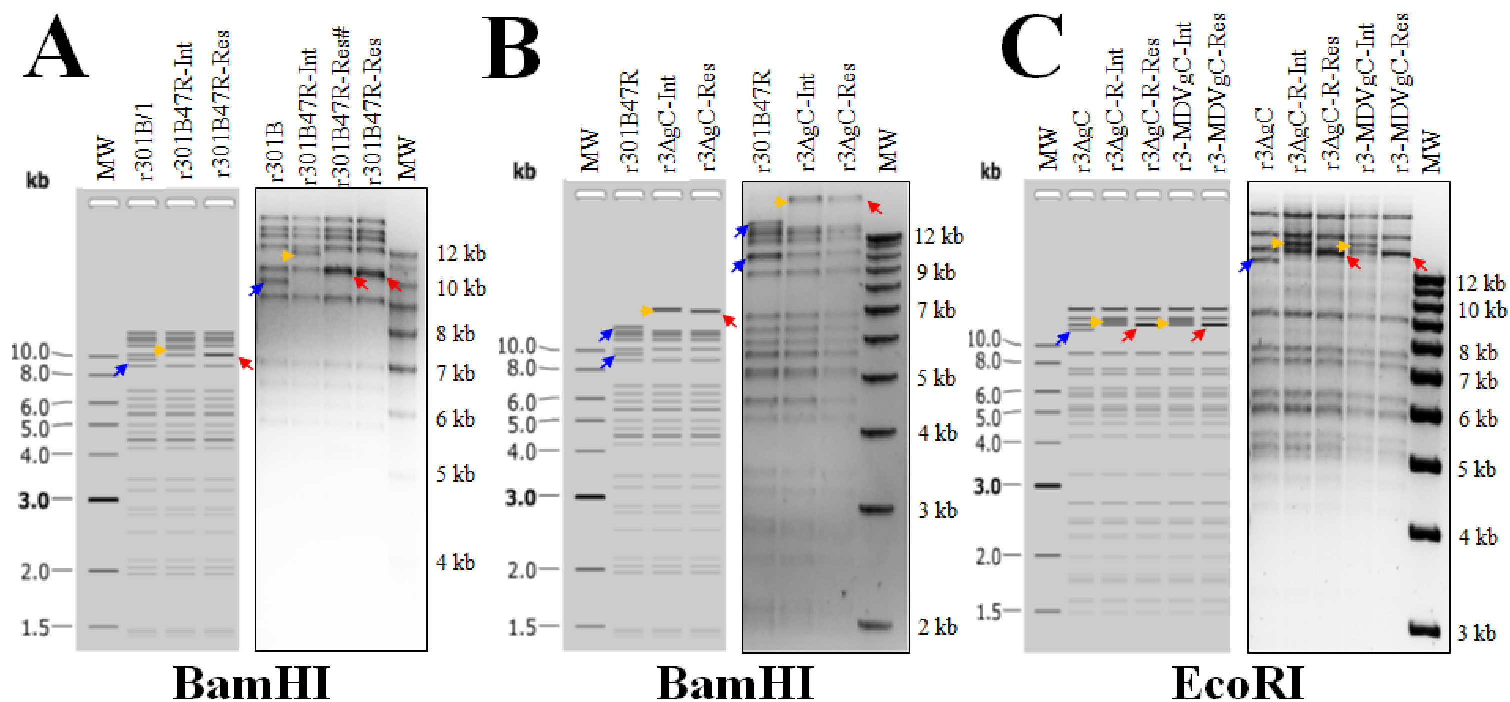


Figure 10. Generation of r301B/1clones. (A) Predicted and actual RFLP analysis of r301B/1 clones. BAC DNA obtained for r301B, r301B47R-integrate clone and two resolved clones were digested with BamHI and electrophoresed through a 1.0% agarose gel. Integration of the mRFP+*AphAI* sequence resulted in an increase in the 9,545 bp (→) fragment to 11,207 bp (→). Resolution by removal of the *AphAI* sequence shifted the 11,207 bp fragment to 10,233 bp (←). One resolved clone (#) was used after this point. (B) Predicted and actual RFLP analysis of r3ΔgC r301B clone. BAC DNA obtained for r301B47R, r3ΔgC-integrate clone and r3ΔgC-resolved clone were digested with BamHI and electrophoresed through a 1.0% agarose gel. Integration of the *AphAI* sequence into this locus removed a BamHI site resulting in combining the 10,223 and 14,854 bp fragments (→) to 24,671 bp (→). Removal of the *AphAI* sequence through resolution reduced the 23,933 bp fragment by 1,028 bp to 23,643 bp (←). (C) Predicted and actual RFLP analysis of r3ΔgC-R and r3-MDVgC clones derived from the r3ΔgC clone. BAC DNA obtained for r3ΔgC, r3ΔgC-R-integrate, r3ΔgC-R-resolved, r3-MDVgC-integrate, and r3-MDVgC-resolved clones were digested with EcoRI and electrophoresed through a 1.0% agarose gel. Integration of 3×Flag301BgC-*AphAI* or MDVgC-*AphAI* sequences into this locus resulted an increase in the 12,879 bp (→) fragment to 15,420 bp (→) or 15,403 bp (→), respectively. Removal of the *AphAI* sequence from r3ΔgC-R-Int reduced the 15,420 bp fragment by 1,038 bp to 14,382 bp (←) to generate r3ΔgC-R-Res. Removal of the *AphAI* sequence from r3-MDVgC-Int reduced the 15,403 bp fragment by 1,034 bp to 14,369 bp (←) to generate r3-MDVgC-Res.The molecular weight marker used was the 1 kb Plus DNA Ladder from Invitrogen, Inc. (Carlsbad, CA). No extraneous alterations are evident

Replication of v301B47R in cell culture

Following reconstitution of r301B/1 and r301B47R resulting in v301B/1 and v301B47R, respectively, we first tested *in vitro* replication properties using plaque size assays. Consistent with fusing fluorescent proteins to pUL47 in other herpesviruses, there was no change in replication between parental v301B/1 and v301B47R during cell culture replication in chicken embryo cells (CEC) cultures (Figure 11A). Additionally, while reconstituting r301B47R in DF-1-Cre (data not shown) and propagating in CEC cultures, mRFP expression was abundantly expressed that could be visualized using fluorescent microscopy (Figure 11B). Expression of pUL47mRFP was almost exclusively found in the nucleus, consistent with MDV, though levels appeared to more abundant than observed for MDV (167, 182). Western blotting using anti-mRFP antibody showed mRFP expression was fused to the pUL47 since mRFP alone is ~26 kDa in size, while fused to pUL47 would create ~115 kDa protein (Figure 10C). These results show that fusing mRFP to the C-terminus of pUL47 of 301B/1 resulted in no change in viral replication in tissue culture and allowed the direct visualization of 301B/1 replication in cells. This data is consistent with former studies fusing fluorescent proteins to pUL47 homologous (167, 184, 185, 187).

v301B47R as a tool for tracking virus in chickens

Next, we tested the ability of v301B47R to replicate and horizontal transmission in chickens. To do this, ten chickens were experimentally infected with 4,000 PFU v301B47R and housed with ten naïve contact chickens for nine weeks to measure natural infection (horizontal transmission). First, we were interested in whether we could directly identify infected birds based on fluorescence in plucked feathers as has been previously done in our laboratory for MDV (171). Like our former results with fluorescently tagged MDV, feather follicles were

easily identified from feathers in most experimentally infected chickens at 14 to 28 days pi (Figure 11D) with a total of 90% of birds positive for v301B47R by 21 dpi (Figure 11E). Staining of FFs showed feathers positive for pUL47mRFP were also positive for anti-GaHV-3 glycoprotein B (Y5.9), while negative for anti-HVT glycoprotein B (L78.2). These results show that fusing mRFP to 301B/1 pUL47 can be an effective tool to track v301B/1 in chickens.

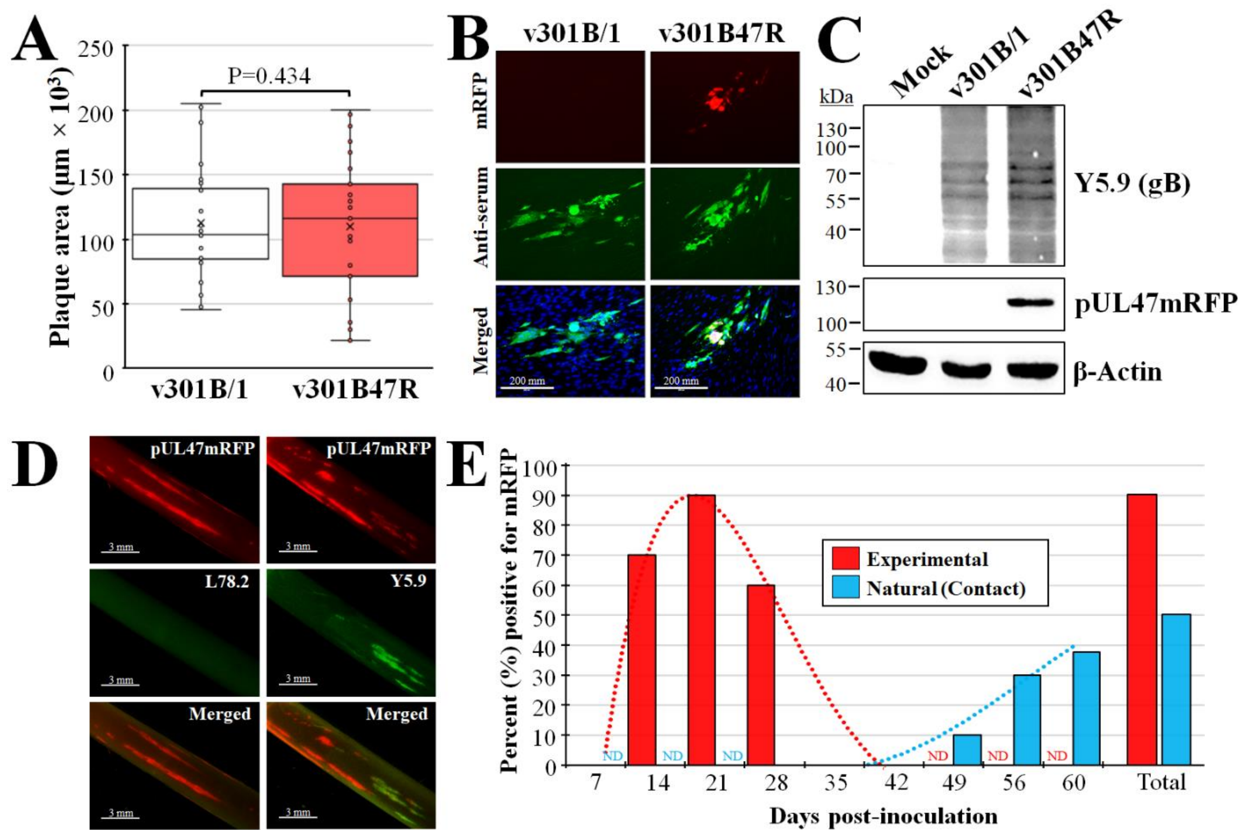
v301B47R can spread from chicken to chicken.

Over the course of 9 weeks, 50% of the naïve contact chickens housed with experimentally infected chickens became positive by the time the experiment was terminated (Figure 11E). There was a delay of about three weeks before naïve contact chickens began to show fluorescent feathers compared to experimentally infected chickens, which is consistent with the time it takes for MDV to horizontal transmission. These results confirm that 301B/1 can horizontal transmission from chicken to chicken.

Generation of r301B/1 lacking gC or expressing 3×Flag301B gC or MDV gC

Now that we had a tool to track 301B/1 in cell culture and chickens, we wanted to test two hypotheses. First, we hypothesized that 301B/1 gC, like MDV gC, would be required for horizontal transmission in chickens. Second, we hypothesized that, since both GaHV-3 and MDV are chicken herpesviruses with similar pathogeneses, that MDV gC would compensate for 301B/1 replication and transmission. Therefore, we removed the complete UL44 ORF from r301B47R to generate r3ΔgC (Figure 9 and Figure 10). To generate a rescuant virus, 301B/1 gC was inserted back into the viral genome where it was originally removed but included a 3×Flag tag at the N-terminus (r3-ΔgC-R) that should allow us to identify 301B/1 gC in downstream studies. In addition, we inserted MDV gC in its place to generate r3-MDVgC. RFLP analysis confirmed the integrity of the BAC clones as the predicted banding pattern was observed

(Figures 10B and 10C). In addition, DNA sequencing was used to confirm that each clone was correct at the nucleotide level (data not shown) using primers specific for each gene (Table 5).



[4]**Figure 11. Replication and fluorescent protein expression in tissue culture cells.** (A) Plaque areas of viruses reconstituted from r301B/1 and r301B47R were measured and the results are shown as box & whisker plots. There were no significant differences in plaque sizes between the two viruses using Student's *t* tests. (B) Representative plaques for v301B/1 and v301B47R are shown. Plaques were stained with polyclonal chicken anti-GaHV-3 antibody and goat anti-chicken-IgY Alexa488 (green) was used as secondary antibody to identify plaques. Fluorescent expression of mRFP (red) was directly visualized and cells were counterstained with Hoechst 33342 to visualize nuclei. (C) Western blotting for pUL47mRFP using anti-mRFP antibody. The anti-GaHV-3 antibody Y5.9 (188) was used to show the relative level of infection in the cultures. Antibody against chicken β-actin is shown as a loading control. (D) Fluorescent protein expression in feather follicles (FFs) infected with v301B47R at 21 dpi. FFs were also stained with anti-HVT L78.2 or -GaHV-3 Y5.9 plus anti-mouse IgG-Alexa488 (green) and images were collected with a fluorescent stereoscope. (E) Percent of chickens positive for pUL47mRFP in experimental and naturally (contact) infected chickens over 60 days.

[4]Figure 11C was generated by Dr. Nagendra Prabhu Ponnuraj.

Replication of v301B/1 lacking gC (v3ΔgC) or expressing MDV gC (v3-MDVgC) in cell culture

Following reconstitution of r301B/1 clones with UL44 removed (v3ΔgC) and replaced with Flag-tagged 301B/1 gC (r3ΔgC-R) or MDV gC (r3-MDVgC), we tested replication in CEC cultures using plaque size assays (Figure 12A). Removal of 301B/1 gC resulted in significantly large plaque sizes, which is consistent with what is observed for MDV (109, 178). Adding 3×Flag301B gC restored smaller plaque sizes (v3ΔgC-R), while adding MDV gC also restored smaller plaque sizes that were significantly different tov3ΔgC. Virus replication kinetics showed not significant differences (Figure 12B). Figure 12C shows western blotting of total protein and media from infected cells using anti-MDV gC and -Flag antibodies. The rescued 301B/1 gC could be detected using the anti-Flag antibody in both cellular protein extracts and infected cell media, suggesting 301B/1 gC is also secreted as has been previously shown for MDV gC (109, 189).

We also confirmed MDV gC expression in both infected cells and was in the media of infected cells. Immunofluorescence assays (IFA) was used to examine expression in cells and showed that Flag-tagged 301B/1 gC (Figure 12D) and MDV gC proteins (Figure 12D) were detected as expected. These results show that adding the 3×Flag epitope to the N-terminus 301B/1 gC did not affect 301B/1 replication in cell culture and allowed us to identify its expression *in vitro*. Also, 301B/1 expressing MDV gC did not affect replication based on plaque size assays, and MDV gC was expressed in v3-MDVgC.

