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"Novel genetic factors in Marek's disease virus pathogenesis and establishment of a cell culture-based viral integration system"

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4. Abbreviations

AAV-2	Adeno-associated virus 2
ILTV	Infectious laryngotracheitis virus
b-ZIP	Basic leucine zipper
BAC	Bacterial artificial chromosome
bp	Base pairs
CEC	Chicken embryo cells
circRNA	Circular rnas
d.p.i	Days post-infection
DCs	Dendritic cells
DDR	DNA damage repair
DEV	Duck enteritis virus
DR	Direct repeat
EBV	Epstein–Barr virus
eGFP	Enhanced green fluorescent protein
FFE	Feather follicle epithelia
FISH	Fluorescence in situ hybridization
GaAHV-2	Gallid alphaherpesvirus 2
GaAHV-3	Gallid alphaherpesvirus 3
gC	Glycoprotein C
HCMV	Human cytomegalovirus
HHV-6A	Human herpesvirus 6A
HHV-6B	Human herpesvirus 6B
HHV-7	Human herpesvirus 7
HR	Homologous recombination
HSV-1	Human herpesvirus 1
HSV-2	Human herpesvirus 2
HVT	Herpesvirus of turkey
IRL	Internal repeat long
IRS	Internal repeat short
kpb	Kilobase pair
KSHV	Kaposi's sarcoma-associated herpesvirus
LATs	Latency-associated transcripts
lncRNA	Long noncoding rnas
MD	Marek’s disease
MDV	Marek’s disease virus

MeAHV-1	Meleagrid alphaherpesvirus 1
MeHV-1	Meleagrid alphaherpesvirus 1
Meq	Marek's ecori Q fragment
miRNA	Microrna
mTMR	Multiple telomeric repeats
NK	Natural killer
ORFs	Open reading frames
pp14	Phosphoprotein 14
pp38	Phosphoprotein 38
RFLP	Restriction fragment length polymorphism
RLORF4	Repeat long open reading frame 4
SPF	Specific-pathogen-free
SSA	Single-strand annealing
sTMR	Short telomeric repeats
TK	Thymidine kinase
TMRs	Telomeric repeats
TRL	Terminal repeat long
TRS	Terminal repeat short
UL	Unique long
US	Unique short
vIL-8	Viral interleukin 8
vLIP	Viral lipase
vTR	Viral telomerase RNA subunit
VZV	Varicella-zoster virus

5. Introduction

5.1. Herpesviruses

Herpesviruses have been discovered not only in vertebrates, such as humans, mammals, birds, and fishes, but also in animals of lower taxa, including molluscs. These infections are associated with various clinical manifestations, ranging from asymptomatic or mild symptoms to severe diseases, such as lymphoma.

5.1.1. Classification of herpesviruses

The classification of herpesviruses is complex. All herpesviruses share a typical virion morphology and harbor the linear, double-stranded DNA genomes, comprising the order *Herpesvirales* (Davison et al. 2009). *Herpesvirales* encompasses three distinct virus families: the *Herpesviridae,* which infect mammals, birds, and reptiles, and the *Alloherpesviridae,* which include the herpesviruses of fish and amphibians; and the *Malacoherpesviridae,* which contain the herpesviruses of invertebrates. The *Herpesviridae* family was further classified into three subfamilies: *Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae* defined primarily based on genetic properties (Gatherer et al. 2021). Alphaherpesviruses have a variable mammalian host range and also infect avian and reptilian hosts, with a relatively rapid reproductive cycle. They are able to establish latent infection primarily but not exclusively in sensory ganglia (Bloom 2016). Betaherpesviruses have a restricted host range and are characterized by a long reproductive cycle (over seven days) and latency in secretory glands, lymphoreticular cells, kidneys, and other tissues (Flamand 2018; Aimola et al. 2020). Gammaherpesviruses also have a limited natural host spectrum, specifying replication and latency in T or B lymphocytes (Lieberman 2013).

At present, nine herpesviruses infect humans (Gatherer et al. 2021), and among them are representatives from each of the three subfamilies: human herpesvirus 1 and 2 (also termed as Herpes simplex viruses 1 and 2, HSV-1 and HSV-2), human herpesvirus 3 (also termed as varicella-zoster virus, VZV), human herpesvirus 4 (also termed as Epstein–Barr virus, EBV), human herpesvirus 5 (also termed as human cytomegalovirus, HCMV), human herpesvirus 6A and 6B (HHV-6A and HHV-6B), human herpesvirus 7 (HHV-7), and human herpesvirus 8 (also termed as Kaposi's sarcoma-associated herpesvirus, KSHV). Additionally, some crucial diseases among domestic animal species are caused by herpesvirus. Avian herpesviruses (AHV) are enzootic among the poultry and even cause hemorrhagic or neoplastic diseases. AVH, such as Marek’s disease virus (MDV), Duck enteritis virus (DEV), and Infectious laryngotracheitis virus (ILTV), are of great importance to the commercial poultry industry because they are causing substantial economic losses from disease and/or the cost of vaccination (Ou and Giambrone 2012; Devlin et al. 2016; Dhama et al. 2017).

5.1.2. Virion structure and genome organization

The morphology of herpesviruses is characteristic and complex, distancing from other viruses. A typical herpesvirus consists of a core, capsid, tegument, and envelope, forming a spherical to pleomorphic, 120 – 250 nm diameter virion (Figure 1). The size variation is due to variability in the thickness of the tegument and the state of the envelope [10]. The core of the mature virion contains a linear, double-stranded DNA ranging from 124 – 295 kilobase pair (kbp) in length, which is packed in an orderly manner in the form of a torus (Liu and Zhou 2007). The virus core was protected by the nucleocapsid, which is a T=16 icosahedron containing 162 capsomers arranged with 150 hexons, 11 pentons, and one portal (Gatherer et al. 2021). Surrounding the capsid is the tegument, consisting of inner and outer layers. Teguments contain more than 20 proteins, some of which are present in hundreds of copies per virion (Newcomb et al. 2012). The envelope, the outer covering of the virion, is composed of an altered membrane and some virally encoded glycoproteins as short spike structures on the surface (Mettenleiter et al. 2009).

Herpesvirus DNAs are linear and double-stranded. Features of herpesviruses genomes vary from the size of genomes, base composition to sequence arrangements: (1) the length of the genome is 125 to 295 kbp; (2) the composition of guanine plus cytosine content (G+C) varies from 31% to 77%; (3) the genomes can be divided into six groups designated as class A to F. Except for class F genome, other genome classes possess terminal and internal repeated sequences (Osterrieder et al. 2014; Davison 2007). Class A genomes consist of a unique sequence flanked by a direct repeat region (DR) with several kbp in size. Class B genomes also have directly repeated sequences at the termini, containing a variable number of tandem repeated. Class C genomes harbor terminal sequences as class B, but consist of an internal set of direct repeats that is unrelated to the terminal sequences. Class D and E genomes contain two unique regions (U_L and U_S), each flanked by terminal and internal inverted repeats (TR_L/IR_L and TR_S/IR_S). The class E genomes are the most complex, TR_L/IR_L of which is much larger and which contains the a-like sequences at internal and terminal repeat junctions.

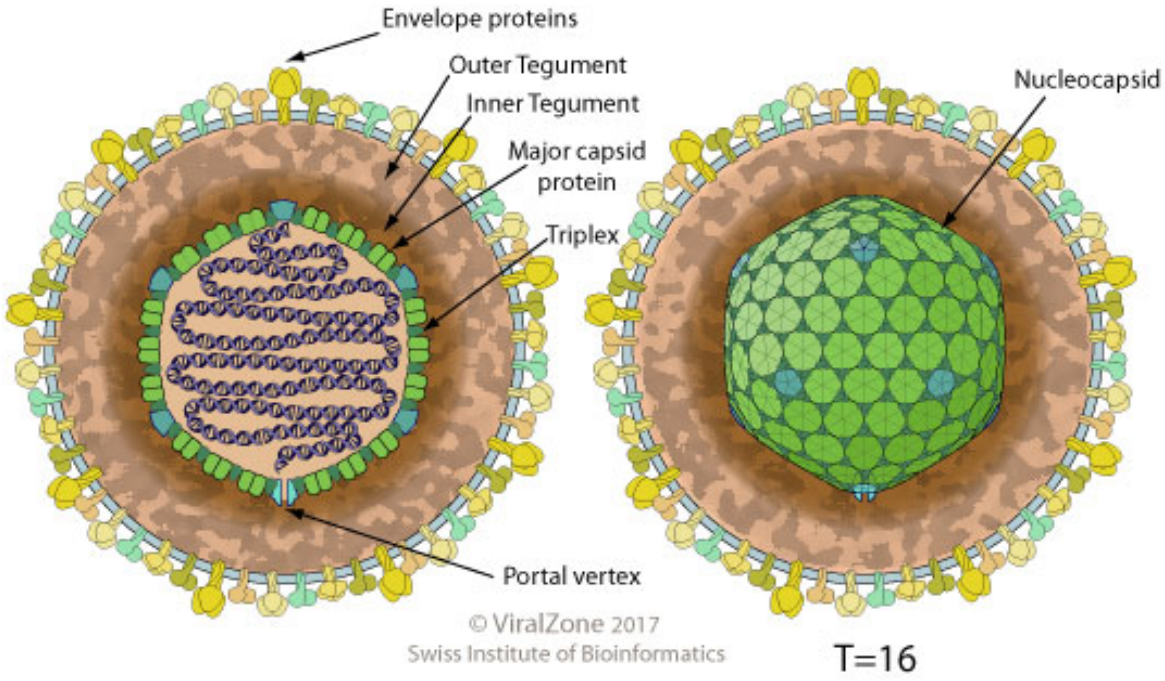

Figure 1: Schematic representation of the alphaherpesvirus virion.