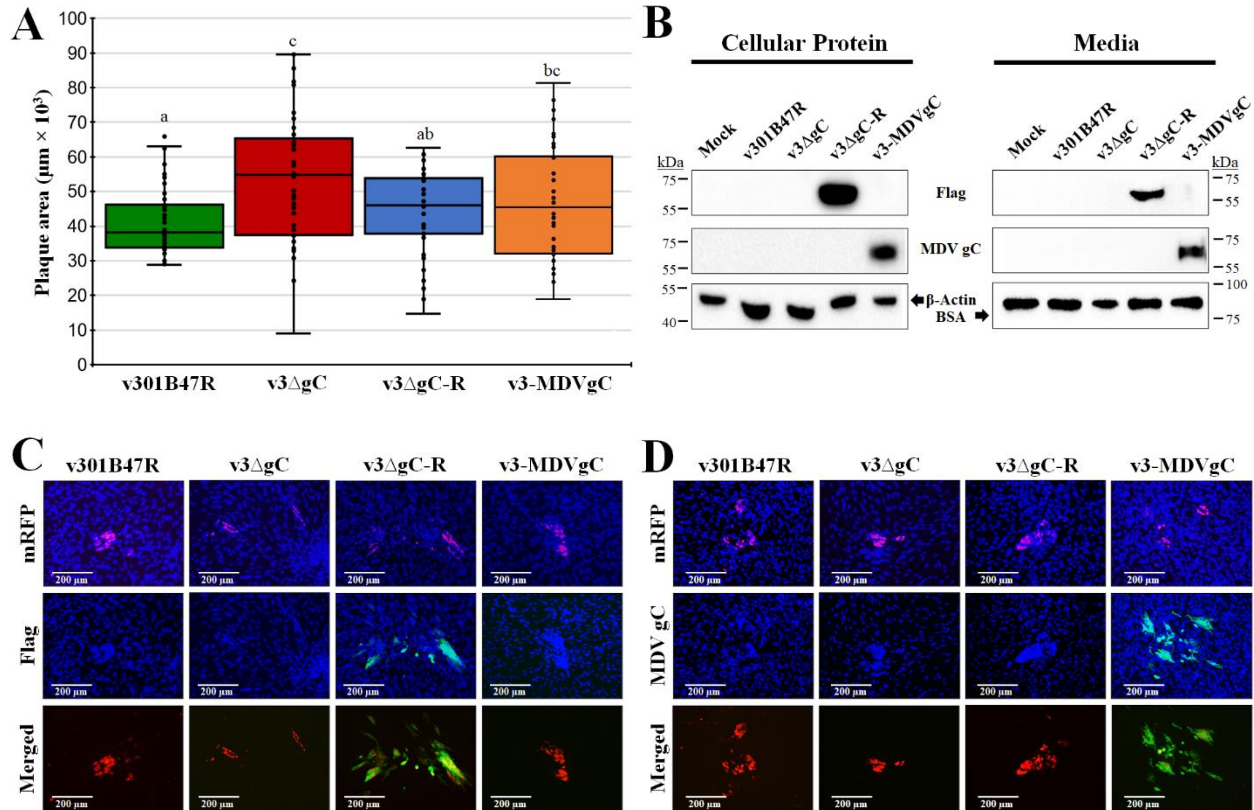


Figure 12. Replication and expression of proteins in cell culture. (A) Plaque areas for viruses reconstituted from r301B47R, r3ΔgC, r3ΔgC-R, and r3-MDVgC were measured and shown as box & whisker plots. Averages with different letters are significantly different using Student's *t*-tests ($P \leq 0.05$). (B) Western blotting to confirm 301B/1 gC and MDV gC expression. Both total cellular protein and infected cell culture media were used to detect 3×Flag tagged 301B/1 gC and MDV gC. Anti-Flag® M2 was used to detect 301B/1 gC, while anti-MDV gC A6 antibody was used to confirm MDV gC expression. For protein loading control, mouse anti-β-actin was used for total protein, while anti-BSA was used for infected cell media. (C and D) Expression of 301B/1 gC in v3ΔgC-R and MDV gC in v3-MDVgC. Representative plaques for all four viruses were stained with anti-Flag® (C) or -MDV gC A6 antibodies with goat anti-mouse-Alexa488 (green) as secondary antibody. Fluorescent expression of mRFP (red) was directly visualized and cells were counterstained with Hoechst 33342 to visualize nuclei. Only anti-Flag (C) or -MDV gC (D) and pUL47mRFP are shown in the merged images.

301B/1 gC is required for horizontal transmission

To test our hypotheses that GaHV-3 gC, like MDV gC, would be required for horizontal transmission in chickens, we tested our newly derived v3ΔgC using our experimental and natural infection model for horizontal transmission. To do this, 8-10 chickens were inoculated with 10,000 PFU of each virus and housed with 6-10 uninfected chickens over the course of 8 weeks.

Using qPCR assays to measure 301B/1 replication in the blood of experimentally infected chickens (Figure 13A) and presence in FFs (Figure 13B), no differences were seen between v301B47R, v3ΔgC, and v3ΔgC-R. However, when contact chickens were monitored for natural infection, no chickens housed with v3ΔgC-infected birds became infected compared to 88% and 60% of contact chickens were infected with v301B47R and v3ΔgC-R, respectively (Figure 13B). Following termination, whole blood was collected from all contact chicken, serum was tested for anti-GaHV-3 antibodies using IFAs and blood was used to measure 301B/1 viral DNA. It was confirmed all chickens negative for fluorescent FFs were also negative for anti-GaHV-3 antibodies and 301B/1 viral DNA in the blood (data not shown). IFA (Figure 14A) and western blotting (Figure 14C) was used to confirm the 3×Flag epitope remained fused to the 301B/1 gC protein. With these results, we can conclude that 301B/1 gC is required for horizontal transmission of the 301B/1 MD vaccine strain and the addition of a 3×Flag epitope at the N-terminus of 301B/1 gC does not affect its function during horizontal transmission.

MDV gC can compensate for 301B/1 gC during 301B/1 horizontal transmission.

To test our second hypothesis that MDV gC would compensate for 301B/1 replication and transmission, we also tested v3-MDVgC *in vivo*. There was no difference in virus replication in the blood using qPCR assays (Figure 13A) nor the ability to reach the FFs (Figure 13B). Interestingly, v3-MDVgC was able to naturally infect chickens similar to v301B47R and v3ΔgC-R showing that MDV gC can compensate for 301B/1 in this essential function *in vivo*. IFA (Figure 14B) and western blotting (Figure 14C) were used to confirm MDV gC expression was maintained during replication in FFE cells. These results show that MDV gC can compensate for 301B/1 gC during 301B/1 MD vaccine strain horizontal transmission in chickens.

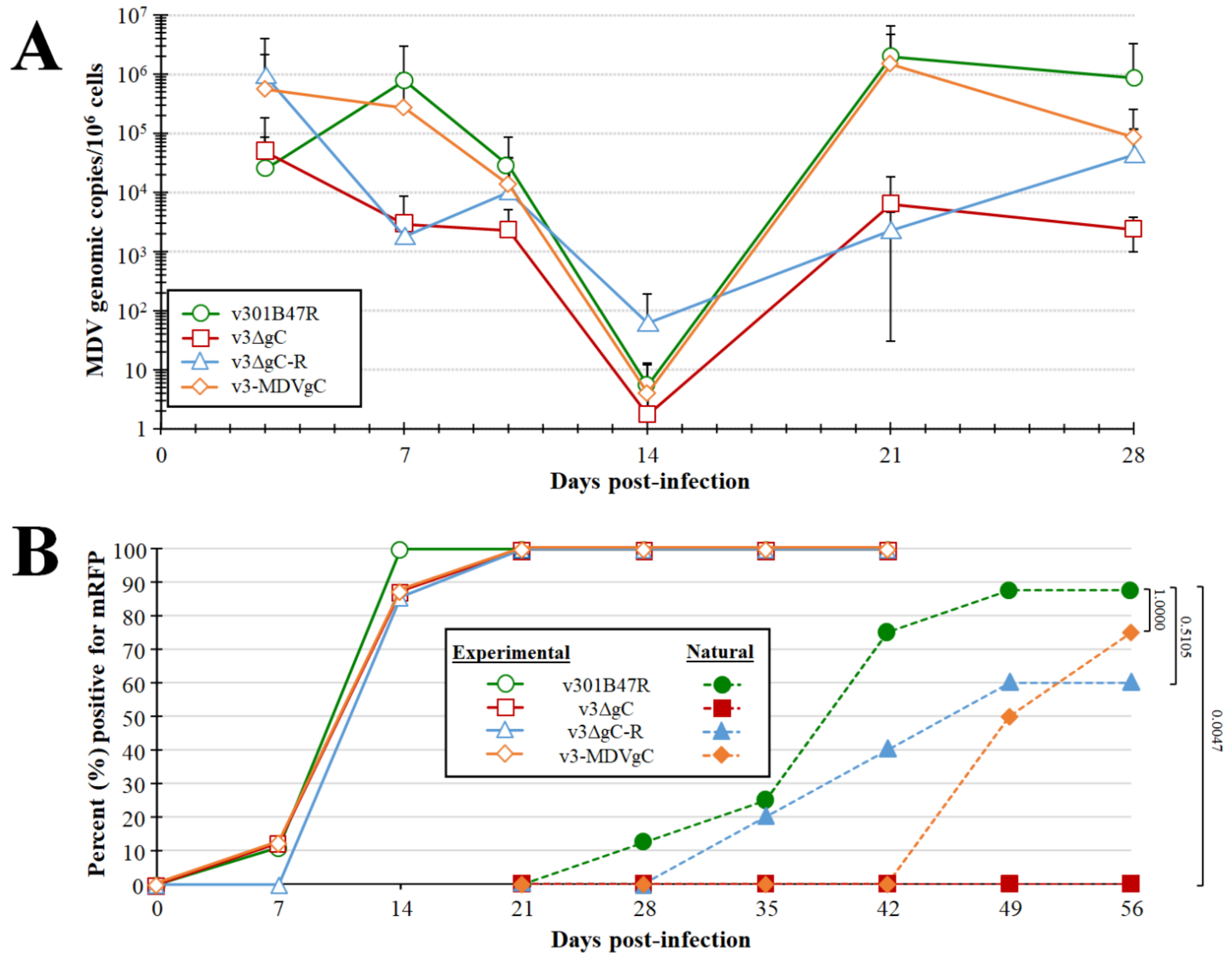
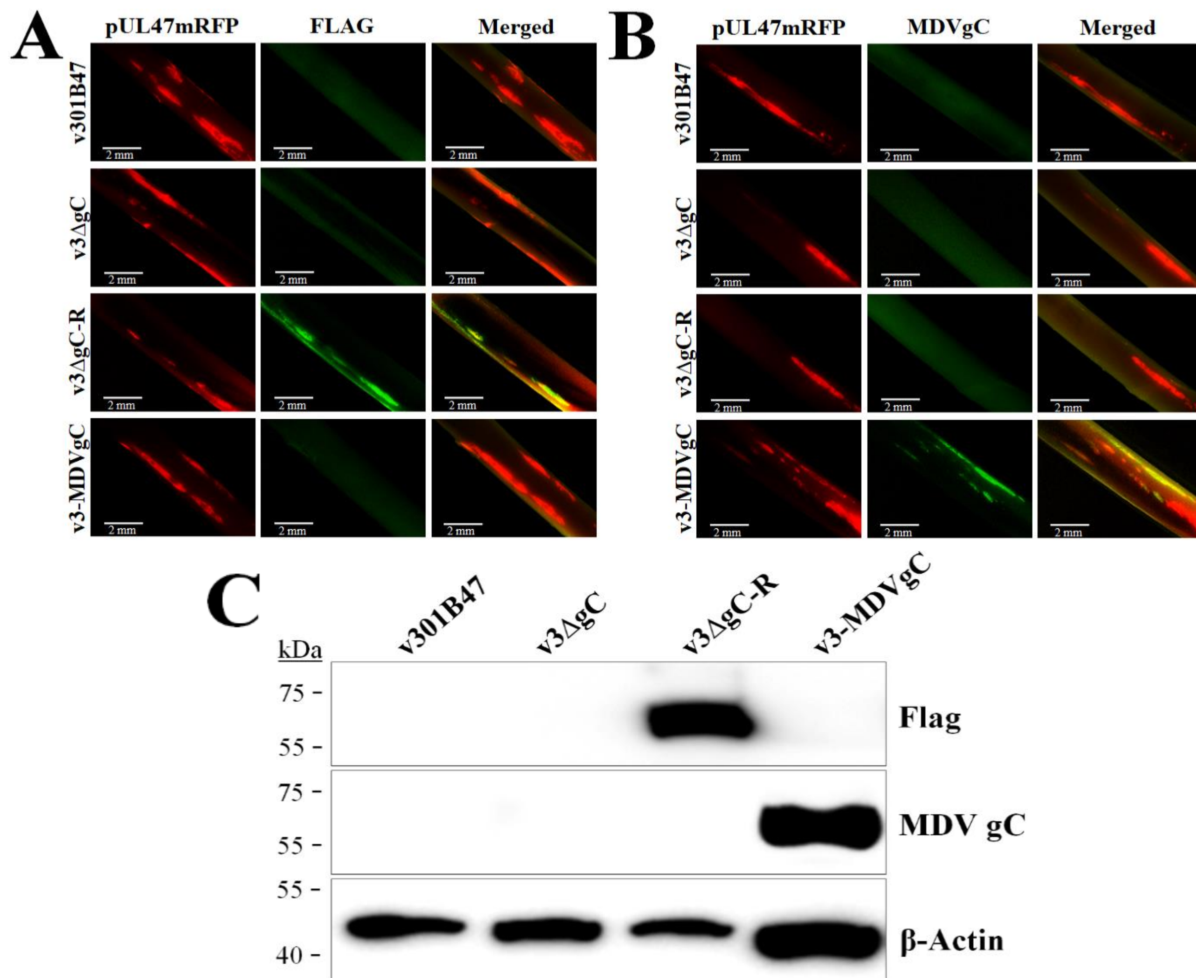


Figure 13. Replication and horizontal transmission of r301B/1 viruses in chickens. Pure Columbian chickens were experimentally infected with v301B47R, v3ΔgC, v3ΔgC-R, or v3-MDVgC as described in the Materials and Methods for 56 days. (A) Replication was monitored in experimentally infected chickens by quantification of 301B/1 genomes in the blood over the first 4 weeks of infection. Shown are the mean 301B/1 genomic copies per 10^6 blood cells ± standard error of means. No significant differences were observed between all groups and time points using Student's *t*-tests. (B) Quantitative analysis of the percent of birds positive for pUL47mRFP in FFs over the course of the experiment. Using Fisher's exact test at $p<$ 0.05, there was no significant difference in the total number of chickens positive for experimentally infected chickens with 100% positive by 21 days pi. No naïve contact chickens housed with v3ΔgC were naturally infected, while 88, 75, and 60% of contact chickens were naturally infected with v301B47R, v3-MDVgC, or vΔgC-R, respectively. Using Fisher's exact test at $p<0.05$, there was no significant difference between v3ΔgC-R (P = 0.5105) and v3-MDVgC (P = 1.0000) compared to v301B47R, while v3ΔgC was significantly different ($P = 0.0047$).



[5]**Figure 14. Expression of gC in r301B/1 viruses in chickens.** Pure Columbian chickens were experimentally infected with v301B47R, v3ΔgC, v3ΔgC-R, or v3-MDVgC as described in the Materials and Methods for 56 days. (A and B) Feathers were plucked from v301B47R, v3ΔgC, v3ΔgC-R, and v3-MDVgC at 28 dpi, fixed, then stained using anti-Flag M2 (A) or anti- MDV gC (B) antibodies. FFs obtained from v3ΔgC-R-infected birds were positive for 3×Flag301B gC, while FFs from v3-MDVgC -infected chickens were positive for MDV gC protein. (C) Western blot analysis for 3×Flag301B and MDV gC in FFEs. Whole-cell protein lysates were collected from FFE cells scraped from infected FFs, electrophoresed through a 10% SDS-PAGE gel, transferred to nitrocellulose membranes, and probed for Flag or MDV gC as described in the Materials and Methods. Anti-β-actin antibody was used as internal cellular control.

DISCUSSION

In this report, we tested the importance of the alphaherpesvirus conserved gC protein for horizontal transmission of the MD vaccine strain 301B/1. In addition, we tested whether MDV

[5] Figure 14C was generated by Huai Xu.

gC could compensate for 301B/1 gC in transmission and whether N-terminal tagging of 301B/1 gC would affect its function during horizontal transmission. We were able to confidently conclude that 301B/1 gC is required for horizontal transmission of 301B/1 virus and the addition of a 3×Flag epitope at the N-terminus did not affect its function during transmission. We were also able to conclude that MDV gC can compensate 301B/1 gC during horizontal transmission of 301B/1 virus. This data, combined with our former work on MDV (20, 108), suggests the essential role for the alphaherpesvirus conserved gC during horizontal transmission is a conserved function of avian herpesviruses.

The exact role of MDV and 301B/1 gC during horizontal transmission is not completely understood, but the absolute requirement during natural infection suggests it may be involved in virus-cell binding to cells. For HSV-1, glycoprotein D (gD) binds to the herpesvirus entry mediator (HVEM), nectin-1, nectin-2, or modified heparin sulfate on the surface of cells providing a mechanism for cell tropic binding (115) and it is believed gD of other members of the *Alphaherpesvirinae* perform similar functions. However, formerly we showed that gD is not required for MDV horizontal transmission (20). The absolute requirement for gC in this process suggests it plays may be required for binding to cells during MDV natural infection. Homologues of gC perform multiple functions *in vitro* that include primary attachment of cell-free virus to proteoglycans on the surface of cells (97, 175, 190, 191) but is not required for specific interactions on cells where gD normally performs this function (13, 15). Although gC is not essential for HSV-1 entry *in vitro*, it significantly increases the efficiency of HSV-1 infection by providing an additional binding mechanism (107) and helps shield gB from antibody neutralization (176). Based on the ability of 301B/1 to naturally infect chickens when expressing 301B/1 or MDV gC and the close sequence homology (72.655% protein identity) between the

two proteins (Figure 15), both proteins may target the same chicken cellular protein and cell type to initiate infection. We are currently performing studies to elucidate potential binding partners for gC.