The alphaherpes-virus contains a double-stranded DNA genome that is monopartite and has a size of 120 - 240 kbp, protected by the nucleocapsid that consists of 162 capsomers (150 are hexameric and 12 pentameric). An outer and inner amorphous tegument surrounds the capsid. The outer layer consists of glycoprotein spikes embedded in the lipid bilayer of the envelope. (Image from https:// viralzone.expasy.org/178?outline=all_by_species)

5.2. Marek's disease virus (MDV)

The herpesvirus of interest in this thesis is MDV, an oncogenic alphaherpesvirus that causes neurological disorders, immunosuppression, and visceral tumors in chickens (Osterrieder et al. 2006). MDV is widely disseminated and ubiquitous, causing significant losses in the poultry industry due to increased morbidity and mortality of chickens.

MDV belongs to the genus *Mardivirus* in the subfamily *Alphaherpesviruses*. The genus *Mardivirus* have birds as natural hosts, and are best known for causing severe and sometimes fatal infections on domesticated poultry such as ducks and chickens. Presently, members of the genus *Mardivirus* can be divided into six species (Gatherer et al. 2021). Marek's disease virus, in the species *Gallid alphaherpesvirus 2* (GaAHV-2), consists of all pathogenic strains, which are variable in their pathogenic and oncogenic potential and are classified as mildly virulent (m) to virulent (v), very virulent (vv) and very virulent plus (vv+) (Calnek 2001). Gallid alphaherpesvirus 3 (GaAHV-3), in the species *Gallid alphaherpesvirus 3*, contains avirulent and non-oncogenic strains, such as the SB-1 strain. Meleagrid alphaherpesvirus 1 (MeAHV-

1), also known as herpesvirus of turkey (HVT), is in the species *Meleagrid alphaherpesvirus 1* and a ubiquitous, nonpathogenic virus of domestic turkeys (Gatherer et al. 2021). The sequence similarity between the three serotypes ranges from 50% to 80% (Davison 2001; Kingham et al. 2001).

5.2.1. History of Marek's disease virus

József Marek, a Hungarian veterinary pathologist, firstly reported a generalized polyneuritis disease in chickens in 1907 (Osterrieder et al. 2006). Two decades later, Pappenheimer and colleagues proposed that visceral lymphoma was part of the symptoms of this disease, and they suggested to term "neurolymphomatosis gallinarum" (Pappenheimer et al. 1929b; Pappenheimer et al. 1929a). In the 1960s, it had developed into a much more severe disease with a commonly 10-30% mortality rate, causing severe economic losses to the poultry industry (Biggs 1968). A cell-associated herpesvirus was identified in tumor cells, as the causative agent of Marek's disease (MD) (Churchill and Biggs 1967). In the following decades, the devastating Marek's disease came under control after introducing vaccines and widespread vaccination with a live attenuated virus HPRS16 or HVT in the early 1970s (Churchill et al. 1969; Okazaki et al. 1970). Over time, the virulence of MDV gradually increased, with breakthroughs in the vaccine protection. A bivalent or polyvalent vaccine formulation, combined HVT and SB-1, was introduced to provide enhanced protection as the second-generation vaccine against MDV (Calnek et al. 1983). However, they provided less protection for the emergence of highly virulent MDV, such as the RB-1B strain (Schat 2016). Dr. Rispens, coming from the Dutch Central Veterinary Institute, firstly isolated, tested, and established the third generation MDV vaccine, which is the gold standard vaccine—CVI988-Rispens, an attenuated MDV-1 strain (Rispens et al. 1972a; Rispens et al. 1972b). All MDV vaccines provide efficiency against disease progressions, such as neurological deficits and tumorigenesis, but do not induce sterile immunity, thereby establishing infection and exposing mature viruses to the environment. Notably, the modified-live virus vaccine significantly reduced the incidence of MD but allowed infection, replication, and transmission of viruses, potentially prompting the evolution of more virulent pathogens (Gandon et al. 2001; Read et al. 2015). Considering this, MD remains a threat to the poultry industry.

5.2.2. MDV pathogenesis

MDV has a very complex lifecycle, involving different cell types and targeting many organs. The "Cornell model of MDV pathogenesis," a widely accepted MDV lifecycle model, delineated the natural *in vivo* infection into four interlacing main phases: (i) entry, (ii) replication, (iii) latency, and (iv) spread (Figure 2) (Calnek 2001; Bertzbach et al. 2020).

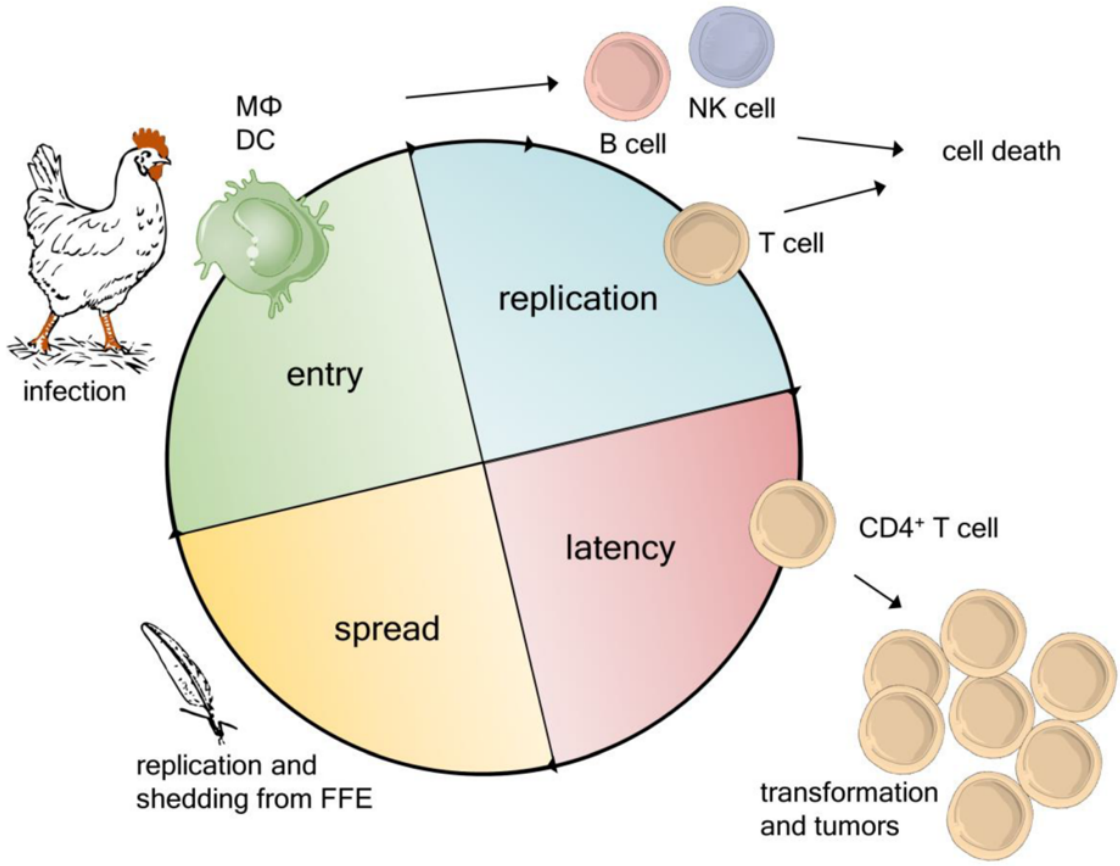

Figure 2: Schematic illustration of the MDV lifecycle

MDV infection is initiated by inhalation of infectious dust. The virus is transmitted by mononuclear phagocytes to lymphoid organs such as the spleen, thymus and bursa, where it multiplies lytically in lymphocytes. A latency period can be established by infected T-cells. Latently and/or lytically infected T cells transport the virus to the skin and follicular epithelium (FFE), where cell-free MDV is produced. Additionally, MDV can transform latently infected T cells, leading to malignant lymphomas. The figure was obtained from (Bertzbach et al. 2020).

5.2.2.1. MDV primary infection and lytic replication

The natural route of MDV infection starts with the inhalation of dust and feather dander containing cell-free virus particles (Calnek et al. 1970). After intratracheal inoculation, initial virus replication is evident in mononuclear phagocytes, mainly macrophages or dendritic cells (DCs), and pulmonary B cells (Barrow et al. 2003; Baaten et al. 2009), which then deliver the virus to primary lymphoid tissues- the bursa of Fabricius, thymus, and spleen. MDV encodes a secreted CXC chemokine, termed vIL-8 or vCXCL13, which can recruit B cells and a subset of $CD4^+$ T cells (Engel et al. 2012; Haertle et al. 2017). Early cytolytic replication occurs mainly in B cells, peaking between 3-7 days post-infection (d.p.i) (Shek et al. 1983; Baigent et al. 1998). However, B cells are completely unnecessary for MDV replication. In the absence of B

cells, $CD4^+$ and $CD8^+$ T cells are compensated for the loss of B cells and facilitate efficient virus cytolytic replication in these organs (Schermuly et al. 2015; Bertzbach et al. 2018b). In addition, primary chicken endothelial cells and natural killer (NK) cells are also shown to be susceptible to cytolytic infection (Lion et al. 2018; Bertzbach et al. 2019b). During the early cytolytic replication, MDV causes a transient thymus and bursa of Fabricius atrophy (Berthault et al. 2018). MDV-induced apoptosis affects infected cells in the thymus but also non-infected cells in the bursa, which causes a massive depletion of T- and B-cells in these organs (Morimura et al. 1996; Berthault et al. 2018). However, MDV-infected bursal B-cells are associated with a drastic delay in proliferation/cell cycle progression, leading to a prolonged B-cell survival (Trapp-Fragnet et al. 2021). The depletion of lymphocytes caused by MDV ultimately leads to immunosuppression (Berthault et al. 2018).

5.2.2.2. MDV latency

The establishment of latency is a hallmark for all herpesviruses, enabling them to escape from the host immune system and maintain life-long viral genetic material (Cohen 2020). From approximately 7 d.p.i, MDV enters the latent infection when lytic replication can no longer be detectable, and tumors are not yet formed. Latently infected cells are mainly activated $CD4^+$ T cells, although B cells, CD8+ cells, and $CD4^-CD8^-$ T cells can also be involved (Schat et al. 1982; Schat et al. 1991; Lee et al. 1999). Few proteins and RNAs are produced during the MDV latency, most of which originate from both the long and short repeat regions (Sugaya et al. 1990). Three regions of transcripts have been mainly studied: latency-associated transcripts (LATs), the 1.8-kb family of transcripts and transcripts originating from the *meq* (MDV004) gene (Osterrieder et al. 2006). Latency is associated with viral genome integration into the telomeres of the host cells, ensuring the lifelong persistence of the virus in the host. The genomic DNA of MDV harbors TMRs identical to host telomere sequences $(TTAGGG)_n$, which facilitates viral integration into the telomeres of host chromosomes to maintain their genetic material (Kaufer et al. 2011; Greco et al. 2014; Osterrieder et al. 2014). This will be discussed in section 5.3.

5.2.2.3. MDV shedding

Latently and/or lytically infected T cells transport the virus to the skin and feather follicle epithelia (FFE), where is also the site of MDV replication (Couteaudier and Denesvre 2014; Bertzbach et al. 2020; Davidson 2020). Viral DNA can be detected as early as 5-7 d.p.i in FFE and increase substantially until 28 d.p.i, reaching a plateau (Baigent et al. 2005; Islam and Walkden-Brown 2007). Notably, infectious mature virions have been observed in FFE, from which cell-free infectious virions can be purified (Calnek et al. 1970; Couteaudier and Denesvre 2014). Form infected feather follicles, MDV is shed into the environment via

releasing infectious dander and feather debris at 12-14 d.p.i, causing horizontal disease spread (Jarosinski et al. 2014). MDV shedding makes the virus ubiquitous and causes environmental contamination. The infectious MDV can last up to 16-28 weeks in the environment (Carrozza et al. 1973).

5.2.3. MDV genome structure

The double-stranded MDV DNA genome is approximately 180 kilo base pair and belongs to class E consisting of a unique long (U_L) and a unique short (U_S) sequence that is flanked by the terminal (TR_L and TR_S) and internal (IR_L and IR_S) inverted repeat regions (Tulman et al. 2000; Osterrieder et al. 2006). Two virus species in the genus *Mardivirus*, GaAHV-3 and HVT, share significant sequence homology throughout the genome except within the repeat-long regions (Figure 3). More than 100 open reading frames (ORFs) have been annotated in the MDV genome. The unique regions mainly harbor genes that are conserved and have homologs in other alphaherpesviruses, typically involved in DNA replication, production of progeny virus, and various other processes critical to the viral lifecycle (Lee et al. 2000). In addition, several MDV-unique genes in these regions are essential for viral replication and pathogenesis, such as viral lipase (vLIP, encoded by LORF2) and MDV012 (Kamil et al. 2005; Schippers et al. 2015; Liao et al. 2021). In contrast, the repeat regions harbor many MDV-specific genes, RNAs, and other sequence fragments that are important for MDV pathogenesis, tumorigenesis, and latency (Bertzbach et al. 2018a).

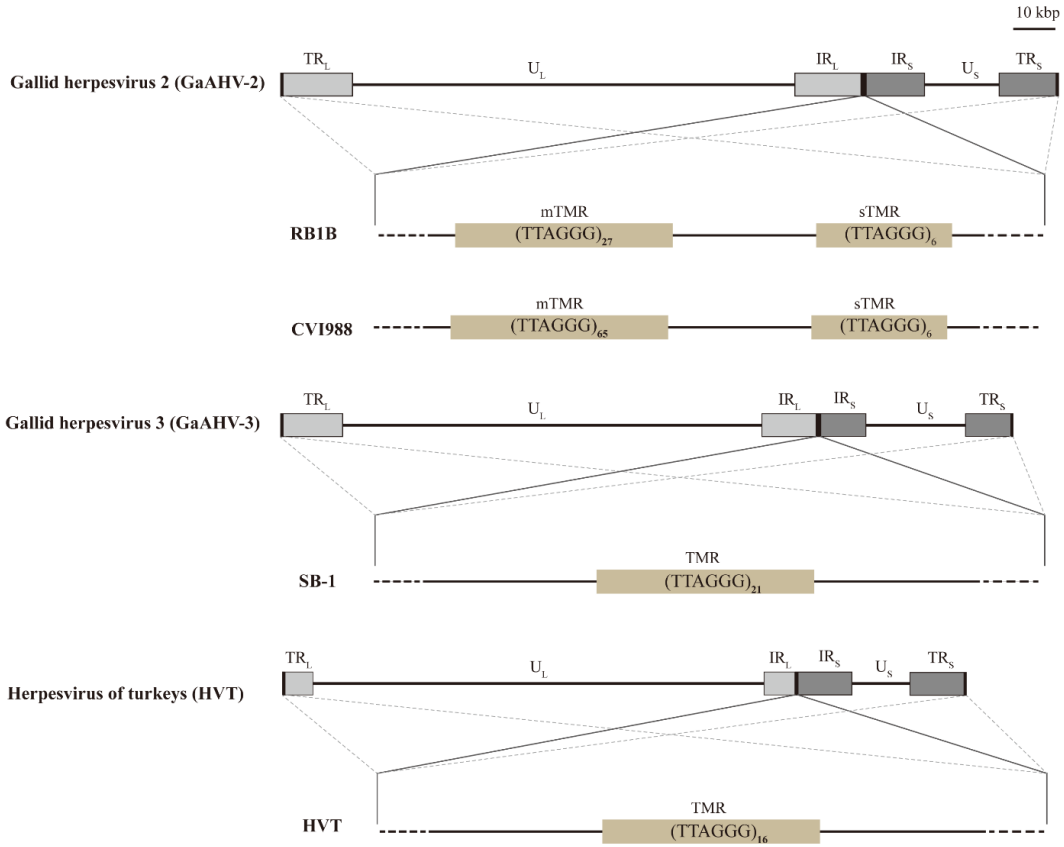

Figure 3: Overview of the vvMDV strain RB1B and MDV vaccine genomes that harbor telomeric repeat (TMR) arrays

These viruses have a class E genome, consisting of a unique long (U_L) and short (U_S) that are flanked by terminal (TR_L and TR_S) and internal (IR_L and IR_S) inverted repeat regions. A focus on the regions harboring TMRs is shown.

5.2.4. MDV virulence factors

MDV encodes about 100 proteins that orchestrate the virus life cycle and/or contribute to pathogenesis. Several viral factors involved in MDV pathogenesis and/or tumorigenesis have been identified, and some of the mechanisms have been deciphered (Figure 4). Research on deciphering the mechanistic descriptions of these viral factors is still ongoing to completely understand the mechanisms for MDV pathogenesis. To date, the most crucial oncoprotein in MDV pathogenesis is the major oncoprotein Meq (Marek's EcoRI Q fragment), a basic leucine zipper (b-ZIP) transcription factor (Jones et al. 1992). Meq is constitutively expressed in lytic and latent infection and even in MDV-driven lymphoblastoid cells (Arumugaswami et al. 2009a; Arumugaswami et al. 2009b) and can regulate cellular and viral gene expression, such as p53, retinoblastoma protein, and vTR (Levy et al. 2003; Brown et al. 2006; Osterrieder et al. 2006; Zhao et al. 2009; Conradie et al. 2019). Moreover, few amino acid changes in the *meq* gene significantly alter virulence, vaccine resistance, and viral shedding ability, providing fundamental insights into evolutionary adaptations (Conradie et al. 2019; Conradie et al. 2020).