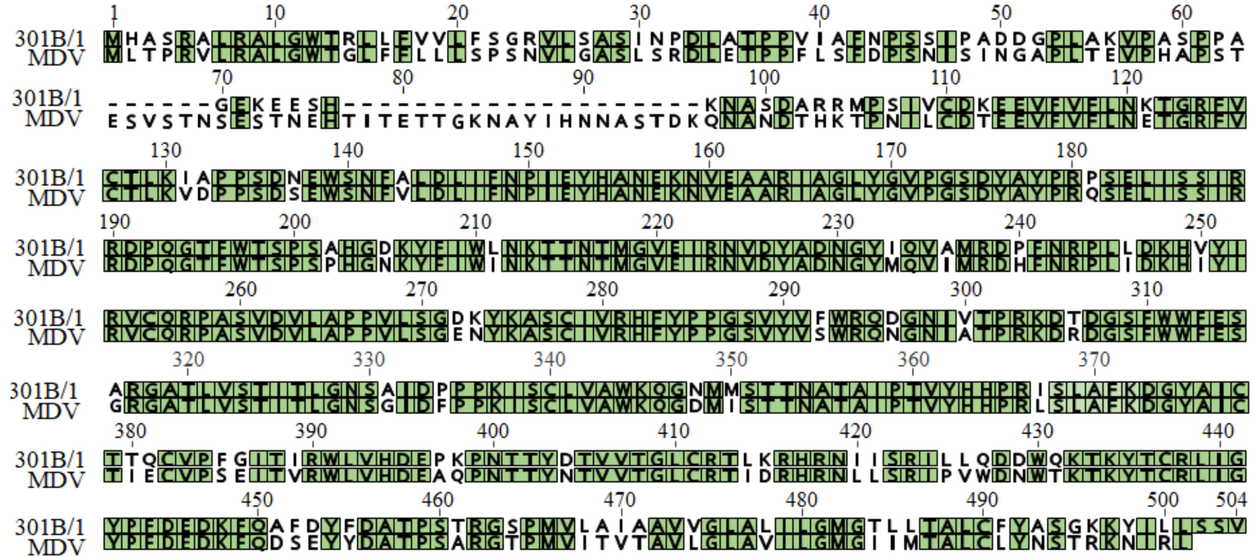


Figure 15. Avian gC protein alignment. Alignment of 301B/1 and MDV (RB-1B strain) gC protein using MUSCLE Alignment in Geneious Prime 2021.0.3 (Biomatters, Inc., San Diego, CA). Green highlighted amino acids are conserved between the two proteins.

Currently, most MD vaccines do not transmit efficiently in chickens and thus cannot compete with virulent virus that does spread efficiently. Read et al. (137) showed that current MD vaccines can enhance transmission of virulent MDV in the field, possibly because they are unable to block infection of chickens and shedding of virus. The fact that 301B/1 is as effective as traditional vaccine strains (181) and is able to efficiently transmit in chickens suggest this vaccine strain may better protect unvaccinated or “missed” chickens in a flock and potentially compete with virulent MDV for replication in the skin. On top of that, swapping MDV gC for 301B/1 will most likely provide better immunogenic responses to MDV as gC is a major antigenic target against MDV (192) and could increase its protective index.

Another important result in this report was that both 301B/1 and MDV gC proteins were secreted into the infected culture cell media suggesting alternative splicing of 301B/1 and MDV

gC occurs in 301B/1 as has been shown for MDV gC during MDV cell culture propagation (109). Further analysis on the splicing of the gC mRNA during 301B/1 replication is warranted; however, this data suggests the splicing of gC transcripts is also conserved in 301B/1 and the mechanism of gC transcript splicing, such as ICP27 and pUL47 (171, 193), may also be conserved among the avian herpesviruses.

In summary, our results support our hypothesis that the absolute requirement of gC during horizontal transmission is conserved among alphaherpesviruses. This report extends our work on MDV gC requirement to the MD vaccine strain 301B/1, and we can conclude at least for some avian herpesviruses with similar pathogeneses, that the functional importance of gC during horizontal transmission is conserved. Further studies are warranted to determine whether gC homologues of other alphaherpesvirus are required for natural infection, although there are limited natural animal models to perform such studies making the avian herpesvirus models important for understanding conserved herpesvirus genes during natural infections.

CHAPTER 4: THE REQUIREMENT OF GLYCORPOTEIN C FOR HORIZONTAL TRANSMISSIONIS FUNCTIONALLY CONSERVED WITHIN THE ALPHAHERPESVIRUS GENUS (*MARDIVIRUS*), BUT NOT THE HOST (*GALLID*)[6]

ABSTRACT

Marek's disease (MD) in chickens is caused by *Gallid alphaherpesvirus* 2, better known as MD alphaherpesvirus (MDV). Current vaccines do not block horizontal transmission from chicken-to-chicken, therefore, understanding MDV horizontal transmission provides important information for the development of potential therapies to protect against MD, while also providing a natural host to study herpesvirus dissemination. It has long been thought that glycoprotein C (gC) of alphaherpesviruses evolved with their host based on their ability to bind and inhibit complement in a species-selective manner. Here, we tested the functional importance of gC during horizontal transmission and host specificity using the natural model system of MDV in chickens through classical compensation experiments. We determined that another chicken alphaherpesvirus (*Gallid alphaherpesvirus* 1 or infectious laryngotracheitis virus) gC could not compensate for chicken MDV gC during horizontal transmission, while turkey alphaherpesvirus (*Meleagrid alphaherpesvirus* 1 or HVT) gC could compensate for MDV gC. ILTV and MDV are *Gallid alphaherpesviruses*; however, ILTV is a member of the *Iltovirus* genus, while MDV is classified as a *Mardivirus* along with HVT. These results suggest that gC is

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[6] This chapter has been previously published on *Viruses 13*(8), p.1419.
Vega-Rodriguez, W., Ponnuraj, N., Garcia, M. and Jarosinski, K.W., 2021. The Requirement of Glycoprotein C for Horizontal Transmission Is Functionally Conserved within the Alphaherpesvirus Genus (Mardivirus), but Not the Host (Gallid). As per the Journal's specification, the author retains the right to reuse this article as a chapter in this thesis without the need to provide signed documentation.

functionally conserved based on the virus genera (*Mardivirus* vs. *Iltovirus*) and not the host (*Gallid* vs. *Meleagrid*).

INTRODUCTION

All avian herpesviruses are members of the *Alphaherpesvirinae* within the *Herpesviridae* (194) and include *Gallid alphaherpesviruses* (GaHV-) 1, 2, and 3 and *Meleagrid alphaherpesvirus* 1 (MeHV-1), better known as infectious laryngotracheitis virus (ILTV) or GaHV-1, Marek's disease alphaherpesvirus (MDV) or GaHV-2, GaHV-3, and turkey herpesvirus (HVT), respectively. MDV, GaHV-3, and HVT are classified into the genus *Mardivirus*, while ILTV is classified as an *Iltovirus* based on genomic sequencing (139).

ILTV (GaHV-1) is the prototypic member of the *Iltovirus* genus that also includes *Psittacid alphaherpesvirus* 1 (PsHV-1) (139). ILTV is highly contagious and results in significant losses to the poultry industry due to severe respiratory disease including conjunctivitis, sinusitis, oculo-nasal discharge, bloody mucus and overall high morbidity (195). For the most part, ILTV is localized to the respiratory tract and horizontal transmission is through shedding of respiratory secretions and transmitted by inhalation of infectious material. Similarly, MDV and HVT infection is initiated in the lungs of chickens or turkeys, respectively. However, infection in the respiratory system is initiated by inhalation of infectious material in dander and dust that contains infectious virus previously shed from feather follicle (FF) epithelial (FFE) skin cells of infected birds. The most well studied *Mardivirus* is MDV where transmission of MDV can be through direct bird-to-bird contact or through indirect contact with infected feathers, dust, or dander. It is not completely understood what mechanism is used by MDV to spread in a flock; however, MDV glycoprotein C (gC) is essential for horizontal transmission (20, 108, 109, 178). The requirement of gC for horizontal transmission was recently confirmed to

be conserved for another *Mardivirus*, GaHV-3 (196) showing the functional importance of gC in *Mardivirus* horizontal transmission. To date, only shedding of infectious virus using a gC-null ILTV was shown to be attenuated *in vivo*, but horizontal transmission was never directly addressed (197).

MDV gC, previously referred to as the “A-antigen”, is encoded by the UL44 gene in the MDV genome, and it is conserved among the *Alphaherpesvirinae*. MDV gC is highly expressed during *in vitro* and *in vivo* propagation; however, its expression is reduced following serial passage in tissue culture cells (108, 148-153). Alphaherpesvirus gC proteins have been shown to be important for multiple functions during herpesvirus infection including primary attachment of cell-free virus to heparin sulfate- and chondroitin-like glycosaminoglycans on the surface of cells (97, 175) and the late steps of egress from cultured cells (97, 99). gC homologues have also been shown to have immune evasion functions. For example, herpes simplex virus 1 (HSV-1), HSV-2, *Suid alphaherpesvirus* 1 (SuHV-1) or pseudorabies virus (PRV), *Equid alphaherpesvirus* 1 (EHV-1), *Saimiriine alphaherpesvirus* 1 (SaHV-1), and *Bovid alphaherpesvirus* 1 (BoHV-1) gC proteins are thought to have immune evasive functions by binding and inhibiting the complement components C3 (100-104, 198, 199).

Following numerous studies examining *Mardivirus* gC proteins during horizontal transmission in chickens (20, 108, 109, 171, 178, 196), we asked whether other alphaherpesvirus gC proteins can compensate for MDV gC during horizontal transmission. It has long been thought that gC proteins evolved with the host based on the species-selective interaction of different alphaherpesvirus gC proteins and complement C3 in which the ability to bind and inhibit C3 was conserved within the virus and host genera (104). That is, HSV-1 and -2 gC efficiently bound and inhibited human C3 but not equine or chicken C3. To test the hypothesis

that gC proteins evolved with the host, we tested the ability of ILTV (GaHV-1) and HVT (MeHV-1) gC to compensate for GaHV-2 (MDV) gC during horizontal transmission. To do this, we replaced MDV gC with ILTV or HVT gC and tested the ability of each virus to spread from chicken to chicken. Interestingly, MDV expressing ILTV gC was unable to spread, while MDV expressing HVT gC efficiently spread from chicken to chicken. Collectively, these results show that the function of gC during horizontal transmission is conserved among the virus genera (*Mardivirus* vs. *Iltovirus*), but not within the host (*Gallid* vs. *Meleagrid*). Our results suggest the evolution of avian gC was, at least partially, based on the pathogenesis of the virus and not through evolution with the host.

MATERIALS AND METHODS

Cell culture and cells

Chick kidney cells (CKCs) were prepared from 2-4 weeks-old specific-pathogen-free (SPF) chickens, obtained from the University of Illinois at Urbana-Champaign (UIUC) Poultry Farm, following standard methods (165) and seeded in growth medium consisting of Medium 199 (Cellgro, Corning, NY, USA) supplemented with 10% tryptose-phosphate broth (TPB), 0.63% $NaHCO_3$ solution, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 4% fetal bovine serum (FBS). Confluent CKCs were maintained in F10.199 medium consisting of a 1:1 mixture of Ham's F10 (Cellgro) and Medium 199 supplemented with 7.5% TPB, 0.63% $NaHCO_3$, 0.2% FBS and antibiotics.

Chicken embryo cells (CECs) were prepared from 10-11-day-old SPF embryos obtained from the UIUC Poultry Farm following standard methods (165). Briefly, primary CECs were seeded in growth medium consisting of Medium 199 supplemented with 10% TPB, 0.63%

$NaHCO_3$ solution, antibiotics, and 4% FBS. Confluent CECs were maintained in Medium 199 supplemented with 7.5% TPB, 0.63% $NaHCO_3$, 0.2% FBS, and antibiotics.

The chicken DF-1-Cre fibroblast cell line (166) was cultivated in a 1:1 mixture of Leibovitz L-15 and McCoy 5A (LM) media (Gibco, Gaithersburg, MD, USA) supplemented with 10% FBS and antibiotics, and maintained in 50 µg/ml Zeocin (Invitrogen, Carlsbad, CA). All cells were maintained at 38°C in a humidified atmosphere of 5% CO_2.

Generation of recombinant (r)MDVs

To clone HVT gC, the HVT UL44 ORF was cloned from a previously described HVT bacterial artificial chromosome (BAC) clone (200) into pcDNA3.1 (Invitrogen) using standard techniques. Briefly, primers overlapping the start and stop codon were designed with HindIII and XbaI sites on the ends and amplified by PCR using Dream Taq PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) using primers shown in Table 6, gel purified, and cloned into the multiple clone site of pcDNA3.1. The ILTV expression construct has been previously described (201).

To generate HVT and ILTV gC transfer vectors, the I-SceI-AphAI cassette from pEP-KanSII was amplified by PCR with Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) using primers shown in Table 6 and inserted into the coding sequence of HVT and ILVT gC using BamHI or AflII, generating pcHVTgC-in and pcILTVgC-in, respectively. To generate pcHVTgC with a C-terminal Myc-His tag, Gibson assembly reaction mixture (New England Biolabs, Inc., Ipswich, MA, USA) was used to insert the Myc-His sequence into pcHVTgC-in to generate pcHVTgC-MycHis-in (pcHVTgC*-in) using primers in Table 6. Subsequently, the gC proteins were amplified from their respective transfer vectors by PCR with Phusion Flash High-Fidelity PCR Master Mix using primers shown in Table 7 and inserted into

rΔgC (178) for mutagenesis in GS1783 *Escherichia coli* cells. Restriction fragment length polymorphism (RFLP) analyses, analytical PCR, and DNA sequencing confirmed all clones were correct. Primers used for sequencing have been previously published (109, 178).

rMDVs were reconstituted by transfecting DF-1-Cre cells, which efficiently remove the mini-F BAC sequences from the viral genome (166), with purified BAC DNA plus Lipofectamine 2000 (Invitrogen) using the manufacturers' instructions. Transfected DF-1-Cre cells were mixed with fresh primary CKCs or CECs until plaques formed, then further propagated in CKCs or CECs until virus stocks could be stored. All rMDVs were used at ≤ 5 passages for *in vitro* and *in vivo* studies.

Table 6. Primers used for cloning genes and generation of shuttle vectors.

Construct[1]	**Direction[2]**	**Sequence (5'→3')[3]**
pcHVTgC	For	CGTAAGCTTTGTGTTTTATTGAGCGGTCG
	Rev	CGTTCTAGATTTGGCCGCTGCGTGATACC
pcHVTgC-in	For	CGACGGGATCCCCAGGGTTCTTTCTGGACTAGTCCTACACCCCGTG GAAA**TAGGGATAACAGGGTAATCGATTT**
	Rev	TTAACGGATCC**GCCAGTGTTACAACCAATTAACC**
pcILTVgC-in	For	TCGCACTTAAGTGTTGAAGCGCTTGGCGCTTATCCTCCATCTGCTGC GCTGGGTA**TAGGGATAACAGGGTAATCGATTT**
	Rev	TTAACCTTAAG**GCCAGTGTTACAACCAATTAACC**
pcHVTgC*-in	Vector For	TGAGTTTAAACCCGCTGATCGTTTAAACCCGCTGATCAGCCT
	Vector Rev	TCGAAGGGCCCTCTAGACTCATTCCGCCCCGGTAGG
	Insert For	TTTACCTACCGGGGCGGAATGAGTCTAGAGGGCCCTTCGAACAAAA
	Insert Rev	GCTGATCAGCGGGTTTAAACGATCAGCGGGTTTAAACTCAATGGT

[1]Construct generated with the set of primers.
[2]Directionality of the primer.
[3]Underlined sequences indicated restriction enzymes used in the cloning. Bold nucleotides indicate priming sites within the mutagenesis template plasmid pEP-KanSII.

Table 7. Primers used for generation of recombinant Marek's disease alphaherpesvirus (MDV).

Modification[1]	Direction[2]	Sequence (5'- 3')[3]
MDV-ILTVgC	Forward	ATACTAAACGATGGAGTTGTGTTTTATGAGCGTTGAAAAC**GATCCACTAGTAACGGCCGCCAG**
	Reverse	TCACGTTTCTCCACTATTGCATTATTGTCTGACAAATAAA**AGCTCTAGCATTTAGGTGACACT**
MDV-HVTgC	Forward	ATACTAAACGATGGAGTTGTGTTTTATGAGCGTTGAAAAC**TGTGTTTTATTGAGCGGTCG**
	Reverse	TCACGTTTCTCCACTATTGCATTATTGTCTGACAAATAAA**TTTGGCCGCTGCGTGATACC**
MDV-HVTgC*	Forward	ATACTAAACGATGGAGTTGTGTTTTATGAGCGTTGAAAAC**TGTGTTTTATTGAGCGGTCG**
	Reverse	TCACGTTTCTCCACTATTGCATTATTGTCTGACAAATAAA**CTGATCAGCGGGTTTAAACG**

[1]Modification to the MDV genome using two-step Red-mediated recombination.
[2]Directionality of the primer.
[3]Bold indicates the template-binding region of the primers for PCR amplification with each respective transfer plasmid.

Measurement of plaque areas

Plaque areas were measured as previously described (163). Briefly, CECs were seeded in 6-well dishes and infected with 100 plaque-forming units (PFU) per well. After 5 days, cells were washed once with phosphate buffered saline (PBS), fixed and permeabilized with PFA buffer (2% paraformaldehyde, 0.1% Triton X-100) for 15 min, and washed twice with PBS. Immunofluorescence assays (IFAs) were performed as previously described (163) using anti-MDV chicken sera and goat anti-chicken IgY-Alexa Fluor® 568 or 488 secondary antibody (Molecular Probes, Eugene, OR). Digital images of 50 individual plaques were collected using an EVOS FL Cell Imaging System (Thermo Fisher Scientific) and compiled using Adobe Photoshop 21.0.1 release. Plaque areas were measured using ImageJ (170) version 1.51k software, and means were determined for each plaque population. Box and Whisker plots were generated using Microsoft® Excel® for Microsoft 365.