Furthermore, other protein-coding genes, including vIL-8, RLORF4 (repeat long open reading frame 4), phosphoprotein 14 (pp14), and phosphoprotein 38 (pp38), have been identified as virulence factors of which deletion mutants severely impaired disease development and tumor formation (Parcells et al. 2001; Reddy et al. 2002; Jarosinski et al. 2005; Engel et al. 2012; Tahiri-Alaoui et al. 2013).

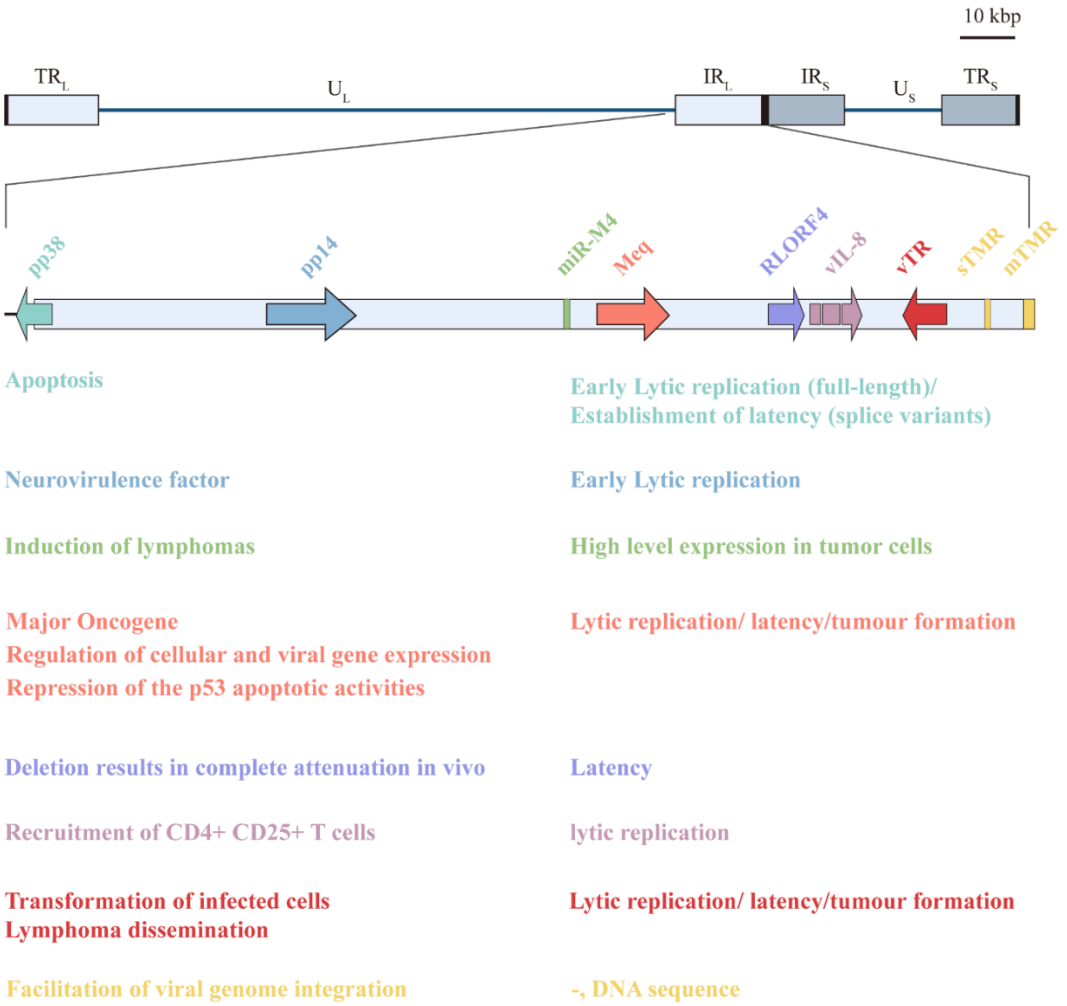

Figure 4: Schematic representation of the MDV genome with a focus on the viral factors involved in pathogenesis and tumorigenesis.

The repeat regions contain MDV-specific genes encoding for proteins or RNA. Deletion one of them has been shown that are responsible for pathogenesis, cellular tropism, tumorigenesis, and/or latency. The positions of the following genes or DNA sequences are shown in the IRL: pp38, pp14, miR-M4, major oncogene *meq*, RLORF4, viral chemokine vIL-8, viral telomerase RNA (vTR), and TMR. The corresponding functions and expression stages of viral virulence factors are respectively listed.

Usually, with strict size limitations, the majority of herpesvirus transcripts are unspliced, but a few genes are also known to be spliced. Moreover, viruses, including herpesvirus, utilize host alternative splicing machinery to generate multiple proteins from their limited genome (Berget

et al. 1977; Toth 2008; Nojima et al. 2009). Notably, tumor viruses produce spliced RNA isoforms from bicistronic or polycistronic RNA transcripts, which is essential for diversifying their transcriptomes during virus infection and oncogenesis (Zheng 2010; Shuda et al. 2011; Ajiro and Zheng 2015; Ajiro and Zheng 2019). For MDV, a small proportion of genes containing introns have already been identified and characterized, such as vIL-8, glycoprotein C (gC), pp38, and MDV012, which play crucial roles in the viral lifecycle (Parcells et al. 2001; Li et al. 2006; Jarosinski and Osterrieder 2012; Schippers et al. 2015). Furthermore, alternative RNA splicing occurs when several splice donors and/or acceptors are eligible and facultative, based on which multiple spliced transcripts have been identified in the region containing *meq*, RLORF4/5a, and vIL-8 (Jarosinski and Schat 2007; Okada et al. 2007). An alternative spliced transcript, Meq/vIL-8, has been reported that it was as a negative regulator of Meq and showed different nuclear mobilities (Anobile et al. 2006). The abrogation of the Meq/vIL-8 isoform in the very virulent plus (vv+) MDV 686 strain slightly accelerated the onset of disease and increased the prevalence of persistent neurological disease, suggesting that it could be a self-controller to avoid early mortality of infected chickens (Liao et al. 2020). Comprehensive transcriptome analyses of MDV-infected cells revealed extensive splicing of viral genes resulting in coding and non-coding RNA transcripts. However, most of their biological relevance remains poorly understood (Bertzbach et al. 2019a; Sadigh et al. 2020).

The MDV genome also encodes for a rich repertoire of non-coding RNAs, including viral microRNAs (miRNA), the viral telomerase RNA subunit (vTR), long noncoding RNAs (lncRNA), and circular RNAs (circRNA) (Fragnet et al. 2003; Yao and Nair 2014; Bertzbach et al. 2020; Chasseur et al. 2022). MDV has been discovered to encode 14 miRNA precursors that produce 26 mature miRNAs. MDV-miR-M4, a human miRNA miR-155 ortholog, plays a critical role in the induction of tumors (Zhao et al. 2011; Bondada et al. 2019; Zhang et al. 2019). miR-M7-5p, which originated from the LAT-cluster, may contribute to the establishment and maintenance of latency (Strassheim et al. 2012). A well-study non-coding RNA is vTR which plays a crucial role in MDV-driven tumor formation (Fragnet et al. 2003; Trapp et al. 2006). vTR shares high sequence identity with chicken telomerase RNA and the conserved stem-loop structure (Fragnet et al. 2003). Furthermore, MDV harbors a specific sequence in its genome, called telomeric repeat (TMR) arrays, which facilitate viral integration to establish latency in infected-CD4+ cells and tumor cells (Kaufer et al. 2011; Greco et al. 2014; Osterrieder et al. 2014).

5.3. Herpesvirus genome integration

The herpesviruses' hallmark is mainly their ability to establish lifelong latency and reactivate to infect other hosts. The key to successful latency establishment is that the viral genome is

stably maintained in the nuclei of latently infected cells. Most herpesviruses maintain their genome as circular episomes in one or multiple copies, which are or are not physically associated with host chromosomes (Osterrieder et al. 2014; Cohen 2020). Intriguingly, HHV-6A/B and MDV have been found to integrate their genome into the telomeres of host chromosomes (Delecluse and Hammerschmidt 1993; Luppi et al. 1993; Arbuckle et al. 2010). Telomeres are specialized and conserved structures at the ends of vertebrate chromosomes, consisting of tandem hexanucleotide $(TTAGGG)_n$ associated with numerous proteins. Analysis of these genomes revealed that HHV-6A/B and MDV harbor arrays of TMRs, identical to those of the host (Osterrieder et al. 2014).

5.3.1. MDV Telomeric repeats

TMRs were identified in all sequenced MDV genomes to date. The number and arrays of TMRs are flexible and variable among different strains and species (Figure 3). TMRs are located within the *a*-like sequences, which are present at both ends of the linear genome and in an inverted orientation at the IR_L-IR_S junction. For MDV, each *a*-like sequence harbors two TMR arrays: multiple telomeric repeats (mTMR), with specific variable lengths, and short telomeric repeats (sTMR), with a fixed number of six repeats. For GaAHV-3 and HVT, they only harbor mTMR.

5.3.2. Mechanism of MDV integration

The presence of viral TMRs in some herpesviruses, including HHV-6 and MDV, facilitates directed integration into host telomeres and then ensures faithful viral maintenance in host cells during cell division (Kaufer et al. 2011; Greco et al. 2014; Wallaschek et al. 2016b). Previously, studies have demonstrated that the exact TMR sequences are crucial for MDV integration. Mutation of viral TMRs, replaced with either structurally similar $(TAAGGC)_n$ or completely scrambled repeats $(ACGACA)_n$, preclude efficient integration into host telomeres (Kaufer et al. 2011). Tumor cells driven from these MDV mutants-infected animals harbored only a single MDV integration site that was not located in host telomeres, but elsewhere in the chromosome. Functional analysis of the individual TMR arrays revealed that either mTMR or sTMR affects MDV integration (Kaufer et al. 2011; Greco et al. 2014). sTMR show a dual role in the MDV life cycle. Complete deletion of sTMR abrogates virus replication, while extensive mutation of sTMR does not, indicating that the sTMR arrays likely serve as spacers between the packaging signal pac-1 and the DR-1 cleavage site to ensure viral replication (Volkening and Spatz 2013; Greco et al. 2014). Mutation of the sTMR alone slightly reduced viral integration, which still occurred in up to four chromosomes, indicating that the sTMR plays a relatively minor role in integration. On the other hand, deletion of the mTMR alone severely impaired the integration frequency (Kaufer et al. 2011). Analysis of tumor cells derived from

mTMR deletion mutant-infected animals demonstrated that viral integration only occurred in a single locus that was random integration as concatemers, indicating that the mTMR plays a significant role in the MDV genome integration into host telomeres (Kaufer et al. 2011).

Viral TMRs and homology to the host telomeres play a crucial role in HHV-6 and MDV integration, indicating that the insertion of the virus genome into host telomeres most likely occurs via the homologous recombination (HR) pathway (Osterrieder et al. 2014; Aimola et al. 2020). Many cellular recombinases have been proposed to be involved in this integration, such as Rad51, Rad52, which are essential for DNA damage repair (DDR) and single-strand annealing (SSA) by HR, respectively. Although the inhibition of Rad51 did not alter the HHV-6 integration efficiency, more studies are required to decipher this process (Wallaschek et al. 2016a; Wight et al. 2018). Beyond the cellular pathways, several viral factors also could be involved in the homologous recombination. MDV encodes a putative recombinase complex consisting of pUL12 and the HSV ICP8 ortholog encoded by UL29, which are also named U41 and U70 in HHV-6A/B. Surprisingly, even though UL12 or U70 aids in the SSA DNA repair pathway, silencing these genes using shRNA did not affect virus genome maintenance or viral telomere integration (Wight et al. 2018; Previdelli et al. 2019). Moreover, HHV-6A/B encodes another putative viral recombinase U94, unique to other telomere herpesviruses, which is an orthologue of the adeno-associated virus 2 (AAV-2) integrase (Rep68). The absence of U94 did not impair HHV-6A integration in several commonly used cell lines, suggesting that U94 is dispensable for HHV-6A/B integration at least under these conditions or other factors can complement the loss of U94 (Wallaschek et al. 2016a). However, studying for MDV integration could only be investigated qualitatively upon infection of animals due to the lack of an in vitro integration assay, leading to a lag behind HHV-6 research. Therefore, establishing a cell culture-based system would shed further light on the MDV integration mechanism.

5.3.3. The importance of integration in MDV tumorigenesis

For oncogenic MDV strains, viral integration efficiency is directly correlated with pathogenesis and tumor formation. Deletion or mutation of TMRs severely impaired MDV-induced tumor formation (Kaufer et al. 2011; Greco et al. 2014). The integration occurred in all MDV-driven tumor cells even upon TMR mutation. Moreover, analysis of tumor cells obtained from different organs of an animal showed a similar integration pattern (Delecluse and Hammerschmidt 1993; Osterrieder et al. 2014). These demonstrated that viral integration occurs at a very early stage of the lymphocytes' transformation and is likely the initial step in the process of tumor formation, which ensures faithful maintenance of the viral genome and expression of oncogenic factors, such as Meq and vTR (Lee et al. 2008; Kaufer et al. 2010; Conradie et al. 2019).

Of note, three vaccine viruses strains that protect chickens against oncogenic MDV, CVI988, SB-1 and HVT, also harbor TMRs in their genome. Currently, all these vaccines do not provide sterilizing immunity, allowing pathogenic strain infection, replication, and transmission (Read et al. 2015; Conradie et al. 2020). In vaccinated chickens, these vaccines can be detected in a latent form and in very few metaphase chromosomes harboring the virus genome (McPherson et al. 2016; McPherson et al. 2018). It is unclear whether these vaccines also establish latency by integrating their genome into the host and utilizing their TMRs to facilitate this process. On the other hand, vaccination with CVI988 did reduce not only the pathogenicity and tumor formation but also the viral telomere-integration (McPherson et al. 2018). Therefore, it is worthy to investigate whether the mechanism of vaccinal protection is associated with the disrupted latency or transformation of oncogenic MDV, and whether the latency of vaccines contributes to the protection.

5.4. Project introduction

Despite many years of MDV research, many critical questions remain unanswered and need extensive studies. Comprehensive MDV transcriptome analyses revealed that the coding capacity of the MDV genome is greater than previously anticipated, identifying some novel MDV genes and splice variants. Functional characterization of MDV genes is essential to shed light on the complex virus life cycle and MDV pathogenesis. In this cumulative dissertation, I addressed the contribution of two novel proteins to MDV pathogenesis and tumorigenesis. Furthermore, the establishment of de novo *in vitro* integration assay system that will provide an optimal plateform for in vitro research on the integration mechanism of MDV into host telomeres. Work from both aims has been published, and the results are summarized:

1. **To identify and investigate the contribution of three putative MDV-1-specific protein-coding genes**

   Recently, comprehensive MDV transcriptome analyses have identified a couple of potential protein-coding genes, such as MDV082, RLORF11, and SORF6, which are also unique to MDV-1 based on sequence alignment. Using FLAG-tagged mutants, we concluded that only the protein of MDV082 is produced during viral infection. Moreover, our data revealed that pMDV082 contributes to the rapid onset of Marek's disease but is dispensable for replication, dissemination, and tumor formation in infected chickens. The paper was published in the Journal of Virology, 'The Marek's Disease Virus Unique Gene MDV082 Is Dispensable for Virus Replication but Contributes to a Rapid Disease Onset' and is presented in Section 7 of this thesis.

2. **To characterize the contribution of a novel vIL-8 splice variant**

   MDV encodes a CXC chemokine, also named viral interleukin 8 (vIL-8), which consists of two introns and three exons. Comprehensive transcriptome analyses recently revealed a novel alternative vIL-8 splice junction within intron II. This splicing event would result in a novel exon 3 (E3′) containing the last 16 base pairs (bp) of intron II and a stop codon. While the splice variant was clearly detectable in the transcriptome of B cells and CECs, it remained unknown whether this spliced transcript encodes a protein. Using FLAG-tagged mutants, we demonstrated that the novel splice form is expressed as a protein. This protein is dispensable for virus replication, and its absence does not affect the expression of the secreted vIL-8 chemokine. Only minor effects were observed in MDV pathogenesis and tumor formation. This study provides novel insights into the splice forms of the CXC chemokine of this highly oncogenic alphaherpesvirus. The paper was published in Microorganisms, 'Characterization of a Novel Viral Interleukin 8 (vIL-8) Splice Variant Encoded by Marek's Disease Virus' and is presented in Section 8 of this thesis.

3. **To develop an in vitro quantitative system to study MDV integration**

   MDV integrates its genome into the host telomeres, which is crucial for efficient tumor formation. Telomeric repeat arrays present at the ends of the MDV genome facilitate this integration into host telomeres; however, the integration mechanism remains poorly understood. Moreover, MDV integration could only be investigated qualitatively upon the infection of chickens. Therefore, we developed a cell-based quantitative integration assay that was used to investigate the integration efficiency of MDV in vitro. This quantitative integration system provides an optimal basis for investigating the role of viral and cellular factors in integrating MDV into the host telomeres. The paper was published in Microorganisms, 'A Cell Culture System to Investigate Marek's Disease Virus Integration into Host Chromosomes' and is presented in Section 9 of this thesis.