Viral replication kinetics in cell culture

To measure viral replication kinetics of viruses in cell culture, qPCR assays were used to measure the relative level of replication as previously described (163). Briefly, CECs were prepared in 6-well tissue culture plates and the next day inoculated with 100 PFU/well. Total DNA was collected from the inoculum and at 0, 24, 48, 72, 96 and 120 h following infection, using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD). Quantification of MDV genomic copies in CECs was performed using primers and probe to MDV ICP4 and chicken iNOS in duplex reactions as previously described (163, 172). All qPCR assays were performed as Absolute Quantification using standard curves in an Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) and the results were analyzed using the QuantStudio Design & Analysis Software v1.4.2. The coefficient of regression was >0.99 for standard curves.

Animal experiments

In Trial 1, commercial Pure Columbian (PC) × New Hampshire (NH) cross chickens were used, while in Trial 2, PC chickens were used. All birds were obtained from the UIUC Poultry Farm (Urbana, IL) and were from MD-vaccinated parents and considered maternal antibody positive at hatch.

In Trial 1, five-day old PC × NH chicks were experimentally infected by intra-abdominal inoculation of 2,000 PFU for each rMDV and housed in separate rooms (n=6/group). For each group, another group of chickens (n=11/group) were left uninfected to act as contact controls to determine whether rMDVs were able to horizontal transmission. In Trial 2, seven-day old PC chickens were infected as in Trial 1 (n=10/group) and housed with uninfected contact chickens (n=9/group). In this trial, experimentally infected birds were sacrificed to collect tissue samples, therefore, only 4-7 chickens were available to measure MD incidence. For both trials, chickens

were evaluated daily, euthanized when birds showed clinical signs of MD (e.g., lethargy, depression, paralysis, torticollis, etc.), and examined for gross MD lesions. Chickens positive for MD included birds succumbing to disease prior to the experimental termination date and birds positive for MD-related lesions at termination of the experiment.

Viral replication kinetics *in vivo*

Whole blood was collected by wing-vein puncture (20) at different time points and DNA was extracted using the E.Z. 96 blood DNA kit from Omega Bio-tek, Inc. (Norcross, GA) using the manufacturer's instructions. Quantification of MDV genomes in blood was performed exactly as described for viral replication kinetics in cell culture.

Monitoring rMDVs in feather follicles (FFs)

To monitor the time at which each rMDV reached the FFs, two flight feathers were plucked from the right and left wings (4 total) of experimentally infected birds weekly starting at 7 days post-infection (pi) for pUL47eGFP expression. A Leica M205 FCA fluorescent stereomicroscope with a Leica DFC7000T digital color microscope camera (Leica Microsystems, Inc., Buffalo Grove, IL) was used to document pUL47eGFP expression. Feather plucking for all experimentally infected birds in Trial 2 was discontinued after 42-days pi because only a few experimentally infected birds remained.

Immunofluorescence Assay (IFA) of FFs

Whole feathers plucked from chickens infected with different rMDVs were fixed using PFA buffer, washed twice with PBS, and then blocked in 10% neonatal calf serum. Fixed FFs were stained with primary anti-gC monoclonal A6 antibody (178), anti-ILTV gC monoclonal mAb8 antibody (201), or anti-Myc (Sigma-Aldrich), and anti-mouse Ig Alexa Fluor 568 (Molecular Probes, Eugene, OR) was used as secondary antibody. Digital images were taken

with Leica DFC7000T digital color microscope camera mounted on Leica M205 FCA fluorescent stereomicroscope. All images were compiled using Adobe Photoshop 21.0.1 release.

Statistical analyses

IBM SPSS Statistics Version 27 software (SPSS Inc., USA) was used for statistical analyses. Plaque size assays were analyzed using one-way analysis of variance (ANOVA) with virus included as fixed effect and the plaque size used as a dependent variable. The normalized data for viral replication (qPCR) were analyzed using two-way ANOVA followed by LSD and Tukey's post hoc tests; virus (V) and time (T) and all possible interactions ($V \times T$) were used as fixed effects. The genomic copies were the dependent variable. Fisher's exact tests were used for infection and transmission experiments. Statistical significance was declared at $p < 0.05$. Mean tests experiments associated with significant interaction ($p < 0.05$) were separated using Tukey's test.

RESULTS

Generation of rMDV expressing ILTV or HVT gC

In order to generate rMDV expressing either ILTV or HVT gC, the coding sequence of both ILTV and HVT UL44 open reading frames (ORFs) were inserted into rΔgC, previously described (178), using two step Red-mediated recombination. Figure 16A shows a schematic representation of rWT, rΔgC, and the newly generated rILTVgC, rHVTgC, and rHVTgC*. Restriction Fragment Length Polymorphism (RFLP) analysis of rWT, rΔgC, rILTVgC, rHVTgC, and rHVTgC* confirmed the integrity of the BAC clones as the predicted banding patterns were observed (Figure 16B). PCR and DNA sequencing were used to confirm that each clone was

correct at the nucleotide level (data not shown) using previously published primers flanking MDV UL44 (20, 109).

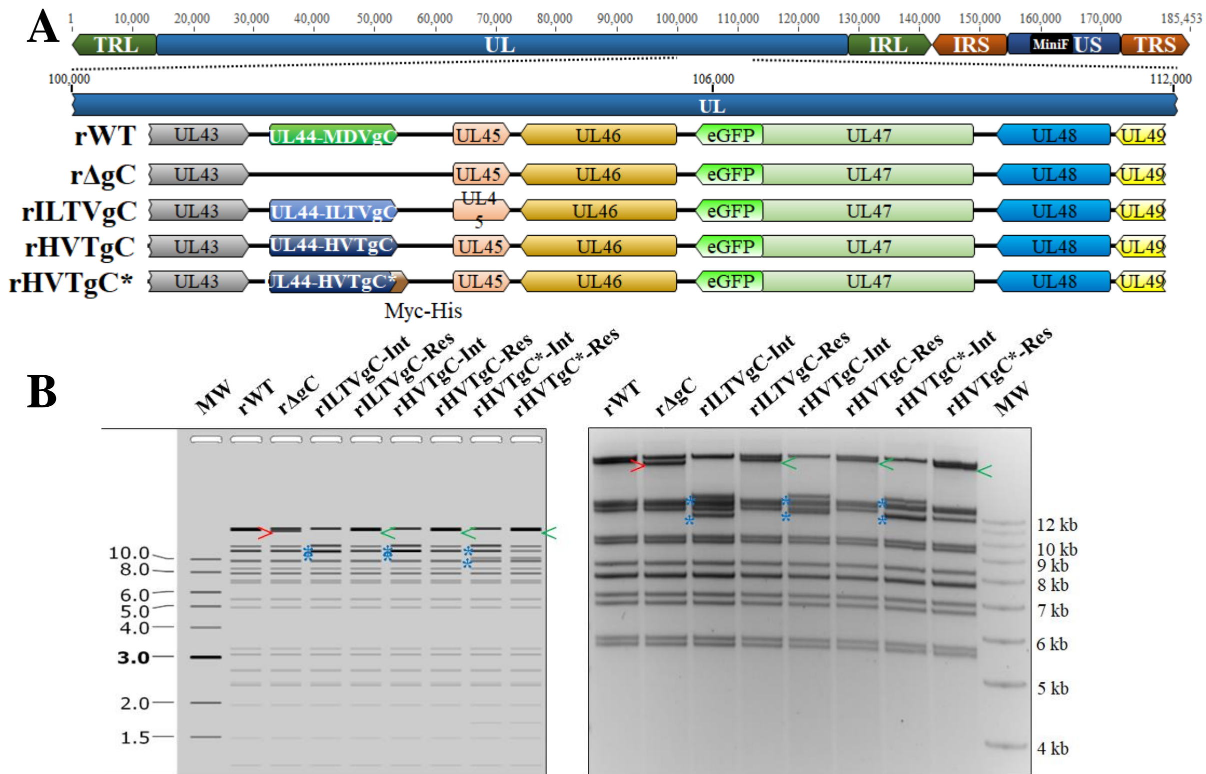

Figure 16. Generation of rMDVs. The MDV genome in schematic form (not to scale) depicting the locations of the terminal (TRL) and internal repeat long (IRL), terminal (TRS) and internal repeat short (IRS), unique long (UL) and unique short (US) regions. A portion of the UL region is expanded to show the relevant genes within this region including UL44 and UL47 (eGFP). Differences between the rMDVs are shown with changes in color representing ILTV UL44 (gC) and HVT UL44 in the MDV UL44 locus. rΔgC shows deletion of MDV UL44 (gC). (B) Predicted RFLP diagram was generated using SnapGene software (from Insightful Science; available at snapgene.com) and is shown in the left panel. BAC DNA obtained for rWT (MDV gC), rΔgC (ΔMDV gC), rILTVgC, rHVTgC, and rHVTgC* integrate and resolved clones were digested with HindIII and used in RFLP analysis. For the generation of rILTVgC, insertion of the AphAI cassette into the HindIII fragment incorporates two additional HindIII sites resulting in the reduction of the HindIII fragment (>) from 23,369 bp to 13,915 (*), and 11,660 bp (*). Following resolution, removal of the AphaI cassette results in a 24,537 bp HindIII site (<). For the generation of rHVTgC, insertion of the AphAI cassette into the HindIII fragment incorporates two additional HindIII sites resulting in 13,997 (*) and 12,003 bp (*) fragments. Following resolution, removal of the AphaI cassette results in a 24,967 bp HindIII site (<). For the generation of rHVTgC*, insertion of the AphAI cassette into the HindIII fragment reduces the HindIII fragment (>) from 23,369 bp to 13,997 (*) and 11,937 bp (*) fragments. Following resolution, removal of the AphAI cassette results in a 24,885 bp HindIII site (<). The molecular weight marker used was the GeneRuler 1 kb Plus DNA Ladder from Thermo Scientific (Carlsbad, CA). Differences between the rMDVs are shown with changes in color representing ILTV UL44 (gC) and HVT UL44 in the MDV UL44 locus.

Replication of rMDV expressing ILTV or HVT gC in cell culture

Following reconstitution of each virus in CECs, plaque size assays were performed to measure the ability of the rMDVs to replicate in cell culture (Figure 17A). As it has been previously described, vΔgC generated significantly larger plaques than vWT (108, 151, 178). Interestingly, vILTVgC and vHVTgC also generated significantly larger plaques than vWT. Addition of the C-terminal tag on HVTgC (vHVTgC*) resulted in reduction of plaque sizes similar to vWT. Multi-step viral replication kinetics showed that vWT was significantly different to all other viruses at 24 hours pi. At 120 hours pi, vWT and vHVTgC were both significantly different to vILTVgC and vHVTgC*, and vHVTgC was significantly different to vΔgC. vΔgC was significantly different to all viruses at this timepoint. (Figure 17B). In all, these results are consistent with the inhibitory effect MDV gC has on MDV cell-to-cell spread and suggests ILTV and HVT gC do not have this inhibitory effect on MDV replication.

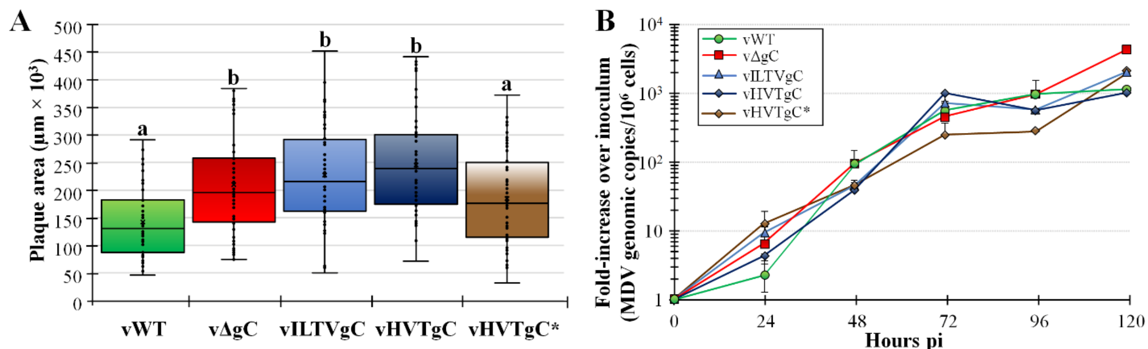

Figure 17. Replication of rMDVs in tissue culture cells. (A) Mean plaque areas for viruses reconstituted from BAC clones described in Figure 16 were measured and shown as box & whisker plots. Significant differences were determined using one-way ANOVA ($p < 0.05$, $n = 250$). Mean plaque areas with different letters are significantly different using LSD and Tukey's post hoc tests ($P \leq 0.05$). (B) Multi-step replication kinetics was used to measure virus replication in CECs. Total viral genome copies were measured for each virus at 24, 48, 72, 96, and 120 h pi. Shown is the fold-increase over inoculum at day 0. vWT was significantly different to all other viruses at 24 hours pi. At 72 hours pi, vHVTgC was significantly different to vΔgC and vHVTgC*, while vHVTgC* was significantly different to vHVTgC. At 96 hours pi, vWT and vΔgC were significantly different to vHVTgC*, and vHVTgC* was significantly different to both, vWT and vΔgC, as well. At 120 hours pi, vWT and vHVTgC were both significantly different to vILTVgC and vHVTgC*. vΔgC was significantly different to all viruses at this timepoint. ($p < 0.05$, two-way ANOVA, LSD and Bonferroni, $n = 3$). Differences between the rMDVs are shown with changes in color representing ILTV UL44 (gC) and HVT UL44 in the MDV UL44 locus.

Replication of rMDVs during experimental infection

Next, we tested the ability of vILTVgC, vHVTgC, and vHVTgC* to replicate in experimentally infected chickens. To measure *in vivo* replication, we determined the MDV genomic copies in blood from experimentally infected chickens over 35 (Trial 1) and 21 (Trial 2) days pi. Results showed that vΔgC was significantly different to all viruses at all time points except for 3- and 35-days pi in Trial 1, while vWT was significantly different to all viruses at 35 days pi (Figure 18A). In Trial 2, no significant differences were measured between all viruses at the same time point (Figure 19A). Since each rMDV expressed pUL47eGFP (97, 99, 102, 150, 151, 153, 175), we monitored the ability of each virus to infect FFE cells required for horizontal transmission. To do this, feathers were plucked from each experimentally infected bird and expression of pUL47eGFP was visualized using a fluorescent micro-stereoscope. There were no significant differences in the total number of birds that were infected in each group ranging from 100% for vΔgC to 83% positive for all other groups in Trial 1 (Figure 18B). In Trial 2, pUL47eGFP positivity ranged from 89% for vHVTgC to 50% for vHVTgC* (Figure 19B).

MD induction of rMDVs during experimental infection

There were no significant differences in MD incidence among the experimentally infected groups in both trials (Figures 18C and 19C). At termination of the experiment, blood cells and serum were collected from all birds that remained negative for pUL47eGFP expression in FFs and did not develop MD. Blood cells were used in qPCR assays to detect viral genomes and serum was used to detect anti-MDV antibodies and all were negative for both assays. Figures 18D and 19D summarize all data combined showing all but one experimentally infected chicken in the vILTVgC group in Trial 2 were infected. The results

show that MDV, ILTV, and HVT gC play no role in virus replication and MD induction in experimentally infected chickens.