6. References

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7. The Marek's Disease Virus Unique Gene MDV082 Is Dispensable for Virus Replication but Contributes to a Rapid Disease Onset

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8. Characterization of a Novel Viral Interleukin 8 (vIL-8) Splice Variant Encoded by Marek's Disease Virus

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9. A Cell Culture System to Investigate Marek's Disease Virus Integration into Host Chromosomes

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10. Discussion

10.1. General discussion

Marek's disease is a contagious, deadly lymphoproliferative disease of chickens caused by MDV. The virus induces immunosuppression and severe neurological symptoms and typically results in mortality rates of up to 100% in unvaccinated chickens, which causes substantial economic losses in poultry production worldwide (Bertzbach et al. 2020). Therefore, billions of chickens are vaccinated every year, effectively decreasing its natural incidence (Schat 2016). Unfortunately, all the generations of vaccines do not induce sterile immunity, thereby establishing infection and exposing mature viruses to the environment, allowing the viruses to become resistant and potentially prompting the evolution of more virulent pathogens (Read et al. 2015). In-depth characterization of the genetic factors of MDV is an essential step toward understanding the complex viral lifecycle, from viral primary infection, lytic replication, latency, to tumor formation eventually. Many studies have demonstrated the roles of several viral factors involved in viral pathogenesis, including the oncoprotein Meq, the viral chemokine vIL-8/vCXCL13, RLORF4, RLORF5a, pp14, and pp38 (Bertzbach et al. 2018; Bertzbach et al. 2020). The functional analyses of these virus-specific virulent genes have provided much insight into the molecular mechanisms responsible for MDV pathogenesis. However, many aspects have not been assessed, and an update of the status quo would help identify knowledge gaps to fill them. Moreover, deciphering the mechanisms of viral factors involved in MDV pathogenesis and lymphomagenesis will also provide new strategies for efficiently engineering new vaccines against this deadly pathogen.

Comprehensive MDV transcriptome analyses recently revealed that the coding capacity of the MDV genome is greater than previously anticipated and identified additional hypothetical coding sequences, such as SORF6 and RLORF11 (Bertzbach et al. 2019; Sadigh et al. 2020). mRNA levels of these transcripts were high in lytically infected cells. Intriguingly, although the very virulent strain RB1B has high sequence similarity (>99%) with the vaccine strain CVI988, their transcriptome showed some minor differences in the viral messenger RNA (mRNA) levels, reflecting that either change in the protein expression or functional differences in virulence factors might be responsible for the difference in pathogenesis between RB1B and the vaccine. One of the notable transcripts is MDV082, which has significantly higher expression in CVI988-infected cells compared to cells that were infected with RB1B. These three transcripts (SORF6, RLORF11 and MDV082) all possess an upstream TATA box and a downstream poly(A) cleavage cluster, resembling protein-encoding genes. Moreover, they are unique to MDV based on sequence alignment. Therefore, investigating their coding potentials and the functions of their gene products could shed more light on the complex viral lifecycle.

RNA alternative splicing is an essential and common process for eukaryotic gene expression, occurring when several splice donor sites (GT) and/or acceptor sites (AG) are eligible and facultative, which allows the construction of different RNA transcript isoforms from a single pre-mRNA (Bonnal et al. 2020). Despite its prevalence in eukaryotic cells, viruses, such as adenovirus and herpesvirus, have been demonstrated to utilize alternative splicing machinery of the host to generate diverse sets of transcripts (Berget et al. 1977; Toth 2008; Nojima et al. 2009). For MDV, some of the introns and associated spliced genes have been previously described, most notably the spliced variants of MDV oncogene *meq*, the viral lipase (vLIP), glycoprotein C (gC), vIL-8 and pp14 (Kamil et al. 2005; Bertzbach et al. 2018). In addition, in the *meq* to vIL8 region, multiple spliced transcripts have been identified via the nucleotide sequencing of a limited number of cDNA products, including fusion proteins of Meq, RLORF4, and RLORF5a with exons II and III of vIL-8 (Jarosinski and Schat 2007). The complex transcriptional landscape of MDV revealed the presence of 71 introns, giving rise to hundreds of splice forms, including a novel vIL-8 splice junction, which was detected at about 13–40-fold lower levels than previously published vIL-8 transcripts (Bertzbach et al. 2019; Sadigh et al. 2020). This new splice site was predicted to lead to a novel ORF that could also splice with other transcripts through canonical splicing motifs. Recent studies have indicated that a spliced transcript of vIL-8 and *meq* play a role in MDV pathogenesis (Liao et al. 2020). Consequently, there is an immense need to better understand the role of splicing in the MDV lifecycle and in MDV pathogenesis.

Except for several virulence genes and gene products, MDV integration efficiency is directly correlated with the pathogenesis and lymphomagenesis (Kaufer et al. 2011b; Greco et al. 2014; Osterrieder et al. 2014). Even though the natural MDV-chicken model has demonstrated that TMRs present at the ends of the MDV genome facilitates directed integration into host telomeres, the deciphering of the integration mechanism has been seriously hampered by the lack of an in vitro integration assay. To shed further light on the MDV integration mechanism, we tested several REV-transformed chicken T cell lines for their ability to facilitate MDV latency, genome maintenance, and integration. Combined and compared with in vivo information, we established a cell culture-based system to investigate MDV integration.

Taken together, we first identified a novel protein encoded by MDV082 and the spliced vIL-8-E3′protein, and then investigated the role of these novel proteins on MDV pathogenesis. Also, we developed and validated a novel in vitro integration assay as an optimal basis for investigating the MDV integration mechanism. All will be discussed in greater detail below.

10.2. Characterization and contribution of three putative genes in MDV pathogenesis

As most MDV genes have homologues in other alphaherpesviruses, their function is mainly involved in DNA replication, envelopment, and many other processes essential for the virus lifecycle (Liao et al. 2021). In addition, extensive studies have demonstrated that MDV encodes some specific protein-coding genes, RNAs, and other sequence elements, mainly located in the repeat regions, that contribute to viral pathogenesis, tumorigenesis, and latency (Bertzbach et al. 2018). While these studies have undoubtedly provided insights into the viral lifecycle and MDV pathogenesis, some unique and as of yet uncharacterized genes existing in the MDV genome may also be critical to understanding viral pathogenicity. Based on previous transcriptome analyses and bioinformatic predictions, some MDV-specific transcripts, such as MDV082, RLORF11 and SORF6, are potential ORFs and could encode proteins (Bertzbach et al. 2019; Sadigh et al. 2020). We, therefore, inserted FLAG cassettes into the N- and/ or C- terminals of these putative genes based on the vvMDV strain RB1B, to be able to detect their expression using commercial anti-FLAG antibodies (Schippers et al. 2015).

Regarding the expression, we concluded that only the pMDV082 is produced during viral infection. There are some reasons why the expression of RLORF11 and SORF6 proteins were not detectable in our experiments. Firstly, they might be expressed at low levels so that WB and IFA cannot detect them; Secondly, they might be expressed in other phases of the viral lifecycle, such as latency. A previous study, for example, showed that the mRNA transcript of RLORF11 was detected during the lytic and latent infection (Hunter 2012). Last but not least, they could even represent potentially non-coding RNAs. It is not surprising that MDV encodes numerous linear non-coding RNAs, including viral microRNAs (miRNAs), the viral telomerase RNA subunit (vTR), and long noncoding RNAs (lncRNAs), and even circular RNAs (circRNAs) (Kaufer et al. 2010; Kaufer et al. 2011a; Zhao et al. 2011; Zhang et al. 2019; Chasseur et al. 2022).. Therefore, the hypothetical expression of proteins or non-coding RNAs remains to be addressed further.

We, next, mutanted the start code of MDV082 in a very virulent RB1B MDV, thereby abrogating its expression. We characterized this mutant virus in vitro and vivo to investigate the contribution of pMDV082 in viral replication. As with most of the unique-MDV genes, we demonstrated that pMDV082 is completely dispensable for the in vitro replication and cell-to-cell spread. Also, pMDV082 did not show specific localization in CEFs and B cells, but appeared very late in MDV-induced plaques, even up to 4 dpi. Intriguingly, pMDV082 potentially share the same transcriptional start site with the immediate-early gene ICP4, forming a bicistronic transcript (Bertzbach et al. 2019; Sadigh et al. 2020), but they seem to show different temporal expression. ICP4 transcripts are terminated by four alternative

polyadenylation signals, which play a major role in post-transcriptional regulation, particularly during latency (Heidari et al. 2008; Rasschaert et al. 2018). Nevertheless, it remains unknown whether the transcription and expression of MDV082 are regulated by these alternative polyadenylation signals of ICP4.

Furthermore, we infected chickens with the pMDV082-deficient recombinant virus to evaluate the contribution of MDV082 to viral replication in vivo, MD incidence and tumor formation. Coherent to the in vitro data, we did not a observe significantly difference in viral replication between mutant and wild type virus. The absence of pMDV082 did not alter diseases and tumors incidence but delayed the onset of the disease. Intriguingly, MDV082 mRNA is one of only a few transcripts that are higher expressed in the live attenuated virus strain CVI988-infected cells than vvMDV strain RB1B-infected.