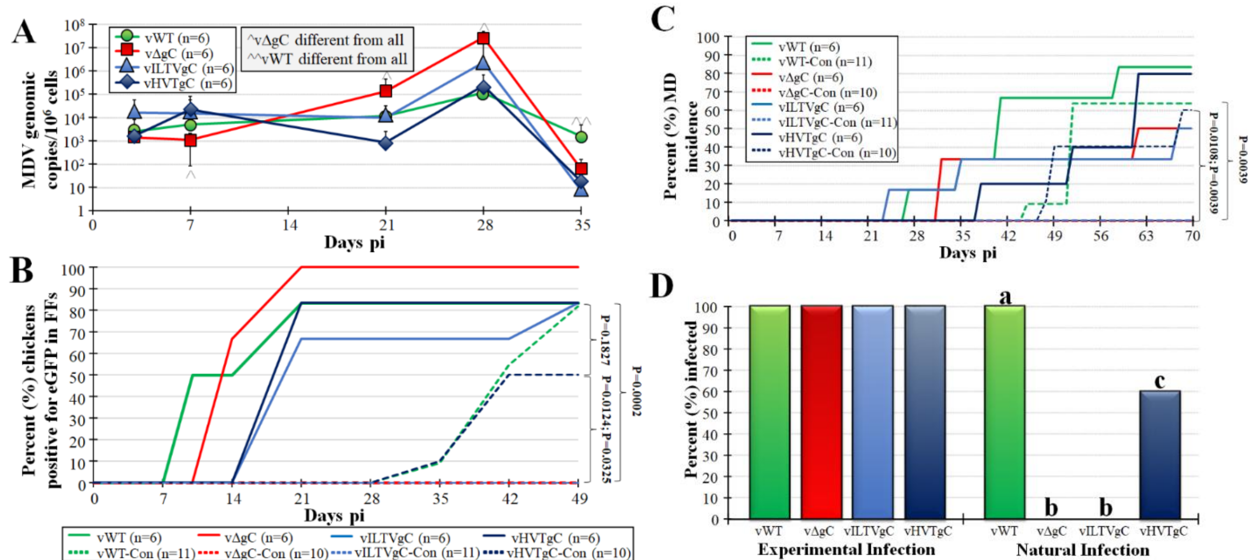

Figure 18. Replication of rMDVs in experimentally and naturally infected chickens - Trial 1. PC × NH chickens were inoculated with vWT, vΔgC, vILTVgC or vHVTgC as described in the Materials and Methods for 70 days. (A) MDV replication was monitored in experimentally infected chickens by quantification of MDV genomes in the blood over the first 3-5 weeks of infection. Shown are the mean MDV genomic copies per 10^6 blood cells ± standard deviations. Only vΔgC was significantly different to all other viruses at 7-, 21-, and 28-days pi, while vWT was significant to all other viruses at 35 days pi ($p < 0.05$). (B) Quantitative analysis of the percent of birds positive for pUL47eGFP in FFs over the course of the experiment. Using Fisher's exact test at $p < 0.05$, there was no significant difference in the total number of chickens positive for experimentally infected chickens. No naïve contact chickens housed with vΔgC or vILTVgC were naturally infected, while all other viruses were able to infect contact chickens. P values for naturally infected chickens are shown using Fisher's exact tests and bolded if significant. (C) Total MD incidence was determined by identification of gross lesions in dead or euthanized chickens. There were no significant differences in the total number of chickens developing MD in experimentally infected chickens. P values for naturally infected chickens are shown using Fisher's Exact Tests. (D) The total number of chickens infected based on viral genomes in the blood, pUL47eGFP positivity in FFs, MD, and anti-MDV antibodies in serum when all data was combined. Fisher's exact tests determined there were no significant differences between all rMDVs in experimentally infected birds. Both vΔgC and vILTVgC were unable to spread to contact chickens. Groups with different letters are significantly different using Fisher's exact tests ($P \leq 0.05$).

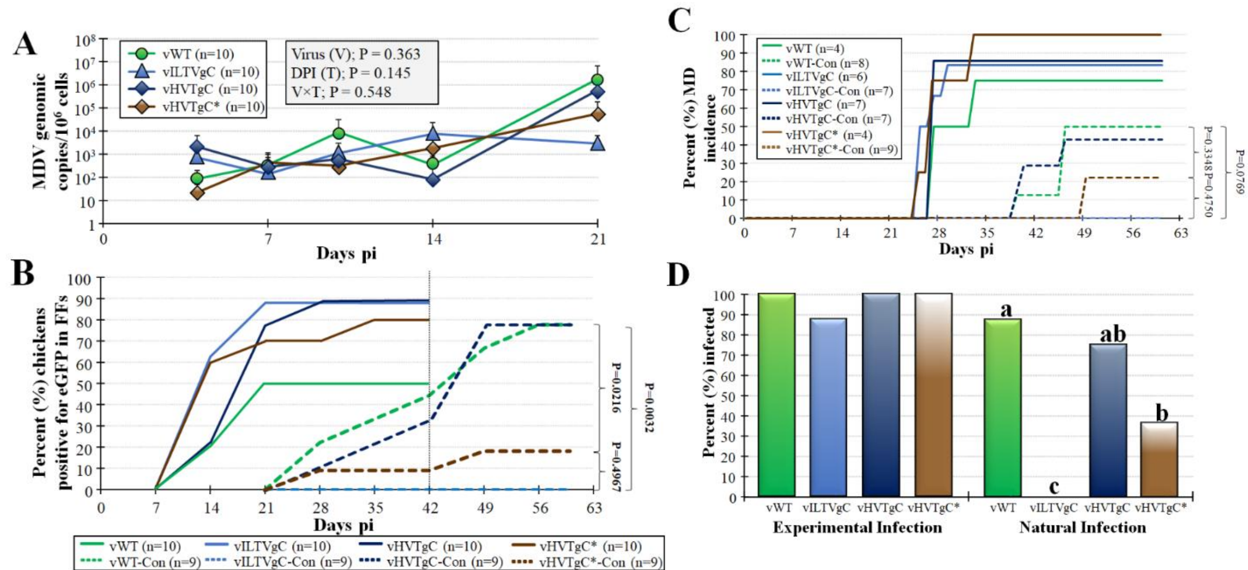

Figure 19. Replication of rMDVs in experimentally and naturally infected chickens - Trial 2. PC chickens were inoculated with vWT, vILTVgC, vHVTgC, or vHVTgC* as described in the Materials and Methods for 63 days. (A) MDV replication was monitored in experimentally infected chickens by quantification of MDV genomes in the blood over the first 3-5 weeks of infection. Shown are the mean MDV genomic copies per 10^6 blood cells ± standard deviations. No significant differences ($p > 0.05$, n = 109) were determined between all viruses at the same time points. (B) Quantitative analysis of the percent of birds positive for pUL47eGFP in FFs over the course of the experiment. Using Fisher's exact test at $p < 0.05$, there was no significant difference in the total number of chickens positive for experimentally infected chickens. No naïve contact chickens housed with vILTVgC were naturally infected, while all other viruses were able to infect contact chickens. P values for naturally infected chickens are shown using Fisher's exact tests and bolded if significant. (C) Total MD incidence was determined by identification of gross lesions in dead or euthanized chickens. There were no significant differences in the total number of chickens developing MD in experimentally infected chickens. P values for naturally infected chickens are shown using Fisher's Exact Tests. (D) The total number of chickens infected based on viral genomes in the blood, pUL47eGFP positivity in FFs, MD, and anti-MDV antibodies in serum when all data was combined. Fisher's exact tests determined there were no significant differences between all rMDVs in experimentally infected birds. vILTVgC was unable to spread to contact chickens. Groups with different letters are significantly different using Fisher's exact tests ($P \leq 0.05$).

Expression of gC proteins in FFs

To determine whether rMDV expressed their respective gC proteins following experimental infection of chickens, feathers plucked during the experiment were stained with specific antibodies. FFs obtained from Trial 1 showed positive staining for MDV gC in the vWT-infected FFs using anti-MDV gC antibody, while vΔgC, vILTVgC, and vHVTgC were negative for MDV gC (Figure 20A). Using anti-ILTV gC antibody, positive staining was observed from vILTVgC-infected FFs showing vILTVgC maintained its expression during in vivo replication (Figure 20B). Since we did not have an antibody specific for HVT

gC, we utilized the C-terminal Myc-His tag on HVT gC to confirm HVTgC* expression was maintained in vHVTgC*-infected FFs in Trial 2 (Figure 20C). These results confirm each rMDV maintained expression of their respective gC protein during experimental infection in chickens.

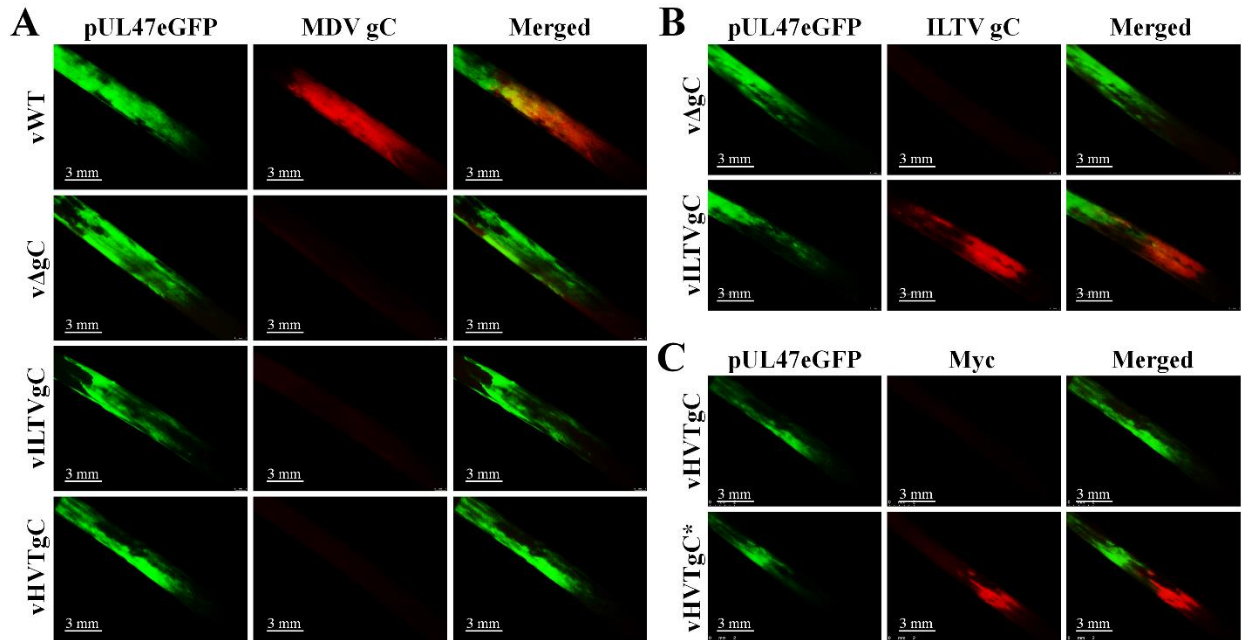

Figure 20. gC protein expression in experimentally infected chickens. Feathers were plucked from vWT, vΔgC, vILTVgC, vHVTgC, and vHVTgC* at 28 days pi from both experimental infection trials. Representative FFs were fixed, then stained using anti-MDV gC (A), -ILTV gC (B), or -Myc (C) antibodies. Expression of pUL47eGFP was used to identify infected FFs.

gC proteins are functionally conserved among the *Mardivirus* and not the *Gallid* genera

Natural infection with rMDVs expressing ILTV or HVT gC

To determine whether each rMDV could horizontal transmission in chickens, both infection in FFs and MD incidence in naturally infected (contact) chickens housed with the experimentally infected groups was determined. No contact chickens were infected in the vΔgC-Con group based on the presence of MDV in the FFs (Figure 18B) and MD incidence (Figure 18C) that is consistent with the requirement of MDV gC for horizontal transmission (20, 108, 178). Interestingly, no contact chickens in the vILTVgC-Con group were naturally

infected based on pUL47eGFP in the FFs (Figures 18B and 19B) and MD incidence (Figures 18C and 19C), while contacts in the vHVTgC-Con (Trials 1 and 2) and vHVTgC*-Con (Trial 2) groups were infected. To confirm contacts negative for pUL47eGFP in FFs or MD were never infected with MDV, qPCR assays for viral DNA in the blood and IFAs for anti-MDV antibodies in their serum were used and these birds were confirmed to be negative for infection (data not shown). A summary of all naturally infected birds is shown in Figures 18D and 19D. All contact birds in the vΔgC-Con (Trial 1) and vILTVgC-Con (Trials 1 and 2) groups were negative for infection. These data show that both HVT gC and HVT gC with a C-terminal Myc-His tag (vHVTgC*) were able to facilitate natural infection of MDV, while ILTV gC (vILTVgC) was unable to compensate for MDV gC. We can conclude that turkey HVT gC is functionally conserved with chicken MDV gC, while chicken ILTV gC is not conserved.

DISCUSSION

It has been shown that alphaherpesvirus gC proteins bind and inhibit complement C3 through species-selective interactions based on their ability to bind to C3 from their respective hosts (104). Thus, it has been suggested the gC proteins evolved with their respective hosts. Utilizing our model for examining the essential role gC proteins play during horizontal transmission in chickens (20, 103, 108, 109, 178, 195) we sought to test the specificity of avian gC proteins. To do this, we exchanged chicken MDV gC with chicken ILTV gC or turkey HVT gC and tested the ability of each gC protein to compensate for MDV gC in MDV horizontal transmission. Our results conclusively showed that MDV expressing ILTV gC was defective during horizontal transmission in chickens, while MDV expressing HVT gC readily spread. Both MDV and ILTV are *Gallid alphaherpesviruses*, but

belong to the *Mardivirus* and *Iltovirus* genera, respectively. HVT is a *Meleagrid alphaherpesvirus* but is characterized in the *Mardivirus* genus with MDV. These results show gC proteins did not necessarily evolve with the host in a species-selective mechanism-based chicken ILTV gC being unable to compensate for chicken MDV gC, while turkey HVT gC was able to compensate for MDV gC during horizontal transmission.

Former studies on MDV plaque sizes generated from gC-null viruses showed significantly increased plaque sizes (20, 109, 149). The mechanism for the increased replication is not understood. Interestingly, MDV expressing ILTV or HVT gC also generated increased plaque sizes (Figure 17A) suggesting both proteins lack the inhibitory function that MDV gC possesses in cell culture. Multi-step replication kinetics also showed differences during cell culture propagation, particularly increased replication of vΔgC at 120 hours pi, the same time at which plaque sizes were measured (Figure 17B). Interestingly, no differences were seen in the vILTVgC and vHVTgC groups that conflicts with the plaque size assay data. The reason for this discrepancy is not known but could be due to the sensitivity of each assay and the output being measured. Since one-step replication kinetics are not possible with cell-associated MDV, we are limited in the assays to measure virus replication in cell culture to identify potential replication defects. However, in all, there were no replication defects for all viruses in cell culture and only increased replication compared to vWT.

Currently, it has not been directly shown whether avian herpesviruses bind and inhibit complement C3; however, the *Mardiviruses* MDV, GaHV-3, and HVT gC proteins encode the conserved cysteines predicted to be important for folding and binding C3 (202). In contrast, ILTV gC does not encode all these cysteines, where only C1-C2 and C5-C8 are

present. Figure 21 shows alignment and predicted motifs identified between the MDV, GaHV-3, HVT, and ILTV gC proteins including the conserved cysteines. It can be predicted these differences result in altered structural features between MDV and ILTV gC folding and helps to explain the lack of compensation for MDV gC during horizontal transmission of MDV. We have formerly shown MDV gC can compensate for GaHV-3 gC (196) in chickens, and in the present report, we show that HVT gC can compensate for MDV gC during horizontal transmission (Figure 18 and 19). Although all combinations of gC compensation experiments between the viruses is not complete, the current data strongly suggests highly conserved functions for gC proteins between these three *Mardiviruses*. Further experiments determining whether avian gC proteins bind and inhibit C3 is warranted to address these differences and the potential role gC and complement may play during natural infection in the host.

Despite encoding the Marek_A Superfamily and Ig-like domains that most gC proteins in the conserved domain database (CDD) (203) have, ILTV gC is only 15% conserved to MDV gC (Table 8). In retrospect, based on the protein sequence homology between MDV and ILTV gC (Figure 21), it is not a surprise that ILTV gC would not compensate for MDV gC and undoubtedly has significantly different functions compared to MDV gC. Interestingly, turkey HVT gC is more conserved to MDV gC (75.7%) than chicken GaHV-3 that is 72.7% identical MDV. Although there is only a small difference between HVT and GaHV-3 gC protein identities to MDV gC (75.7 to 72.7%), this is further evidence that the alphaherpesvirus gC proteins did not necessarily evolve with the host. It is likely the gC protein of ILTV evolved very early during the divergence of the *Gallid alphaherpesviruses* and possibly MDV, GaHV-3, and HVT evolved

after this divergence. These results also suggest the potential cellular targets for MDV, GaHV-3, and HVT gC proteins may be conserved between *Gallid* and *Meleagrid* host genera.

In conclusion, this report suggests the functional conservation of gC during horizontal transmission is not conserved within the host but is conserved within the virus genus and host specificity of MDV in chickens. Future studies in our laboratory are directed at understanding the conserved and divergent functions of gC proteins in the context of natural infection to better understand the evolution of host specificity and disease pathogeneses.

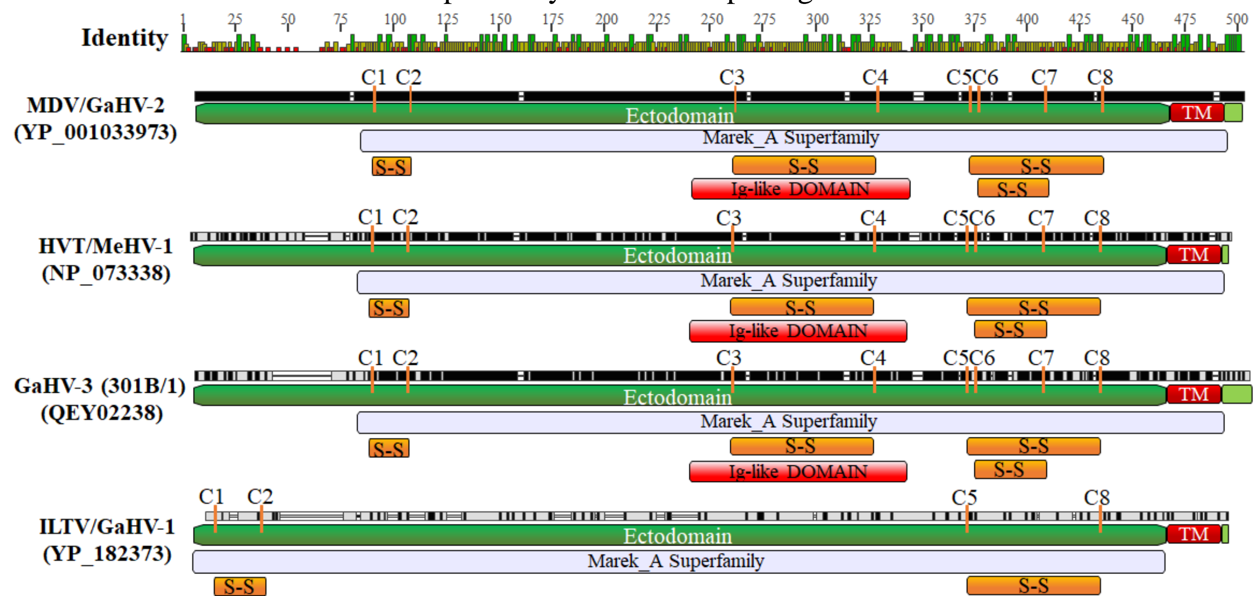

Figure 21. Alignment of MDV (YP_001033973; RB-1B), HVT (NP_073338; FC126), GaHV-3 (QEY02238; 301B/1), and ILTV (YP_182373; USDA ref) gC proteins using MUSCLE Alignment in Geneious Prime 2021.0.3 (Biomatters, Inc., San Diego, CA). The predicted signal sequence was removed from each protein before alignment that represents the ectodomain, transmembrane domain (TM), and short cytoplasmic domain. The eight cysteines predicted to be important for disulfide binding (S-S) and folding are shown, as well as predicted motifs for each protein using MyHits motif scan (204).

Table 8. Protein identities for *Gallid alphaherpesviruses* 1, 2, 3, and *Meleagrid alphaherpesvirus* 1.

Identities[1]	GaHV-1 (ILTV)	GaHV-2 (MDV)	GaHV-3	MeHV-1 (HVT)
GaHV-1 (ILTV)	-	15.2%	18.3%	16.4%
GaHV-2 (MDV)	15.2%	-	72.7%	75.7%
GaHV-3	18.3%	72.7%	-	69.6%
MeHV-1 (HVT)	16.4%	75.7%	69.6%	-

[1]Percent (%) protein identities between *Gallid alphaherpesvirus* (GaHV) 1 (GaHV-1) or infectious laryngotracheitis virus (ILTV), GaHV-2 or Marek's disease virus (MDV), GaHV-3, and *Meleagrid alphaherpesvirus* 1 or turkey herpesvirus (HVT) using MUSCLE Alignment in Geneious Prime 2021.20.3.

CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Current vaccination to prevent MD controls clinical disease but does not induce sterilizing immunity resulting in virus infection and evolution towards greater virulence (135, 205). This emphasizes the need for novel vaccines or therapies to block MD. MDV has not shown a significant change in pathogenesis over the last decade, partially because the virus has not evolved to transmit earlier. However, the emergence of new strains remains a concern. We believe the best way to accomplish sterilizing immunity is by using recombinant DNA vaccines or therapies where genes that are important in the MDV transmission are targeted.

As mentioned in Chapter 2, MDV gC expression is significantly reduced after serial passaging in tissue culture cells. We found that RLORF4 was not responsible for this reduced expression. However, we have also identified another viral protein called the infected cell protein 27 (ICP27) that is involved in regulation of gC. In fact, ICP27 is not only important for MDV replication in chickens, but just like gC, it is important for horizontal transmission of the virus (171). It has also been shown that gC expression is affected by the deletion of ICP27 *in vitro*, where no differences were observed in gC mRNA levels; however, ICP27 is required for gC protein production, as it has been shown for HSV-1 gC (171, 206-208). Further research is necessary; however, we hypothesize that the majority of MDV gC that is secreted *in vitro* is due to a lack of ICP27 intron retention, or possibly ICP27 promoting mRNA splicing as it has been seen for KSHV (209). Additionally, the viral protein VP13/14 (UL47) is an essential factor of horizontal transmission of MDV (193). It has also been shown that VP13/14 enhances the splicing ability and expression of MDV gC (193). Further studies are necessary to understand the complex regulation of MDV gC by ICP27 and VP13/14 during horizontal transmission of MDV.

Interestingly, our preliminary data suggests that GaHV-3 gC is also secreted into the media of infected cells *in vitro* (Chapter 3). Further research is necessary; however, we hypothesize that secretion regulation of gC expression and mRNA splicing of gC proteins is common among avian and mammalian alphaherpesviruses than previously thought.

Here, we also showed that the importance of gC in horizontal transmission is a conserved function among another avian herpesviruses, GaHV-3 strain 301B/1 (Chapter 3). Currently, our laboratory is testing the importance of HVT gC in transmission of HVT in turkeys, their natural host. We hypothesize that HVT gC is also essential for transmission for HVT in turkeys. Consistent with the functional role of gC in transmission, we determined that this conserved function is primarily based on the respective pathogenicity of the viruses and not necessarily through evolution of gC with the host (Chapter 4), as has been previously suggested (104). We believe that these findings will result in better design of vaccines against MD and multivalent recombinant vaccines that express other avian gC antigens (*i.e.*, 301B/1 or HVT vaccines expressing ILTV gC), that could provide higher immunogenic responses and protection than current vaccines.

Collectively, the data presented in this thesis advances our knowledge regarding avian herpesvirus models and their importance in studies that can test conserved genes during natural infection. Moreover, further studies are necessary to determine the precise mechanism involving gC in natural infection including virion binding to the host cell, egress of the virus from the host cell, and immune evasion to improve dissemination of the virus.

REFERENCES

1. Davison AJ. 2002. Evolution of the herpesviruses. Vet Microbiol 86:69-88.
2. International Committee on Taxonomy of V, King AMQ, International Union of Microbiological Societies. Virology D. 2012. Virus taxonomy : classification and nomenclature of viruses : ninth report of the International Committee on Taxonomy of Viruses. Elsevier, London.
3. Cai WZ, Person S, DebRoy C, Gu BH. 1988. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1. An analysis of linker insertion mutants. J Mol Biol 201:575-88.
4. Geraghty RJ, Krummenacher C, Cohen GH, Eisenberg RJ, Spear PG. 1998. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. Science 280:1618-20.
5. Montgomery RI, Warner MS, Lum BJ, Spear PG. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 87:427-36.
6. Shukla D, Liu J, Blaiklock P, Shworak NW, Bai X, Esko JD, Cohen GH, Eisenberg RJ, Rosenberg RD, Spear PG. 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell 99:13-22.
7. Spear PG. 2004. Herpes simplex virus: receptors and ligands for cell entry. Cell Microbiol 6:401-10.
8. Deschamps T, Kalamvoki M. 2018. Extracellular Vesicles Released by Herpes Simplex Virus 1-Infected Cells Block Virus Replication in Recipient Cells in a STING-Dependent Manner. J Virol 92.

9. Temme S, Eis-Hubinger AM, McLellan AD, Koch N. 2010. The herpes simplex virus-1 encoded glycoprotein B diverts HLA-DR into the exosome pathway. J Immunol 184:236-43.

10. Chiang HY, Cohen GH, Eisenberg RJ. 1994. Identification of functional regions of herpes simplex virus glycoprotein gD by using linker-insertion mutagenesis. J Virol 68:2529-43.

11. Whitbeck JC, Peng C, Lou H, Xu R, Willis SH, Ponce de Leon M, Peng T, Nicola AV, Montgomery RI, Warner MS, Soulika AM, Spruce LA, Moore WT, Lambris JD, Spear PG, Cohen GH, Eisenberg RJ. 1997. Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry. J Virol 71:6083-93.

12. Cairns TM, Milne RS, Ponce-de-Leon M, Tobin DK, Cohen GH, Eisenberg RJ. 2003. Structure-function analysis of herpes simplex virus type 1 gD and gH-gL: clues from gDgH chimeras. J Virol 77:6731-42.

13. Cocchi F, Fusco D, Menotti L, Gianni T, Eisenberg RJ, Cohen GH, Campadelli-Fiume G. 2004. The soluble ectodomain of herpes simplex virus gD contains a membrane-proximal pro-fusion domain and suffices to mediate virus entry. Proc Natl Acad Sci U S A 101:7445-50.

14. Zago A, Jogger CR, Spear PG. 2004. Use of herpes simplex virus and pseudorabies virus chimeric glycoprotein D molecules to identify regions critical for membrane fusion. Proc Natl Acad Sci U S A 101:17498-503.

15. Krummenacher C, Supekar VM, Whitbeck JC, Lazear E, Connolly SA, Eisenberg RJ, Cohen GH, Wiley DC, Carfi A. 2005. Structure of unliganded HSV gD reveals a mechanism for receptor-mediated activation of virus entry. EMBO J 24:4144-53.

16. Davison AJ, McGeoch DJ. 1986. Evolutionary comparisons of the S segments in the genomes of herpes simplex virus type 1 and varicella-zoster virus. J Gen Virol 67 (Pt 4):597-611.

17. Parcells MS, Anderson AS, Morgan RW. 1994. Characterization of a Marek's disease virus mutant containing a lacZ insertion in the US6 (gD) homologue gene. Virus Genes 9:5-13.

18. Niikura M, Witter RL, Jang HK, Ono M, Mikami T, Silva RF. 1999. MDV glycoprotein D is expressed in the feather follicle epithelium of infected chickens. Acta Virol 43:159-63.

19. Tan X, Brunovskis P, Velicer LF. 2001. Transcriptional analysis of Marek's disease virus glycoprotein D, I, and E genes: gD expression is undetectable in cell culture. J Virol 75:2067-75.

20. Jarosinski KW, Margulis NG, Kamil JP, Spatz SJ, Nair VK, Osterrieder N. 2007. Horizontal transmission of Marek's disease virus requires US2, the UL13 protein kinase, and gC. J Virol 81:10575-87.

21. Walker JD, Maier CL, Pober JS. 2009. Cytomegalovirus-infected human endothelial cells can stimulate allogeneic CD4+ memory T cells by releasing antigenic exosomes. J Immunol 182:1548-59.

22. Zicari S, Arakelyan A, Palomino RAN, Fitzgerald W, Vanpouille C, Lebedeva A, Schmitt A, Bomsel M, Britt W, Margolis L. 2018. Human cytomegalovirus-infected cells release extracellular vesicles that carry viral surface proteins. Virology 524:97-105.
23. Schumacher D, Tischer BK, Reddy SM, Osterrieder N. 2001. Glycoproteins E and I of Marek's disease virus serotype 1 are essential for virus growth in cultured cells. J Virol 75:11307-18.
24. Mo C, Lee J, Sommer M, Grose C, Arvin AM. 2002. The requirement of varicella zoster virus glycoprotein E (gE) for viral replication and effects of glycoprotein I on gE in melanoma cells. Virology 304:176-86.
25. Cohen JI, Nguyen H. 1997. Varicella-zoster virus glycoprotein I is essential for growth of virus in Vero cells. J Virol 71:6913-20.
26. Mallory S, Sommer M, Arvin AM. 1997. Mutational analysis of the role of glycoprotein I in varicella-zoster virus replication and its effects on glycoprotein E conformation and trafficking. J Virol 71:8279-88.
27. Mallory S, Sommer M, Arvin AM. 1998. Analysis of the glycoproteins I and E of varicella-zoster virus (VZV) using deletional mutations of VZV cosmids. J Infect Dis 178 Suppl 1:S22-6.
28. Brack AR, Dijkstra JM, Granzow H, Klupp BG, Mettenleiter TC. 1999. Inhibition of virion maturation by simultaneous deletion of glycoproteins E, I, and M of pseudorabies virus. J Virol 73:5364-72.
29. Brack AR, Klupp BG, Granzow H, Tirabassi R, Enquist LW, Mettenleiter TC. 2000. Role of the cytoplasmic tail of pseudorabies virus glycoprotein E in virion formation. J Virol 74:4004-16.

30. Seyboldt C, Granzow H, Osterrieder N. 2000. Equine herpesvirus 1 (EHV-1) glycoprotein M: effect of deletions of transmembrane domains. Virology 278:477-89.

31. Zuckermann FA, Mettenleiter TC, Schreurs C, Sugg N, Ben-Porat T. 1988. Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. J Virol 62:4622-6.

32. Balan P, Davis-Poynter N, Bell S, Atkinson H, Browne H, Minson T. 1994. An analysis of the in vitro and in vivo phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. J Gen Virol 75 (Pt 6):1245-58.

33. Sussman MD, Maes RK, Kruger JM, Spatz SJ, Venta PJ. 1995. A feline herpesvirus-1 recombinant with a deletion in the genes for glycoproteins gI and gE is effective as a vaccine for feline rhinotracheitis. Virology 214:12-20.

34. Mijnes JD, van der Horst LM, van Anken E, Horzinek MC, Rottier PJ, de Groot RJ. 1996. Biosynthesis of glycoproteins E and I of feline herpesvirus: gE-gI interaction is required for intracellular transport. J Virol 70:5466-75.

35. Yoshitake N, Xuan X, Otsuka H. 1997. Identification and characterization of bovine herpesvirus-1 glycoproteins E and I. J Gen Virol 78 (Pt 6):1399-403.

36. Johnson DC, Frame MC, Ligas MW, Cross AM, Stow ND. 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J Virol 62:1347-54.

37. Nagashunmugam T, Lubinski J, Wang L, Goldstein LT, Weeks BS, Sundaresan P, Kang EH, Dubin G, Friedman HM. 1998. In vivo immune evasion mediated by the herpes simplex virus type 1 immunoglobulin G Fc receptor. J Virol 72:5351-9.

38. Baines JD, Roizman B. 1993. The UL10 gene of herpes simplex virus 1 encodes a novel viral glycoprotein, gM, which is present in the virion and in the plasma membrane of infected cells. J Virol 67:1441-52.

39. Liang X, Tang M, Manns B, Babiuk LA, Zamb TJ. 1993. Identification and deletion mutagenesis of the bovine herpesvirus 1 dUTPase gene and a gene homologous to herpes simplex virus UL49.5. Virology 195:42-50.

40. Dijkstra JM, Visser N, Mettenleiter TC, Klupp BG. 1996. Identification and characterization of pseudorabies virus glycoprotein gM as a nonessential virion component. J Virol 70:5684-8.

41. Osterrieder N, Neubauer A, Brandmuller C, Braun B, Kaaden OR, Baines JD. 1996. The equine herpesvirus 1 glycoprotein gp21/22a, the herpes simplex virus type 1 gM homolog, is involved in virus penetration and cell-to-cell spread of virions. J Virol 70:4110-5.

42. Rudolph J, Seyboldt C, Granzow H, Osterrieder N. 2002. The gene 10 (UL49.5) product of equine herpesvirus 1 is necessary and sufficient for functional processing of glycoprotein M. J Virol 76:2952-63.

43. Masse MJ, Jons A, Dijkstra JM, Mettenleiter TC, Flamand A. 1999. Glycoproteins gM and gN of pseudorabies virus are dispensable for viral penetration and propagation in the nervous systems of adult mice. J Virol 73:10503-7.

44. Tischer BK, Schumacher D, Messerle M, Wagner M, Osterrieder N. 2002. The products of the UL10 (gM) and the UL49.5 genes of Marek's disease virus serotype 1 are essential for virus growth in cultured cells. J Gen Virol 83:997-1003.

45. Marsden HS, Buckmaster A, Palfreyman JW, Hope RG, Minson AC. 1984. Characterization of the 92,000-dalton glycoprotein induced by herpes simplex virus type 2. J Virol 50:547-54.

46. Richman DD, Buckmaster A, Bell S, Hodgman C, Minson AC. 1986. Identification of a new glycoprotein of herpes simplex virus type 1 and genetic mapping of the gene that codes for it. J Virol 57:647-55.

47. Su HK, Eberle R, Courtney RJ. 1987. Processing of the herpes simplex virus type 2 glycoprotein gG-2 results in secretion of a 34,000-Mr cleavage product. J Virol 61:1735-7.

48. Crabb BS, Nagesha HS, Studdert MJ. 1992. Identification of equine herpesvirus 4 glycoprotein G: a type-specific, secreted glycoprotein. Virology 190:143-54.

49. Engelhardt T, Keil GM. 1996. Identification and characterization of the bovine herpesvirus 5 US4 gene and gene products. Virology 225:126-35.

50. Keil GM, Engelhardt T, Karger A, Enz M. 1996. Bovine herpesvirus 1 U(s) open reading frame 4 encodes a glycoproteoglycan. J Virol 70:3032-8.

51. Drummer HE, Studdert MJ, Crabb BS. 1998. Equine herpesvirus-4 glycoprotein G is secreted as a disulphide-linked homodimer and is present as two homodimeric species in the virion. J Gen Virol 79 (Pt 5):1205-13.

52. Hartley CA, Drummer HE, Studdert MJ. 1999. The nucleotide sequence of the glycoprotein G homologue of equine herpesvirus 3 (EHV3) indicates EHV3 is a distinct equid alphaherpesvirus. Arch Virol 144:2023-33.

53. Davison AJ, Scott JE. 1986. The complete DNA sequence of varicella-zoster virus. J Gen Virol 67 (Pt 9):1759-816.

54. Brunovskis P, Velicer LF. 1995. The Marek's disease virus (MDV) unique short region: alphaherpesvirus-homologous, fowlpox virus-homologous, and MDV-specific genes. Virology 206:324-38.