Moreover, MDV pathogenesis and tumorigenesis are associated with immune response induced by the major histocompatibility complex (MHC) (Hearn et al. 2015; Bertzbach et al. 2022). pMDV082 gave rise to two peptides bound to MHC class II molecules, which are unique to CVI988 (Halabi et al. 2021). Although the difference in the virulence between CVI988 and RB1B has been recently attributed to mainly to the *meq* gene that acquired changes during virus evolution (Conradie et al. 2020), it would be worthwhile to further explore the role of MDV082 in the context of immune recognition and MDV latency.

10.3. Novel insights into a novel viral interleukin 8 (vIL-8) splice variant

Although the majority of herpesvirus genes are unspliced, some herpesviruses, including MDV, make extensive use of alternative splicing to generate multiple proteins from their limited genomes (Verma and Swaminathan 2008; Nojima et al. 2009), most of which are uncharacterised and/or not annotated. Recent RNA-seq provided more in-depth knowledge of MDV transcriptome (Bertzbach et al. 2019; Yang et al. 2020). A novel vIL-8 splice junction was identified that was expressed at approximately 13~40-fold lower levels than previously published vIL-8 transcripts. This splice event would result in a novel exon containing the last 16 bp of intron II and a stop codon. We, therefore, inserted the FLAG cassette at 89 bp downstream of the 5′end of vIL-8 intron II based on the vvMDV strain RB1B. We successfully identified this novel vIL-8 spliced variant by WB and IFA. As with the deletion of the whole vIL-8 region, it’s not surprised that the abrogation of this novel spliced vIL-8-E3′ protein has no effect on MDV replication in vitro and in vivo.

Studies on human oncogenic virus, such as human papillomavirus (HPV), Epstein–Barr virus (EBV), Merkel cell polyomavirus (MCV), human T-cell leukemia virus 1 (HTLV-1), Kaposi's

sarcoma-associated herpesvirus (KSHV), have revealed that RNA splicing is an indispensable step for viral oncogene expression, which diversifies their transcriptomes during virus infection and could activate their oncogenic activities (Ajiro and Zheng 2019). Notably, abrogation of vIL8 secretion alone does not fully compensate for the effect of the deletion of the entire vIL-8 gene sequence on disease and tumor formation (Parcells et al. 2001; Cui et al. 2004; Jarosinski and Schat 2007; Engel et al. 2012). Thus, it remains unknown whether the vIL-8 splice variants contribute in some way to MDV-induced disease and tumor formation. Our data demonstrated that the abrogation of the novel vIL-8-E3′ protein does not directly alter MDV pathogenesis and tumorigenesis. Investigating the role of splicing isoforms, especially those involved in viral oncogene transcripts, will be a formidable challenge, but will provide some fundamental insight into cells transformation. EBV LMP1 and lyLMP1 are produced by RNA splicing from two separate transcripts (Renzette et al. 2014). LMP1 can immortalize and transforms human B cells, but lyLMP1 has no transforming activity and even interferes the LMP1-signaling pathways (Wang et al. 1988; Verma and Swaminathan 2008).

On the other hand, viral gene, such as infected-cell protein 27 (ICP27), can also regulate alternatively splice events (Bryant et al. 2001; Tang et al. 2019). HSV-1 ICP27 predominantly transactivated unspliced gC mRNA and promoted the retention of an intron (Perkins et al. 2003; Sedlackova et al. 2008). As described for HSV-1, MDV ICP27 also can interact with splicing factors, which inhibits mRNA splicing of vIL-8 and the cellular chicken telomerase reverse transcriptase (chTERT). Upon ICP27 expression, unspliced vIL-8 transcripts were at low levels during MDV reactivation (Amor et al. 2011). Thus, it would be intriguing to explore why MDV blocks vIL-8 splice variants during reactivation.

10.4. A Cell Culture System Provides an optimal basis for investigating the MDV integration mechanism

TMRs facilitate MDV integration into chicken telomeres. Upon deletion of the viral TMRs, the virus is still able to preserve its DNA in chicken cells in vivo, but the virus integration occurs randomly and the tumor formation is severely impaired. Until now, investigation of the MDV integration mechanism and viral factors that facilitate this process required animal experiments. To overcome this issue, we set to establish a feasible cell culture-based integration assay. We, firstly, tested serval immortalized chicken T cell lines and optimized the MDV infection, and then measured viral genome maintenance by q-PCR and visualized viral integration by FISH, which allow us to establish an in vitro integration assay system. Then, we tested various knockout mutants using in vitro integration assay, the integration efficiency of which was consistent with our previous in vivo studies. With the in vitro system, many potential cellular and viral factors involved in HHV-6 integration have been assessed (Wallaschek et al.

2016a; Wallaschek et al. 2016b; Wight et al. 2018; Aimola et al. 2020). We strongly believe that this assay also allows to investigate the involvement of cellular or other viral factors that might (co-)facilitate genome integration due to their role in MDV replication and tumorigenesis like vTR (viral telomerase RNA), UL30 (MDV polymerase), UL29 (major DNA-binding protein ICP8), UL12 (MDV 5′-3′ exonuclease) (Previdelli et al. 2019). Moreover, MDV integration efficiency is directly correlated with the pathogenesis and lymphomagenesis. MDV-induced tumor cells obtained from different organs showed the same integration pattern, suggesting that it occurs at a very early phase of transformation. Therefore, effective integration not only enables maintenance of viral genome in latently infected T cells, but also is likely the initial step in lymphomagenesis. As reported, only a limited number of latently infected cells are subsequently transformed, resulting in deadly lymphomas (Mwangi et al. 2011; Bertzbach et al. 2020). Deciphering the integration mechanism would provide deeper insights into viral latency, transformation, and reactivation. Additionally, future directions could be investigations into the genome integration properties of MDV vaccine viruses which also possess TMRs in their genomes.

10.5. Concluding remarks and outlook

During my Ph.D. in the *Viral Integration and Tumorigenesis Group* at the Institute of Virology, my project focused on charactering novel putative genes and studying the integration of Marek's disease virus. Despite many years of research, the MDV genetic factors that potentially contribute to viral pathogenesis and tumorigenesis are still far from fully understood (Bertzbach et al. 2018; Bertzbach et al. 2020). Here we identified a novel protein encoded by MDV082 and a novel vIL-8 splice variant. We revealed that the MDV082 protein contributes to the rapid onset of Marek's disease, and represented the first characterization of the novel vIL-8 splice form. Meanwhile, we developed and validated a cell culture-based integration assay system. With the information obtained here, we understand the genetic factors of MDV better and our in vitro assay will allow us to investigate potential viral and cellular factors involved in the integration mechanism.

Exceeding the three manuscripts of this doctoral thesis, we have been working on several other projects that strongly link to these projects in terms of content. Firstly, except identification and characterization of the novel vIL-8 splice form, we also work on the investigation of other vIL-8 splice variants. Recent work showed that vIL-8, a viral CXC chemokine, plays important roles in MDV pathogenesis and tumorigenesis via recruiting B cells and $CD4^+CD25^+$ T cells (Engel et al. 2012; Haertle et al. 2017). The complete abrogation of vIL-8 significantly reduced virus replication and abrogated tumor formation by about 90%, while abrogation of vIL-8 chemokine expression by mutating its start codon or deleting its exon

l “only” reduced tumor incidence by about 60% (Cui et al. 2004; Engel et al. 2012; Bertzbach et al. 2018). Therefore, it is likely that the complete vIL8 gene function consists of secreted vIL-8 and other aspects, such as one or more of the vIL-8 splice variants. Previous studies showed that vIL-8 exons 2 and 3 interact with upstream viral virulence genes, including *meq*, RLORF4 and RLORF5a, to produce multiple alternatively spliced mRNAs. This alternative splicing significantly expands the size of the MDV transcriptome, but its biological significance remains undetermined and in need of further investigation. We generated the vIL-8 intron 1 deletion mutant, which abrogated the multiple alternative spliced mRNAs fused by the splice acceptor A18 but did not affect the secreted vIL-8. Our data obtained from the animal experiments will provide insights into the splice forms of the CXC chemokine of this highly oncogenic alphaherpesvirus.

Integration viral genome into host telomeres is important for the pathogenesis and lymphomagenesis of oncogenic MDV. Of note, three vaccine strains developed against virulent MDV, including CVI988, SB-1, and HVT, also have type E genomes and possess TMRs in their genome. After vaccination, the HVT genome was found integrated into telomeres of spleen cells as detected by a cytogenetic method, while no FISH images were provided to visualize viral integration (McPherson et al. 2016; McPherson et al. 2018). Our cell culture-based integration assay system does not only provide a crucial platform for the analysis of the MDV integration mechanisms but can be used to investigate if other chicken herpesviruses carrying TMRs could integrate their genome into host telomeres. With this, we can investigate whether these three MDV vaccines integrate their genomes into host telomeres during latency and determine whether this process is facilitated by viral TMRs. We recently generated TMR deletion mutants based on these three vaccines, which showed similar replication abilities when compared to their parental virus. Based on our in vitro integration assay, the vaccine viruses were efficiently maintained over time, while their TMR mutants have less efficient maintenance. With our collaborators at the INRA-ISP in France, Dr. Caroline Denesvre and colleagues, we carried out animal experiment to assess the role of TMRs in HVT replication, reactivation, and viral genome maintenance. Moreover, we will also use CVI988, SB-1, and their TMRs mutants to determine whether these viruses carrying TMRs have more efficient viral genome maintenance during latency in vivo, and whether the efficient latency of these vaccines is relative to its protection against oncogenic MDV.

10.6. References

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11. Summary

Marek's disease virus (MDV) is a highly contagious alphaherpesvirus that causes neurological disorders, immunosuppression, and deadly lymphomas in chickens. The virus causes substantial economic losses in poultry production worldwide due to high mortality rates and the costs of the vaccination. Despite the widespread use of live attenuated vaccines that provide excellent protection against clinical disease, the virus still infects vaccinated animals and continuously evolved towards a higher virulence.

MDV encodes many viral factors, from proteins to non-coding RNAs, which orchestrate the complex viral lifecycle and/or contribute to pathogenesis. Several MDV-specific genes have been shown to be involved in viral pathogenesis and tumorigenesis. Comprehensive MDV transcriptome analyses revealed that the coding capacity of the MDV genome is far greater than previously anticipated and identified some putative MDV-specific genes and splice isoforms. In-depth characterization of the genetic factors of MDV is an essential step toward understanding the complex viral lifecycle, from viral infection, lytic replication, latency, to tumor formation. Here, we set out to assess three MDV-specific hypothetical genes (MDV082, RLORF11, and SORF6) and a putative vIL-8 exon (vIL-8-E3′). Using FLAG-tagged recombinant viruses based on the very virulent MDV strain RB1B, we identified a novel protein encoded by MDV082 and a novel spliced vIL-8-E3′protein. We demonstrate that the novel pMDV082 and vIL-8 splice variant are not essential for virus replication, spread, and tumor formation, but pMDV082 contributes to the rapid onset of Marek's disease. These studies shed light on the expression of MDV-specific genes and the splice forms of the CXC chemokine and unraveled the role of pMDV082 in MDV pathogenesis.

In addition to viral virulence factors, the lymphomagenic properties of oncogenic MDV is also directly linked to viral integration, a process which occurs during the establishment of viral latency. Telomeric repeat arrays present at the ends of the MDV genome facilitate this integration into host telomeres, but the integration mechanism remains poorly understood. To shed further light on the MDV integration mechanism, we developed and validated a cell culture-based integration assay, providing an optimal basis for investigating the role of viral and cellular factors that could be involved in the integration of MDV.

12. Zusammenfassung

Das Marek' s Disease Virus (MDV) ist ein hochansteckendes Alphaherpesvirus, das bei Hühnern neurologische Störungen, Immunsuppression und tödliche Lymphome verursacht. Das Virus verursacht aufgrund der hohen Sterblichkeitsrate und der Kosten für die Impfung weltweit erhebliche wirtschaftliche Verluste in der Geflügelproduktion. Obwohl der weit verbreitete abgeschwächten Lebendimpfstoffe einen ausgezeichneten Schutz vor klinischen Erkrankungen bieten, infiziert das Virus immer noch geimpfte Tiere und hat sich kontinuierlich zu einer höheren Virulenz entwickelt.

MDV kodiert für eine viele virale Faktoren, von Proteinen bis hin zu nicht-kodierenden RNAs, die den komplexen viralen Lebenszyklus steuern und/oder zur Pathogenese beitragen. Mehrere MDV-spezifische Gene sind nachweislich an der viralen Pathogenese und Tumorigenese beteiligt. Umfassende MDV-Transkriptomanalysen haben gezeigt, dass die Kodierungs Kapazität des MDV-Genoms weitaus größer ist als bisher angenommen, und einige mutmaßlich MDV-spezifischen Gene und Spleiß-Isoformen identifiziert wurden. Die eingehende Charakterisierung der genetischen Faktoren von MDV ist ein wesentlicher Schritt zum Verständnis des komplexen viralen Lebenszyklus, von der viralen Infektion über die lytische Replikation und Latenz bis hin zur Tumorbildung. Hier haben wir uns vorgenommen, drei MDV-spezifische hypothetische Gene (MDV082, RLORF11 und SORF6) und ein mutmaßliches vIL-8-Exon (vIL-8-E3′) zu untersuchen. Mit Hilfe von FLAG-markierten rekombinanten Viren, die auf dem sehr virulenten MDV-Stamm RB1B basieren, identifizierten wir ein neuartiges Protein, für das MDV082 kodiert, und ein neuartiges gespleißtes vIL-8-E3′-Protein. Wir konnten zeigen, dass das neue pMDV082 und die vIL-8-Spleißvariante für die Virusreplikation, die Ausbreitung und die Tumorbildung nicht essenziell sind, pMDV082 jedoch zum schnelleren Ausbruch der Marek‘schen Krankheit beiträgt. Diese Studien geben Aufschluss über die Expression von MDV-spezifischen Genen und den Spleißformen des CXC-Chemokins und enträtseln die Rolle von pMDV082 in der MDV-Pathogenese.

Neben viralen Virulenzfaktoren sind die lymphomagenen Eigenschaften des onkogenen MDV auch direkt mit der viralen Integration verbunden, einem Prozess, der während der Etablierung der viralen Latenz stattfindet. Telomer Repeat-Arrays an den Enden des MDV-Genoms erleichtern diese Integration in die Wirts-Telomere, aber der Integrationsmechanismus ist nach wie vor kaum verstanden. Um den Integrationsmechanismus näher zu beleuchten, haben wir ein zellkulturbasiertes Integrationstestsystem entwickelt und validiert, das eine optimale Grundlage für die Untersuchung der Rolle viraler und zellulärer Faktoren bietet, die bei der Integration von MDV zum Tragen kommen.

13. List of publications

13.1. Scientific publications

You, Y. †, Vychodil, T. †, Aimola, G., Previdelli, R. L., Göbel, T. W., Bertzbach, L. D., & Kaufer, B. B. (2021). **A Cell Culture System to Investigate Marek's Disease Virus Integration into Host Chromosomes.** *Microorganisms*, *9*(12), 2489.

You, Y., Hagag, I. T., Kheimar, A., Bertzbach, L. D., & Kaufer, B. B. (2021). **Characterization of a Novel Viral Interleukin 8 (vIL-8) Splice Variant Encoded by Marek's Disease Virus.** *Microorganisms*, *9*(7), 1475.

You, Y., Conradie, A. M., Kheimar, A., Bertzbach, L. D., & Kaufer, B. B. (2021). **The Marek's Disease Virus Unique Gene MDV082 Is Dispensable for Virus Replication but Contributes to a Rapid Disease Onset.** *Journal of Virology*, *95*(15), e00131-21.

Kheimar, A., Klinger, R., Bertzbach, L. D., Sid, H., Yu, Y., Conradie, A. M., ... & Schusser, B. (2021). **A Genetically Engineered Commercial Chicken Line Is Resistant to Highly Pathogenic Avian Leukosis Virus Subgroup J.** *Microorganisms*, *9*(5), 1066.

Bertzbach, L. D., Conradie, A. M., You, Y., & Kaufer, B. B. (2020). **Latest insights into Marek's disease virus pathogenesis and tumorigenesis.** *Cancers*, *12*(3), 647.

Previdelli, R. L., Bertzbach, L. D., Wight, D. J., Vychodil, T., You, Y., Arndt, S., & Kaufer, B. B. (2019). **The role of Marek's disease virus UL12 and UL29 in DNA recombination and the virus lifecycle.** *Viruses*, *11*(2), 111.

13.2. Talks and poster presentations

Yu You, Andelé M. Conradie, Ahmed Kheimar, Luca D. Bertzbach and Benedikt B. Kaufer: Poster "**Marek's disease virus unique gene MDV082 is dispensable for virus replication but contributes to a rapid disease onset**" 03/2022, 31th Annual Meeting of the Society for Virology, München

Yu You*, Tereza Vychodil*, Luca D. Bertzbach*, Renato L. Previdelli, Benedikt B. Kaufer: Poster "**A quantitative assay to assess Marek's disease virus genome integration in vitro**" 03/2021, 30th Annual Meeting of the Society for Virology, Berlin

Yu You, Ahmed Kheimar, Luca D. Bertzbach, Caroline Denesvre and Benedikt B. Kaufer: Oral presentation and Poster "**The telomeric repeats of Marek's disease virus vaccines are required for viral integration and genome maintenance**" 09/2022, the European Society for Veterinary Virology, Ghent.

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16. Conflict of interests

There are no conflicts of interests.

17. Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig und ausschließlich unter Zuhilfenahme der genannten Quellen und Hilfen angefertigt habe.

Berlin, am 16.09.2022

Yu You