55. Gomi Y, Sunamachi H, Mori Y, Nagaike K, Takahashi M, Yamanishi K. 2002. Comparison of the complete DNA sequences of the Oka varicella vaccine and its parental virus. J Virol 76:11447-59.

56. Bryant NA, Davis-Poynter N, Vanderplasschen A, Alcami A. 2003. Glycoprotein G isoforms from some alphaherpesviruses function as broad-spectrum chemokine binding proteins. EMBO J 22:833-46.

57. Costes B, Ruiz-Arguello MB, Bryant NA, Alcami A, Vanderplasschen A. 2005. Both soluble and membrane-anchored forms of Felid herpesvirus 1 glycoprotein G function as a broad-spectrum chemokine-binding protein. J Gen Virol 86:3209-3214.

58. Costes B, Thirion M, Dewals B, Mast J, Ackermann M, Markine-Goriaynoff N, Gillet L, Vanderplasschen A. 2006. Felid herpesvirus 1 glycoprotein G is a structural protein that mediates the binding of chemokines on the viral envelope. Microbes Infect 8:2657-67.

59. Viejo-Borbolla A, Munoz A, Tabares E, Alcami A. 2010. Glycoprotein G from pseudorabies virus binds to chemokines with high affinity and inhibits their function. J Gen Virol 91:23-31.

60. Gonzalez-Motos V, Jurgens C, Ritter B, Kropp KA, Duran V, Larsen O, Binz A, Ouwendijk WJD, Lenac Rovis T, Jonjic S, Verjans G, Sodeik B, Krey T, Bauerfeind R, Schulz TF, Kaufer BB, Kalinke U, Proudfoot AEI, Rosenkilde MM, Viejo-Borbolla A. 2017. Varicella zoster virus glycoprotein C increases chemokine-mediated leukocyte migration. PLoS Pathog 13:e1006346.

61. Liu Y, Guan X, Li C, Ni F, Luo S, Wang J, Zhang D, Zhang M, Hu Q. 2018. HSV-2 glycoprotein J promotes viral protein expression and virus spread. Virology 525:83-95.

62. Aubert M, Chen Z, Lang R, Dang CH, Fowler C, Sloan DD, Jerome KR. 2008. The antiapoptotic herpes simplex virus glycoprotein J localizes to multiple cellular organelles and induces reactive oxygen species formation. J Virol 82:617-29.

63. Hutchinson L, Roop-Beauchamp C, Johnson DC. 1995. Herpes simplex virus glycoprotein K is known to influence fusion of infected cells, yet is not on the cell surface. J Virol 69:4556-63.

64. Fuchs W, Klupp BG, Granzow H, Mettenleiter TC. 1997. The UL20 gene product of pseudorabies virus functions in virus egress. J Virol 71:5639-46.

65. Jayachandra S, Baghian A, Kousoulas KG. 1997. Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space. J Virol 71:5012-24.

66. Foster TP, Kousoulas KG. 1999. Genetic analysis of the role of herpes simplex virus type 1 glycoprotein K in infectious virus production and egress. J Virol 73:8457-68.

67. Mo C, Suen J, Sommer M, Arvin A. 1999. Characterization of Varicella-Zoster virus glycoprotein K (open reading frame 5) and its role in virus growth. J Virol 73:4197-207.

68. Dietz P, Klupp BG, Fuchs W, Kollner B, Weiland E, Mettenleiter TC. 2000. Pseudorabies virus glycoprotein K requires the UL20 gene product for processing. J Virol 74:5083-90.

69. Foster TP, Melancon JM, Kousoulas KG. 2001. An alpha-helical domain within the carboxyl terminus of herpes simplex virus type 1 (HSV-1) glycoprotein B (gB) is

associated with cell fusion and resistance to heparin inhibition of cell fusion. Virology 287:18-29.

70. Foster TP, Rybachuk GV, Kousoulas KG. 2001. Glycoprotein K specified by herpes simplex virus type 1 is expressed on virions as a Golgi complex-dependent glycosylated species and functions in virion entry. J Virol 75:12431-8.

71. Foster TP, Melancon JM, Baines JD, Kousoulas KG. 2004. The herpes simplex virus type 1 UL20 protein modulates membrane fusion events during cytoplasmic virion morphogenesis and virus-induced cell fusion. J Virol 78:5347-57.

72. Foster TP, Melancon JM, Olivier TL, Kousoulas KG. 2004. Herpes simplex virus type 1 glycoprotein K and the UL20 protein are interdependent for intracellular trafficking and trans-Golgi network localization. J Virol 78:13262-77.

73. Melancon JM, Fulmer PA, Kousoulas KG. 2007. The herpes simplex virus UL20 protein functions in glycoprotein K (gK) intracellular transport and virus-induced cell fusion are independent of UL20 functions in cytoplasmic virion envelopment. Virol J 4:120.

74. Foster TP, Alvarez X, Kousoulas KG. 2003. Plasma membrane topology of syncytial domains of herpes simplex virus type 1 glycoprotein K (gK): the UL20 protein enables cell surface localization of gK but not gK-mediated cell-to-cell fusion. J Virol 77:499-510.

75. Foster TP, Chouljenko VN, Kousoulas KG. 2008. Functional and physical interactions of the herpes simplex virus type 1 UL20 membrane protein with glycoprotein K. J Virol 82:6310-23.

76. David AT, Baghian A, Foster TP, Chouljenko VN, Kousoulas KG. 2008. The herpes simplex virus type 1 (HSV-1) glycoprotein K(gK) is essential for viral corneal spread and neuroinvasiveness. Curr Eye Res 33:455-67.

77. Chouljenko VN, Iyer AV, Chowdhury S, Chouljenko DV, Kousoulas KG. 2009. The amino terminus of herpes simplex virus type 1 glycoprotein K (gK) modulates gB-mediated virus-induced cell fusion and virion egress. J Virol 83:12301-13.

78. Chouljenko VN, Iyer AV, Chowdhury S, Kim J, Kousoulas KG. 2010. The herpes simplex virus type 1 UL20 protein and the amino terminus of glycoprotein K (gK) physically interact with gB. J Virol 84:8596-606.

79. Jambunathan N, Chowdhury S, Subramanian R, Chouljenko VN, Walker JD, Kousoulas KG. 2011. Site-specific proteolytic cleavage of the amino terminus of herpes simplex virus glycoprotein K on virion particles inhibits virus entry. J Virol 85:12910-8.

80. David AT, Saied A, Charles A, Subramanian R, Chouljenko VN, Kousoulas KG. 2012. A herpes simplex virus 1 (McKrae) mutant lacking the glycoprotein K gene is unable to infect via neuronal axons and egress from neuronal cell bodies. mBio 3:e00144-12.

81. Saied AA, Chouljenko VN, Subramanian R, Kousoulas KG. 2014. A replication competent HSV-1(McKrae) with a mutation in the amino-terminus of glycoprotein K (gK) is unable to infect mouse trigeminal ganglia after cornea infection. Curr Eye Res 39:596-603.

82. Rider PJF, Naderi M, Bergeron S, Chouljenko VN, Brylinski M, Kousoulas KG. 2017. Cysteines and N-Glycosylation Sites Conserved among All Alphaherpesviruses Regulate Membrane Fusion in Herpes Simplex Virus 1 Infection. J Virol 91.

83. Wisner TW, Wright CC, Kato A, Kawaguchi Y, Mou F, Baines JD, Roller RJ, Johnson DC. 2009. Herpesvirus gB-induced fusion between the virion envelope and outer nuclear membrane during virus egress is regulated by the viral US3 kinase. J Virol 83:3115-26.

84. Kato A, Arii J, Shiratori I, Akashi H, Arase H, Kawaguchi Y. 2009. Herpes simplex virus 1 protein kinase Us3 phosphorylates viral envelope glycoprotein B and regulates its expression on the cell surface. J Virol 83:250-61.

85. Robbins AK, Whealy ME, Watson RJ, Enquist LW. 1986. Pseudorabies virus gene encoding glycoprotein gIII is not essential for growth in tissue culture. Journal of virology 59:635-645.

86. Schreurs C, Mettenleiter TC, Zuckermann F, Sugg N, Ben-Porat T. 1988. Glycoprotein gIII of pseudorabies virus is multifunctional. J Virol 62:2251-7.

87. Whealy ME, Robbins AK, Enquist LW. 1988. Pseudorabies virus glycoprotein gIII is required for efficient virus growth in tissue culture. J Virol 62:2512-5.

88. WuDunn D, Spear PG. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J Virol 63:52-8.

89. Mettenleiter TC, Zsak L, Zuckermann F, Sugg N, Kern H, Ben-Porat T. 1990. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. J Virol 64:278-86.

90. Okazaki K, Matsuzaki T, Sugahara Y, Okada J, Hasebe M, Iwamura Y, Ohnishi M, Kanno T, Shimizu M, Honda E, et al. 1991. BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparinlike moiety on the cell surface. Virology 181:666-70.

91. Shieh MT, WuDunn D, Montgomery RI, Esko JD, Spear PG. 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J Cell Biol 116:1273-81.

92. Karger A, Mettenleiter TC. 1993. Glycoproteins gIII and gp50 play dominant roles in the biphasic attachment of pseudorabies virus. Virology 194:654-64.

93. McClain DS, Fuller AO. 1994. Cell-specific kinetics and efficiency of herpes simplex virus type 1 entry are determined by two distinct phases of attachment. Virology 198:690-702.

94. Karger A, Saalmuller A, Tufaro F, Banfield BW, Mettenleiter TC. 1995. Cell surface proteoglycans are not essential for infection by pseudorabies virus. J Virol 69:3482-9.

95. Karger A, Mettenleiter TC. 1996. Identification of cell surface molecules that interact with pseudorabies virus. J Virol 70:2138-45.

96. Herold BC, WuDunn D, Soltys N, Spear PG. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J Virol 65:1090-8.

97. Mettenleiter TC. 1989. Glycoprotein gIII deletion mutants of pseudorabies virus are impaired in virus entry. Virology 171:623-5.

98. Okazaki K, Honda E, Minetoma T, Kumagai T. 1987. Bovine herpesvirus type 1 gp87 mediates both attachment of virions to susceptible cells and hemagglutination. Arch Virol 97:297-307.

99. Osterrieder N. 1999. Construction and characterization of an equine herpesvirus 1 glycoprotein C negative mutant. Virus Res 59:165-77.

100. Friedman HM, Cohen GH, Eisenberg RJ, Seidel CA, Cines DB. 1984. Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. Nature 309:633-5.

101. Eisenberg RJ, Ponce de Leon M, Friedman HM, Fries LF, Frank MM, Hastings JC, Cohen GH. 1987. Complement component C3b binds directly to purified glycoprotein C of herpes simplex virus types 1 and 2. Microb Pathog 3:423-35.

102. Allen GP, Coogle LD. 1988. Characterization of an equine herpesvirus type 1 gene encoding a glycoprotein (gp13) with homology to herpes simplex virus glycoprotein C. J Virol 62:2850-8.

103. Huemer HP, Larcher C, Coe NE. 1992. Pseudorabies virus glycoprotein III derived from virions and infected cells binds to the third component of complement. Virus Res 23:271-80.

104. Huemer HP, Larcher C, van Drunen Littel-van den Hurk S, Babiuk LA. 1993. Species selective interaction of Alphaherpesvirinae with the "unspecific" immune system of the host. Arch Virol 130:353-64.

105. Huemer HP, Nowotny N, Crabb BS, Meyer H, Hubert PH. 1995. gp13 (EHV-gC): a complement receptor induced by equine herpesviruses. Virus Res 37:113-26.

106. Lubinski J, Wang L, Mastellos D, Sahu A, Lambris JD, Friedman HM. 1999. In vivo role of complement-interacting domains of herpes simplex virus type 1 glycoprotein gC. J Exp Med 190:1637-46.

107. Langeland N, Oyan AM, Marsden HS, Cross A, Glorioso JC, Moore LJ, Haarr L. 1990. Localization on the herpes simplex virus type 1 genome of a region encoding proteins involved in adsorption to the cellular receptor. J Virol 64:1271-7.

108. Jarosinski KW, Osterrieder N. 2010. Further analysis of Marek's disease virus horizontal transmission confirms that U(L)44 (gC) and U(L)13 protein kinase activity are essential, while U(S)2 is nonessential. J Virol 84:7911-6.

109. Jarosinski KW, Osterrieder N. 2012. Marek's disease virus expresses multiple UL44 (gC) variants through mRNA splicing that are all required for efficient horizontal transmission. J Virol 86:7896-906.

110. Sievers E, Neumann J, Raftery M, SchOnrich G, Eis-Hubinger AM, Koch N. 2002. Glycoprotein B from strain 17 of herpes simplex virus type I contains an invariant chain homologous sequence that binds to MHC class II molecules. Immunology 107:129-35.

111. Neumann J, Eis-Hubinger AM, Koch N. 2003. Herpes simplex virus type 1 targets the MHC class II processing pathway for immune evasion. J Immunol 171:3075-83.

112. Feenstra V, Hodaie M, Johnson DC. 1990. Deletions in herpes simplex virus glycoprotein D define nonessential and essential domains. J Virol 64:2096-102.

113. Whittaker GR, Taylor LA, Elton DM, Giles LE, Bonass WA, Halliburton IW, Killington RA, Meredith DM. 1992. Glycoprotein 60 of equine herpesvirus type 1 is a homologue of herpes simplex virus glycoprotein D and plays a major role in penetration of cells. J Gen Virol 73 (Pt 4):801-9.

114. Liang X, Pyne C, Li Y, Babiuk LA, Kowalski J. 1995. Delineation of the essential function of bovine herpesvirus 1 gD: an indication for the modulatory role of gD in virus entry. Virology 207:429-41.

115. Spear PG, Eisenberg RJ, Cohen GH. 2000. Three classes of cell surface receptors for alphaherpesvirus entry. Virology 275:1-8.

116. Zelnik V, Majerciak V, Szabova D, Geerligs H, Kopacek J, Ross LJ, Pastorek J. 1999. Glycoprotein gD of MDV lacks functions typical for alpha-herpesvirus gD homologues. Acta Virol 43:164-8.

117. Miriagou V, Stevanato L, Manservigi R, Mavromara P. 2000. The C-terminal cytoplasmic tail of herpes simplex virus type 1 gE protein is phosphorylated in vivo and in vitro by cellular enzymes in the absence of other viral proteins. J Gen Virol 81:1027-31.

118. Peng T, Ponce de Leon M, Novotny MJ, Jiang H, Lambris JD, Dubin G, Spear PG, Cohen GH, Eisenberg RJ. 1998. Structural and antigenic analysis of a truncated form of the herpes simplex virus glycoprotein gH-gL complex. J Virol 72:6092-103.

119. Atanasiu D, Whitbeck JC, Cairns TM, Reilly B, Cohen GH, Eisenberg RJ. 2007. Bimolecular complementation reveals that glycoproteins gB and gH/gL of herpes simplex virus interact with each other during cell fusion. Proc Natl Acad Sci U S A 104:18718-23.

120. Gross ST, Harley CA, Wilson DW. 2003. The cytoplasmic tail of Herpes simplex virus glycoprotein H binds to the tegument protein VP16 in vitro and in vivo. Virology 317:1-12.

121. Avitabile E, Forghieri C, Campadelli-Fiume G. 2007. Complexes between herpes simplex virus glycoproteins gD, gB, and gH detected in cells by complementation of split enhanced green fluorescent protein. J Virol 81:11532-7.

122. Melancon JM, Luna RE, Foster TP, Kousoulas KG. 2005. Herpes simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion, while neither gB and gK nor gB and UL20p function redundantly in virion de-envelopment. J Virol 79:299-313.

123. Gianni T, Amasio M, Campadelli-Fiume G. 2009. Herpes simplex virus gD forms distinct complexes with fusion executors gB and gH/gL in part through the C-terminal profusion domain. J Biol Chem 284:17370-82.

124. Gianni T, Massaro R, Campadelli-Fiume G. 2015. Dissociation of HSV gL from gH by alphavbeta6- or alphavbeta8-integrin promotes gH activation and virus entry. Proc Natl Acad Sci U S A 112:E3901-10.

125. Jons A, Dijkstra JM, Mettenleiter TC. 1998. Glycoproteins M and N of pseudorabies virus form a disulfide-linked complex. J Virol 72:550-7.

126. Mach M, Osinski K, Kropff B, Schloetzer-Schrehardt U, Krzyzaniak M, Britt W. 2007. The carboxy-terminal domain of glycoprotein N of human cytomegalovirus is required for virion morphogenesis. J Virol 81:5212-24.

127. El Kasmi I, Lippe R. 2015. Herpes simplex virus 1 gN partners with gM to modulate the viral fusion machinery. J Virol 89:2313-23.

128. Striebinger H, Funk C, Raschbichler V, Bailer SM. 2016. Subcellular Trafficking and Functional Relationship of the HSV-1 Glycoproteins N and M. Viruses 8:83.

129. Marek J. 1907. Multiple Nervenentzündung (Polyneuritis) bei Hühnern. Deutsche Tierärztliche Wochenschrift 15:417-421.

130. Payne LN. 2004. Pathological responses to infection, p 78-96. *In* Davison TF, Nair VK (ed), Marek's disease: An Evolving Problem. Elsevier Academic Press, Institute for Animal Health, Compton Laboratory, UK.

131. Morrow C, Fehler F. 2004. Marek's disease, p 49-61. *In* Davison F, Nair V (ed), Marek's Disease doi:10.1016/b978-012088379-0/50009-8. Institute for Animal Health, Compton Laboratory, UK.

132. Zelnik V. 2004. Diagnosis of Marek's disease, p 156-ix doi:10.1016/B978-012088379-0/50016-5.

133. Baigent S, Davison F. 2004. Marek's disease virus: Biology and life cycle. Marek's Disease: An Evolving Problem:62-77.

134. Adldinger HK, Calnek BW. 1973. Pathogenesis of Marek's disease: early distribution of virus and viral antigens in infected chickens. Journal of the National Cancer Institute 50:1287-1298.

135. Davison F, Nair V. 2005. Use of Marek's disease vaccines: could they be driving the virus to increasing virulence? Expert Rev Vaccines 4:77-88.

136. Parcells MS, Arumugaswami V, Prigge JT, Pandya K, Dienglewicz RL. 2003. Marek's disease virus reactivation from latency: changes in gene expression at the origin of replication. Poult Sci 82:893-8.

137. Read AF, Baigent SJ, Powers C, Kgosana LB, Blackwell L, Smith LP, Kennedy DA, Walkden-Brown SW, Nair VK. 2015. Imperfect Vaccination Can Enhance the Transmission of Highly Virulent Pathogens. PLoS Biol 13:e1002198.

138. Buckmaster AE, Scott SD, Sanderson MJ, Boursnell ME, Ross NL, Binns MM. 1988. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implications for herpesvirus classification. J Gen Virol 69 (Pt 8):2033-42.

139. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B, Studdert MJ, Thiry E. 2009. The order Herpesvirales. Arch Virol 154:171-7.

140. Lee LF, Wu P, Sui D, Ren D, Kamil J, Kung HJ, Witter RL. 2000. The complete unique long sequence and the overall genomic organization of the GA strain of Marek's disease virus. Proc Natl Acad Sci U S A 97:6091-6.

141. Tulman ER, Afonso CL, Lu Z, Zsak L, Rock DL, Kutish GF. 2000. The genome of a very virulent Marek's disease virus. J Virol 74:7980-8.

142. Izumiya Y, Jang HK, Ono M, Mikami T. 2001. A complete genomic DNA sequence of Marek's disease virus type 2, strain HPRS24. Curr Top Microbiol Immunol 255:191-221.

143. Kingham BF, Zelnik V, Kopacek J, Majerciak V, Ney E, Schmidt CJ. 2001. The genome of herpesvirus of turkeys: comparative analysis with Marek's disease viruses. J Gen Virol 82:1123-35.

144. Spatz SJ, Petherbridge L, Zhao Y, Nair V. 2007. Comparative full-length sequence analysis of oncogenic and vaccine (Rispens) strains of Marek's disease virus. J Gen Virol 88:1080-96.

145. Silva RF, Lee LF, Kutish GF. 2001. The genomic structure of Marek's disease virus. Curr Top Microbiol Immunol 255:143-58.

146. Cui X, Lee LF, Hunt HD, Reed WM, Lupiani B, Reddy SM. 2005. A Marek's disease virus vIL-8 deletion mutant has attenuated virulence and confers protection against challenge with a very virulent plus strain. Avian Dis 49:199-206.

147. Cook ML, Stevens JG. 1968. Labile coat: reason for noninfectious cell-free varicella-zoster virus in culture. J Virol 2:1458-64.

148. Bulow VV, Biggs PM. 1975. Differentiation between strains of Marek's disease virus and turkey herpesvirus by immunofluorescence assays. Avian Pathol 4:133-46.

149. Ikuta K, Ueda S, Kato S, Hirai K. 1983. Monoclonal antibodies reactive with the surface and secreted glycoproteins of Marek's disease virus and herpesvirus of turkeys. J Gen Virol 64 (Pt 12):2597-610.

150. Ikuta K, Ueda S, Kato S, Hirai K. 1983. Most virus-specific polypeptides in cells productively infected with Marek's disease virus or herpesvirus of turkeys possess cross-reactive determinants. J Gen Virol 64 (Pt 4):961-5.

151. Tischer BK, Schumacher D, Chabanne-Vautherot D, Zelnik V, Vautherot JF, Osterrieder N. 2005. High-level expression of Marek's disease virus glycoprotein C is detrimental to virus growth in vitro. J Virol 79:5889-99.

152. Churchill AE, Chubb RC, Baxendale W. 1969. The attenuation, with loss of oncogenicity, of the herpes-type virus of Marek's disease (strain HPRS-16) on passage in cell culture. J Gen Virol 4:557-64.

153. Purchase HG, Burmester BR, Cunningham CH. 1971. Responses of cell cultures from various avian species to Marek's disease virus and herpesvirus of turkeys. Am J Vet Res 32:1811-23.

154. Wilson MR, Southwick RA, Pulaski JT, Tieber VL, Hong Y, Coussens PM. 1994. Molecular analysis of the glycoprotein C-negative phenotype of attenuated Marek's disease virus. Virology 199:393-402.

155. Calnek BW. 2001. Pathogenesis of Marek's disease virus infection. Curr Top Microbiol Immunol 255:25-55.

156. Baaten BJ, Staines KA, Smith LP, Skinner H, Davison TF, Butter C. 2009. Early replication in pulmonary B cells after infection with Marek's disease herpesvirus by the respiratory route. Viral Immunol 22:431-44.

157. Jarosinski KW, O'Connell PH, Schat KA. 2003. Impact of deletions within the Bam HI-L fragment of attenuated Marek's disease virus on vIL-8 expression and the newly identified transcript of open reading frame LORF4. Virus Genes 26:255-69.

158. Spatz SJ, Rue C, Schumacher D, Osterrieder N. 2008. Clustering of mutations within the inverted repeat regions of a serially passaged attenuated gallid herpesvirus type 2 strain. Virus Genes 37:69-80.

159. Spatz SJ, Silva RF. 2007. Polymorphisms in the repeat long regions of oncogenic and attenuated pathotypes of Marek's disease virus 1. Virus Genes 35:41-53.

160. Spatz SJ, Volkening JD, Gimeno IM, Heidari M, Witter RL. 2012. Dynamic equilibrium of Marek's disease genomes during in vitro serial passage. Virus Genes 45:526-36.

161. Liu HC, Soderblom EJ, Goshe MB. 2006. A mass spectrometry-based proteomic approach to study Marek's Disease Virus gene expression. J Virol Methods 135:66-75.

162. Jarosinski KW, Schat KA. 2007. Multiple alternative splicing to exons II and III of viral interleukin-8 (vIL-8) in the Marek's disease virus genome: the importance of vIL-8 exon I. Virus Genes 34:9-22.

163. Jarosinski KW, Osterrieder N, Nair VK, Schat KA. 2005. Attenuation of Marek's disease virus by deletion of open reading frame RLORF4 but not RLORF5a. J Virol 79:11647-59.

164. Spatz SJ, Zhao Y, Petherbridge L, Smith LP, Baigent SJ, Nair V. 2007. Comparative sequence analysis of a highly oncogenic but horizontal spread-defective clone of Marek's disease virus. Virus Genes 35:753-66.

165. Schat KA, Sellers HS. 2008. Cell-culture methods, p 195-203. *In* Dufour-Zavala L, Swayne DE, Glisson JR, Pearson JE, Reed WM, Jackwood MW, Woolcock PR (ed), A laboratory manual for the identification and characterization of avian pathogens, 5th ed. American Association of Avian Pathologists, Jacksonville, FL.

166. Niikura M, Kim T, Silva RF, Dodgson J, Cheng HH. 2011. Virulent Marek's disease virus generated from infectious bacterial artificial chromosome clones with complete DNA sequence and the implication of viral genetic homogeneity in pathogenesis. J Gen Virol 92:598-607.

167. Jarosinski KW, Arndt S, Kaufer BB, Osterrieder N. 2012. Fluorescently tagged pUL47 of Marek's disease virus reveals differential tissue expression of the tegument protein in vivo. J Virol 86:2428-36.

168. Tischer BK, Smith GA, Osterrieder N. 2010. En passant mutagenesis: a two step markerless red recombination system. Methods Mol Biol 634:421-30.

169. Tischer BK, von Einem J, Kaufer B, Osterrieder N. 2006. Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. Biotechniques 40:191-7.

170. Abramoff MD, Magalhaes PJ, Ram SJ. 2004. Image processing with ImageJ. Biophotonics International 11:36-42.

171. Ponnuraj N, Tien YT, Vega-Rodriguez W, Krieter A, Jarosinski KW. 2019. The Herpesviridae conserved multifunctional infected-cell protein 27 (ICP27) is important but not required for replication and oncogenicity of Marek's disease alphaherpesvirus. J Virol 93:e01903-18.

172. Jarosinski KW, Massa PT. 2002. Interferon regulatory factor-1 is required for interferon-gamma-induced MHC class I genes in astrocytes. J Neuroimmunol 122:74-84.

173. Cole RK. 1968. Studies on genetic resistance to Marek's disease. Avian Dis 12:9-28.

174. Lefkowitz EJ, Dempsey DM, Hendrickson RC, Orton RJ, Siddell SG, Smith DB. 2018. Virus taxonomy: the database of the International Committee on Taxonomy of Viruses (ICTV). Nucleic Acids Res 46:D708-D717.

175. Rue CA, Ryan P. 2002. Characterization of pseudorabies virus glycoprotein C attachment to heparan sulfate proteoglycans. J Gen Virol 83:301-9.

176. Komala Sari T, Gianopulos KA, Nicola AV. 2020. Glycoprotein C of herpes simplex virus 1 shields glycoprotein B from antibody neutralization. J Virol 94.

177. Moffat JF, Zerboni L, Kinchington PR, Grose C, Kaneshima H, Arvin AM. 1998. Attenuation of the vaccine Oka strain of varicella-zoster virus and role of glycoprotein C in alphaherpesvirus virulence demonstrated in the SCID-hu mouse. J Virol 72:965-74.

178. Vega-Rodriguez W, Ponnuraj N, Jarosinski KW. 2019. Marek's disease alphaherpesvirus (MDV) RLORF4 is not required for expression of glycoprotein C and interindividual spread. Virology 534:108-113.

179. Osterrieder N, Kamil JP, Schumacher D, Tischer BK, Trapp S. 2006. Marek's disease virus: from miasma to model. Nat Rev Microbiol 4:283-94.

180. Witter RL. 1998. The changing landscape of Marek's disease. Avian Pathology 27:S46-S53.

181. Kim T, Spatz SJ, Dunn JR. 2020. Vaccinal efficacy of molecularly cloned Gallid alphaherpesvirus 3 strain 301B/1 against very virulent Marek's disease virus challenge. J Gen Virol 101:542-552.

182. Jarosinski KW, Vautherot JF. 2015. Differential expression of Marek's disease virus (MDV) late proteins during in vitro and in situ replication: role for pUL47 in regulation of the MDV UL46-UL49 gene locus. Virology 484:213-26.

183. Jarosinski KW. 2012. Dual infection and superinfection inhibition of epithelial skin cells by two alphaherpesviruses co-occur in the natural host. PLoS One 7:e37428.

184. Jarosinski KW, Carpenter JE, Buckingham EM, Jackson W, Knudtson K, Moffat JF, Kita H, Grose C. 2018. Cellular stress response to varicella-zoster virus infection of human skin includes highly elevated interleukin-6 expression. Open Forum Infect Dis 5:ofy118.

185. Donnelly M, Elliott G. 2001. Fluorescent tagging of herpes simplex virus tegument protein VP13/14 in virus infection. J Virol 75:2575-83.

186. Verhagen J, Hutchinson I, Elliott G. 2006. Nucleocytoplasmic shuttling of bovine herpesvirus 1 UL47 protein in infected cells. J Virol 80:1059-63.

187. Jarosinski KW. 2012. Marek's disease virus late protein expression in feather follicle epithelial cells as early as 8 days postinfection. Avian Dis 56:725-31.

188. Lee LF, Liu X, Witter RL. 1983. Monoclonal antibodies with specificity for three different serotypes of Marek's disease viruses in chickens. J Immunol 130:1003-6.

189. Isfort RJ, Stringer RA, Kung HJ, Velicer LF. 1986. Synthesis, processing, and secretion of the Marek's disease herpesvirus A antigen glycoprotein. J Virol 57:464-74.

190. Banfield BW, Leduc Y, Esford L, Visalli RJ, Brandt CR, Tufaro F. 1995. Evidence for an interaction of herpes simplex virus with chondroitin sulfate proteoglycans during infection. Virology 208:531-9.

191. Spear PG, Longnecker R. 2003. Herpesvirus entry: an update. J Virol 77:10179-85.

192. Van Zaane D, Brinkhof JM, Westenbrink F, Gielkens AL. 1982. Molecular-biological characterization of Marek's disease virus. I. Identification of virus-specific polypeptides in infected cells. Virology 121:116-32.

193. Chuard A, Courvoisier-Guyader K, Remy S, Spatz S, Denesvre C, Pasdeloup D. 2020. The Tegument Protein pUL47 of Marek's Disease Virus Is Necessary for Horizontal Transmission and Is Important for Expression of Glycoprotein gC. J Virol 95.

194. McGeoch DJ, Rixon FJ, Davison AJ. 2006. Topics in herpesvirus genomics and evolution. Virus Res 117:90-104.

195. Gowthaman V, Kumar S, Koul M, Dave U, Murthy T, Munuswamy P, Tiwari R, Karthik K, Dhama K, Michalak I, Joshi SK. 2020. Infectious laryngotracheitis: Etiology, epidemiology, pathobiology, and advances in diagnosis and control - a comprehensive review. Vet Q 40:140-161.

196. Vega-Rodriguez W, Xu H, Ponnuraj N, Akbar H, Kim T, Jarosinski KW. 2021. The requirement of glycoprotein C (gC) for interindividual spread is a conserved function of gC for avian herpesviruses. Sci Rep 11:7753.

197. Pavlova SP, Veits J, Blohm U, Maresch C, Mettenleiter TC, Fuchs W. 2010. In vitro and in vivo characterization of glycoprotein C-deleted infectious laryngotracheitis virus. J Gen Virol 91:847-57.

198. Rother RP, Rollins SA, Fodor WL, Albrecht JC, Setter E, Fleckenstein B, Squinto SP. 1994. Inhibition of complement-mediated cytolysis by the terminal complement inhibitor of herpesvirus saimiri. J Virol 68:730-7.

199. Fodor WL, Rollins SA, Bianco-Caron S, Rother RP, Guilmette ER, Burton WV, Albrecht JC, Fleckenstein B, Squinto SP. 1995. The complement control protein homolog of herpesvirus saimiri regulates serum complement by inhibiting C3 convertase activity. J Virol 69:3889-92.

200. Baigent SJ, Petherbridge LJ, Smith LP, Zhao Y, Chesters PM, Nair VK. 2006. Herpesvirus of turkey reconstituted from bacterial artificial chromosome clones induces protection against Marek's disease. J Gen Virol 87:769-76.

201. Mundt A, Mundt E, Hogan RJ, Garcia M. 2011. Glycoprotein J of infectious laryngotracheitis virus is required for efficient egress of infectious virions from cells. J Gen Virol 92:2586-2589.

202. Rux AH, Moore WT, Lambris JD, Abrams WR, Peng C, Friedman HM, Cohen GH, Eisenberg RJ. 1996. Disulfide bond structure determination and biochemical analysis of glycoprotein C from herpes simplex virus. J Virol 70:5455-65.

203. Lu S, Wang J, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Marchler GH, Song JS, Thanki N, Yamashita RA, Yang M, Zhang D, Zheng C, Lanczycki CJ, Marchler-Bauer A. 2020. CDD/SPARCLE: the conserved domain database in 2020. Nucleic Acids Res 48:D265-D268.

204. Pagni M, Ioannidis V, Cerutti L, Zahn-Zabal M, Jongeneel CV, Hau J, Martin O, Kuznetsov D, Falquet L. 2007. MyHits: improvements to an interactive resource for analyzing protein sequences. Nucleic Acids Res 35:W433-7.

205. Churchill AE, Payne LN, Chubb RC. 1969. Immunization against Marek's disease using a live attenuated virus. Nature 221:744-7.

206. Sedlackova L, Perkins KD, Lengyel J, Strain AK, van Santen VL, Rice SA. 2008. Herpes simplex virus type 1 ICP27 regulates expression of a variant, secreted form of glycoprotein C by an intron retention mechanism. J Virol 82:7443-55.

207. Rice SA, Knipe DM. 1990. Genetic evidence for two distinct transactivation functions of the herpes simplex virus alpha protein ICP27. J Virol 64:1704-15.

208. Smith IL, Hardwicke MA, Sandri-Goldin RM. 1992. Evidence that the herpes simplex virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. Virology 186:74-86.

209. Majerciak V, Yamanegi K, Allemand E, Kruhlak M, Krainer AR, Zheng ZM. 2008. Kaposi's sarcoma-associated herpesvirus ORF57 functions as a viral splicing factor and promotes expression of intron-containing viral lytic genes in spliceosome-mediated RNA splicing. J Virol 82:2792-801.